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Pharmacological modulation of a voltage sensitive release mechanism for SR Ca^{2+} in ventricular myocytes

by

Cindy Ann Mason

Submitted to the Faculty of Graduate Studies
in partial fulfillment of the requirements for
the degree of Doctor of Philosophy

at

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Halifax, Nova Scotia

September, 1999

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FACULTY OF GRADUATE STUDIES

The undersigned hereby certify that they have read and recommend to the Faculty of Graduate Studies for acceptance a thesis entitled "Pharmacological Modulation of a Voltage Sensitive Release Mechanism for SR Ca^{2+} in ventricular myocytes"

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This thesis is dedicated to my parents for always believing in me and to Jason B. for giving me the most important things in life... I'll love you always.

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ABSTRACT

Excitation-contraction (EC) coupling refers to a process whereby electrical stimulation of the sarcolemmal (SL) membrane of a myocyte is translated into mechanical contraction. A rapid increase in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) is required for contraction to occur. Most of this rise in $[Ca^{2+}]_i$ occurs through release from the sarcoplasmic reticulum (SR). The following two fundamental mechanisms have been proposed to explain the process by which stimulation of the SL causes release of Ca^{2+} from the SR; 1) calcium-induced calcium release (CICR), whereby transsarcolemmal Ca^{2+} influx stimulates opening of SR ryanodine receptors (RyRs) and release of stored Ca^{2+}, and; 2) a voltage sensitive release mechanism (VSRM), whereby SR release of Ca^{2+} is stimulated by depolarization of the SL and is independent of transsarcolemmal Ca^{2+} influx. The objective of the present study was to evaluate the use of tetracaine and ryanodine, two pharmacological agents known to interact with SR RyRs, as tools to investigate the contribution of each mechanism to cardiac contraction. Single electrode voltage clamp and video edge detection were used to measure membrane currents and contractions respectively, in freshly dissociated guinea-pig ventricular myocytes at 37 °C. Both high resistance (15-20 MΩ) and patch electrodes (1-3 MΩ) were used in the present study. Initial experiments showed that tetracaine preferentially inhibited VSRM contractions at concentrations which also inhibited Na^+ current. However, inhibition of the VSRM by tetracaine persisted in the presence of prior Na^+ channel blockade by lidocaine or lidocaine plus tetrodotoxin. Rapid application of moderate concentrations of tetracaine (≥ 300 μM) selectively inhibited VSRM contractions in undialyzed myocytes. However, longer-duration (~3 mins.) continuous exposure was required for inhibition of VSRM contractions in dialyzed myocytes. Continuous exposure also was associated with an increase in SR Ca^{2+} stores, however this effect was independent of the effects of tetracaine on the VSRM. The tetracaine-insensitive component of contraction was inhibited by rapid application of Cd^{2+}, disruption of SR release, or block of $I_{Ca\text{-}L}$ which suggests that this component represents CICR. Thus, effects of tetracaine on cardiac EC-coupling are opposite to that which has been reported in skeletal muscle where this agent selectively inhibits CICR. The results of the present study show that inhibition of the VSRM by tetracaine is not due to a shift in the properties of activation or inactivation of this mechanism nor is it due to the local anesthetic actions of this agent. Higher concentrations (1 mM) became less selective and inhibited both contractions. The effects of ryanodine on contractions also were examined in the present study. The current view about ryanodine is that inhibition of contractions by low concentrations of this agent results from depletion of SR Ca^{2+} stores. However, the present study demonstrates that inhibition of VSRM contractions by nanomolar concentrations of ryanodine is independent of depletion of SR stores as assessed by rapid application of 10 mM caffeine. The effects of tetracaine on VSRM contractions occur within seconds, therefore rapid application of this agent will be a useful technique for evaluation of the contributions of the VSRM to total contraction. By comparison, ryanodine has a slow mechanism of action and therefore cannot be applied rapidly, however the high selectivity and affinity of this agent for SR RyRs makes it a valuable tool for investigation of the VSRM. In conclusion, the results of this study illustrate that both tetracaine and ryanodine can be used as tools to investigate the relative contributions of the VSRM to cardiac contraction.

LIST OF ABBREVIATIONS

cAMP	cyclic-AdenosineMonoPhosphate
8-Br-cAMP	8-Bromo-cyclic-AdenosineMonoPhosphate
Ca^{2+}	calcium
$I_{Ca\text{-}L}$	L-type calcium current
Na^{+}	sodium
I_{Na}	sodium current
$NaCa_{EX}$	sodium-calcium exchanger
V_{PC}	post-conditioning potential
CV	contraction-voltage
IV	current-voltage
CICR	calcium-induced calcium release
VSRM	voltage-sensitive release mechanism
AP	action potential
mV	millivolts
nA	nanoampere
SR	sarcoplasmic reticulum
SL	sarcolemma
μM	micromolar
μm	micrometer
t-tubule	transverse-tubule
EC-coupling	excitation-contraction coupling

RyR(s)	ryanodine receptor(s)
LA	local anesthetic
AC	adenylate cyclase
PKA	protein kinase A
CamK	Ca^{2+} -calmodulin dependent protein kinase
mg	milligram
ml	milliliter
M	molar
mM	millimolar
Ag/AgCl	silver/silver-chloride
Mg^{2+}	magnesium
Ni^{2+}	nickel
Cd^{2+}	cadmium
fura-2-AM	fura-2-acetoxymethyl ester
DICR	depolarization-induced calcium release
T-type	transient calcium current
L-type	long lasting calcium current
DHPR	dihydropyridine receptor
$[Ca^{2+}]_i$	intracellular calcium concentration

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PUBLICATIONS

Portions of this thesis have previously been published.

1. Mason, C.A. & Ferrier, G.R. (1999). Tetracaine can inhibit contractions initiated by a voltage sensitive release mechanism in guinea pig ventricular myocytes. *Journal of Physiology* **519**, 851-865.

2. Ferrier, G.R., Redondo, I.M, Mason, C.A., Mapplebeck, C. & Howlett, S.E. (1999). Regulation of contraction and relaxation by membrane potential in cardiac ventricular myocytes. *American Journal of Physiology* (In press).

3. Mason, C.A. & Ferrier, G.R. (1999). Role of SR Ca load in inhibition of cardiac contractions by tetracaine. *Biophysical Journal* **76**, A458.

4. Ferrier, G.R., Redondo, I.M., Mason, C.A., Mapplebeck, C. & Howlett, S.E. (1999). Inactivating and non-inactivating components of the cardiac voltage-sensitive release mechanism. *Biophysical Journal* **76**, A457.

5. Mason, C.A., Howlett, S.H. & Ferrier, G.R. (1998). Ryanodine selectively inhibits the voltage-sensitive release mechanism for SR Ca in guinea-pig ventricular myocytes. *Biophysical Journal* **74**, A55.

6. Ferrier, G.R. & Mason, C.A. (1997). Tetracaine blocks a voltage-sensitive release mechanism for contraction in ventricular myocytes. *Biophysical Journal* **72**, A161.

INTRODUCTION

OVERVIEW:

General properties of the heart and cardiovascular system:

Evolution of life from a single cell organism to the more complex life forms found today required the development of multicellular organisms. To survive, the multicellular organism needed to develop a system which could provide nutrients, as well as a mechanism to deliver the nutrients to all cells. In single cell organisms simple diffusion was adequate to provide and deliver nutrients and remove wastes, however, in a multicellular organism this system would be insufficient. Thus, multicellular organisms developed a distribution system that is designed around the movement of a fluid: the blood. Blood is carried to all tissues of the body by the circulatory system. Aside from the transport of nutrients and waste, blood also serves many additional functions such as transport of heat, transmission of hydraulic force and transport of respiratory gases. Thus, a blood circulatory system is important in virtually all organisms more than a few millimeters in size and is a necessity for large animals with high metabolic rates (Schmidt-Nielsen, 1990).

An adequate circulatory system depends on one or more pumps to force blood through a system of tubes which extend throughout the entire body. The organ that serves as a pump for blood is the heart. Knowledge of the importance of this organ can be traced back to the days of Aristotle (384-322 B.C.) who viewed the heart as being the central organ of an organism, containing the seat of the soul and vital heat. Aristotle believed that the heart was important for the production of blood, an idea challenged by

Galen of Pergamon (130-201 A.D.), who believed that the liver was responsible for production of blood. It was Galen, however, who viewed the heart as a muscle and described in detail the structure of the ventricles and valvular mechanisms (Rothschuh, 1973).

In spite of this knowledge of the anatomy of the heart, the idea that blood circulated in the body was not put forth until the 1600's when William Harvey courageously questioned Galen's ideas (Rothschuh, 1973). Although Harvey was influenced by the work of both Galen and Aristotle, there were many questions which he sought to answer. Combining his experimental observations with his own ideas led him to the conclusion that blood moved in a circular motion throughout the body (Rothschuh, 1973). Today we can follow this circular path beginning with the oxygenated blood from the lungs which travels through the pulmonary veins to the left ventricle of the heart via the left atrium. From here blood is pumped to the periphery via the arteries which eventually branch into the small capillaries. Blood returns from the periphery via the venous system to the right atrium then to the right ventricle where it is pumped back to the lungs to be reoxygenated.

Conduction through the heart:

Modern day organ transplantation technology illustrates that the heart is myogenic, that is, the stimulus to contract is generated within the organ and does not require intact nervous pathways. The area of the heart responsible for the stimulus is known as the pacemaker which typically resides in the sinoatrial node (SA) found in the

right atrium. Normal excitation of the heart follows a specific pathway which begins at the SA node then travels throughout the atria to the atrioventricular node. From here the stimulus is carried throughout both ventricles by way of the bundle of His and Purkinje system. This causes the ventricles to contract from the apex of the heart upward. The atria and ventricles do not contract simultaneously but rather in sequence to allow blood to flow between these cavities. Innervation of the heart by the autonomic nervous system allows modulation of the intrinsic functioning of the heart through release of neurotransmitters. In this way, the central nervous system can control the rate and force of contraction to meet the demands of the body at any given time (Berne & Levy, 1997).

Properties of muscle:

The functioning of the heart and circulatory system is based on the ability of muscle to contract or shorten. In general, the primary function of muscle is to generate forces and movements used by multicellular organisms in the regulation of their internal environment and for movement through the external environment. Three types of muscle can been identified in vertebrates based on structure, contractile properties and control mechanisms. These are skeletal muscle, which is attached to bone and is under voluntary control; smooth muscle, which surrounds many organs and vessels; and cardiac muscle, which is the muscle of the heart (Schmidt-Nielsen, 1990; Vander, Sherman & Luciano, 1994).

There are many similarities and differences between all three types of muscle. For example, both skeletal and cardiac muscle fibers are known as striated muscle because

they have characteristic banding when viewed through a microscope. This striated pattern results from the presence of numerous sarcomeres and Z-lines placed end-to-end (Schmidt-Nielsen, 1990). As suggested by its name, smooth muscle lacks the cross-striated banding pattern found in skeletal and cardiac muscle. However, smooth muscle does contain the thick and thin filaments found in striated muscle which are responsible for contraction. Thus, the force generating mechanism which involves cross-bridge formation between these thick and thin filaments is similar in all three types of muscle (Chapman, 1983; Schmidt-Nielsen, 1990; Vander, Sherman & Luciano, 1994; Berne & Levy, 1997).

Although striated and smooth muscles use the same mechanism to generate force, variations in the amount of force generated by each contraction is achieved differently in cardiac muscle compared to smooth and skeletal muscle. An increase in the force generated by skeletal and smooth muscle contractions is achieved by the recruitment of more muscle fibers (Vander, Sherman & Luciano, 1994). By contrast, each beat of the heart involves activation of the whole muscle tissue, therefore an increase in force cannot be achieved through recruitment of more muscle fibers. Instead, changes in the force of cardiac contractions are thought to be achieved through variation in transduction of the stimulus at the cellular level (Vander, Sherman & Luciano, 1994). This variation in activation is thought to be achieved by changes in the affinity of the filaments for calcium (Ca^{2+}) and/or a variation in the amount of Ca^{2+} reaching the myofilaments (Chapman, 1983; Vander, Sherman & Luciano, 1994).

EXCITATION-CONTRACTION (EC) COUPLING:

Initiation of contraction in both striated and smooth muscle begins with conduction of an action potential (AP) along the cell membrane. In striated muscle excitation also spreads into the interior of the muscle fibers via invaginations of the outer sarcolemma (SL) membrane (Peachey, 1965; Fawcett & McNutt, 1969; Bers, 1991). These invaginations, which are referred to as transverse-tubule system (t-tubules or t-system) do not exist in smooth muscle cells (Schmidt-Nielsen, 1990; Vander, Sherman & Luciano, 1994; Berne & Levy, 1997). Conduction of an AP along the SL membrane in both smooth and striated muscle ultimately results in cross-bridge formation and contraction of the muscle. Ca^{2+} is essential for cross-bridge formation, thus a rapid increase in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) is required for contraction to occur (Ebashi & Endo, 1968; Ebashi, 1974; Bers, 1991; Berne & Levy, 1997). The mechanism by which excitation of the membrane by an AP results in an increase in $[Ca^{2+}]_i$ and contraction is referred to as excitation-contraction coupling or EC-coupling (Brady, 1964).

Smooth muscle EC-coupling:

Although EC-coupling in all muscle involves the release of Ca^{2+} from an intracellular storage site, this is not an essential part of EC-coupling in smooth muscle as compared to cardiac and skeletal muscle. In smooth muscle most of the increase in intracellular Ca^{2+} occurs through voltage-gated and receptor-activated Ca^{2+} channels in the outer membrane which allow influx of Ca^{2+} from the extracellular space. Release of

Ca^{2+} from an intracellular storage site simply adds to the overall increase in $[Ca^{2+}]_i$. Once in the cytoplasm, Ca^{2+} binds to calmodulin which, in turn, binds to myosin light chain kinase and the entire complex phosphorylates the thick filaments to activate contraction (Vander, Sherman & Luciano, 1994).

EC-coupling in striated muscle:

In contrast to smooth muscle, in striated muscle release of Ca^{2+} from an intracellular storage site known as the sarcoplasmic reticulum (SR) is essential for contraction (Ríos & Pizarró, 1988; Bers, 1991). S. Ringer (1883) was the first to propose a Ca^{2+} requirement for myocardial contraction, however, the idea that Ca^{2+} was the physiologic factor inducing muscle contraction was only put forth in 1940 by Heilbrunn (Ebashi & Endo, 1964; Ebashi, 1976). Even then, it wasn't until the early 1960's that the Ca^{2+} concept really came into focus (Ebashi, 1961b). Identification of the relaxing factor (Ebashi & Lipmann, 1962) or SR was instrumental in the development of this concept as the strong and rapid Ca^{2+} -binding activity of this organelle provided a means by which Ca^{2+} could be rapidly removed from the myofilaments with each contraction-relaxation cycle (Ebashi, 1961a, 1961b; Ebashi & Endo, 1968; Endo, 1977). Since that time, scientists have been working on determining the steps involved in the transduction of the electrical stimulus to Ca^{2+} release and contraction.

Numerous studies have suggested that the mechanisms by which intracellular Ca^{2+} is released for contraction is different in cardiac as compared to skeletal muscle. However, some studies (Cannell, Berlin & Lederer, 1987; Honore, Adamantidis, Dupuis,

Challice & Guibault, 1987a, 1987b; Reiter, 1988; Isenberg & Wendt-Gallitelli, 1989; Ferrier & Howlett, 1995; Hobai *et al.*, 1997; Howlett, Zhu & Ferrier, 1998; Wasserstrom, 1998) have indicated that EC-coupling in cardiac muscle may include a mechanism which more closely resembles that in skeletal muscle than previously proposed. Additional evidence in favor of similar mechanisms of EC-coupling in striated muscle lies in the fact that the anatomy of both types of muscle is nearly identical.

FOCUS OF THIS STUDY:

A mechanism recently proposed by Ferrier and Howlett (1995), known as the voltage-sensitive release mechanism or VSRM, has functional characteristics which resemble those of the depolarization-induced calcium release (DICR) mechanism in skeletal muscle. A detailed description of the VSRM for cardiac EC-coupling will be provided in the following sections along with a description of the more familiar calcium-induced calcium release (CICR) mechanism. Many of the hypotheses raised in the present study have stemmed from work carried out on skeletal muscle, therefore information on skeletal muscle will be provided throughout for comparison. This study originated with the idea that the pharmacological tools used to study skeletal muscle DICR would be useful to study a similar mechanism in cardiac muscle: the VSRM. Thus, the main focus of this thesis is pharmacological modulation of the VSRM by tetracaine and ryanodine, both of which have been used to study skeletal EC-coupling and interact with both cardiac and skeletal SR Ca^{2+} release channels. One point of caution related to the similarity in EC-coupling in both classes of striated muscle is that, although

recent evidence suggests that EC-coupling mechanisms in skeletal and cardiac muscle share similarities, they are by no means identical. Therefore, it was not expected that pharmacological modulation of the VSRM would be identical to that of the skeletal DICR but rather that similar tools would be useful to evaluate similar mechanisms.

Preparation used to study cardiac EC-coupling:

A single cell preparation is a useful model to study EC-coupling of the heart because many of the properties of the whole organ are maintained in the functioning of single myocytes even though the effects of normal cell-to-cell interactions are absent (Zipes & Jalife, 1990). For example, although removal of the pacemaker of the heart halts spontaneous activity, contraction can be maintained by an external stimulus. Similarly, individual cells in the heart can be stimulated to contract after they are separated from the tissue. This property of single isolated myocytes allows researchers to study the properties of individual cardiac myocytes in the absence of confounding variables that arise with whole organ studies. The work presented in this thesis was carried out on single isolated myocytes, therefore the remainder of this introduction will focus on some of the properties of single cells.

MAJOR CELLULAR STRUCTURES INVOLVED IN EC-COUPLING:

A comprehensive explanation of EC-coupling cannot be given without a brief description of the major cellular structures involved. An important point to note is the numerous similarities in the anatomy of skeletal and cardiac muscle cells. (Refer to

Fawcett & McNutt, 1969 or Bers, 1991 for illustrations).

Sarcolemma/ Transverse tubules:

EC-coupling begins with electrical excitation of the surface membrane or SL which is responsible for propagation of the AP and control of Ca^{2+} ion fluxes into and out of the cell (Wohlfart & Noble, 1982; Bers, 1991). In both skeletal and cardiac muscle this membrane extends into the interior of the muscle fiber forming what is known as the t-system (Endo, 1964; Langer, 1978). An action potential propagating along the outer membrane is carried into the t-system to provide adequate stimulation of the myocyte. The only prominent differences between t-tubules of skeletal and cardiac myocytes is size. Skeletal t-tubules are approximately 40 nm in diameter compared to cardiac t-tubules which are 150-200 nm (Fawcett & McNutt, 1969; Bers, 1991).

Sarcoplasmic reticulum:

Adjacent to the t-system on the inside of skeletal and cardiac cells is the SR, an intracellular membrane bound organelle. The central role of the SR is storage and release of Ca^{2+} for each contraction-relaxation cycle. The SR can be subdivided into several compartments which are continuous with one another. In skeletal muscle these include the terminal cisternae which are the areas of SR that lie adjacent to the t-system, and the longitudinal SR, so named because it is oriented longitudinally over the myofilaments. Two terminal cisternae coupled on either side of a t-tubule form a structure known as a triad (Peachey, 1965). By contrast, the SR of cardiac myocytes is more sparse and the subdivisions are less obvious than those in skeletal myocytes. Thus, cardiac SR tends to form a simple network of tubules of rather uniform size which lie over the myofilaments

and parts of which oppose the t-tubules. The area of SR that is closely associated with the t-tubules in cardiac myocytes is referred to as the junctional SR which forms diads as opposed to the skeletal triads (Fawcett & McNutt, 1969; Bers, 1991).

In skeletal and cardiac muscle, stimulation of the terminal cisternae and junctional SR, respectively, induces opening of Ca^{2+} release channels located there. SR Ca^{2+} release channels have been characterized biochemically (Campbell *et al.*, 1987) and physiologically (Smith, Coronado & Meissner, 1986; Rousseau *et al.*, 1986; Smith, Rousseau & Meissner, 1989) as being Ca^{2+} selective. The characteristics of these channels, which are also known as ryanodine receptors (RyR) due to their high affinity for ryanodine (Fleischer *et al.*, 1985; Inui, Saito & Fleischer, 1987), are similar in both skeletal and cardiac SR (Inui *et al.*, 1988). The channels themselves are homotetramers, each with a membrane spanning region and a region which extends into the cytoplasm. The cytoplasmic extensions, also known as 'foot' projections (Franzini-Armstrong, 1970), span the gap between the SR and the t-tubule membranes (Inui *et al.*, 1988; Bers, 1991). The close anatomical relationship between t-tubules and RyRs was an important factor in the development of the theories of EC-coupling in both cardiac and skeletal muscle.

The terminal cisternae/junctional SR of adjacent t-tubules are joined via the longitudinal SR. The longitudinal SR forms a mesh-like network that surrounds the myofilaments or contractile machinery of the cell (Fawcett & McNutt, 1969; Bers, 1991). Energy dependent pumps located in the membrane of both cardiac and skeletal longitudinal SR are responsible for active reuptake of Ca^{2+} back into the SR from the

cytoplasm. The presence of these pumps along with the anatomical location of this compartment suggest that it is responsible for promoting relaxation of a myocyte following contraction (Bers, 1991). The Ca^{2+} that is taken up into the longitudinal SR probably diffuses to the terminal cisternae/junctional SR to be released again with the next stimulation.

A third compartment of the SR known as corbular SR has all of the same structures as the junctional SR, but differs from the latter in that it sits micrometers away from the SL (Sommer, 1995). The morphology of this compartment suggests that, similar to the terminal cisternae/junctional SR, corbular SR may also be responsible for release of stored Ca^{2+}. However, the mechanism by which release occurs from this site probably differs from that which occurs at the terminal cisternae/junctional SR (Bers, 1991).

Ion pumps and voltage-gated channels:

Within the SL and SR membranes of striated muscle there are many proteins responsible for movement of ions into and out of the cytoplasm of the cell. Some of these proteins form channels through which ions can move while others act as carriers or pumps which actively move ions across the SL. The central pore of many of the channel forming proteins contains several charged amino acids that respond to changes in membrane potential by opening and closing the pore. These channels are referred to as voltage-gated ion channels (Adelman, 1995). Voltage-gated ion channels tend to be selective for a particular ion so Na^+ influx occurs through voltage-gated Na^+ channels while Ca^{2+} and K^+ influx occurs through voltage-gated Ca^{2+} and K^+ channels, respectively. The direction in which ions move through their respective channels depends

on their individual electrochemical gradients. The electrochemical properties of various ions along with the properties of their respective voltage-gated channels determine the shape of an action potential (Hille, 1992; Adelman, 1995).

The SL and SR proteins which act as ion carriers or pumps play an essential role in relaxation of a myocyte by removing Ca^{2+} from the cytoplasm. The majority of the Ca^{2+} removed from the cytoplasm is pumped back into the SR by the action of an energy dependent Ca^{2+} pump known as the Ca^{2+}-ATPase. The action of this pump allows most of the Ca^{2+} released for contraction to be recycled back into the SR so it is available for release with the next stimulation. The Ca^{2+} that is not taken up into the SR is pumped out of the cell by the action of two SL pumps. One of these is a Ca^{2+} -ATPase pump which is analogous to the SR Ca^{2+} -ATPase (Schatzmann, 1989) and the other is a sodium-calcium exchanger ($NaCa_{EX}$) (Reuter & Seitz, 1968; Fozzard, 1991). The $NaCa_{EX}$ is an electrogenic, energy independent pump that moves $3Na^+$ ions with every Ca^{2+} ion (Sheu & Fozzard, 1982). The majority of the Ca^{2+} lost to the extracellular space goes out via the $NaCa_{EX}$ which removes approximately 75 % compared to 25 % which goes out via the SL Ca^{2+}-ATPase (Bers *et al.*, 1989; Bers, 1991).

Along with the Ca^{2+} regulating pumps there are pumps responsible for setting up the ionic gradients across cell membranes at any given time. A Na^+/K^+-ATPase pump is largely responsible for maintaining the polarity of the SL membrane at rest. The Na^+/K^+ -ATPase uses the energy from ATP hydrolysis to move Na^+ out of the cell and K^+ in, against their respective concentration gradients (Berne & Levy, 1997).

Contractile proteins or myofilaments:

The proteins responsible for contraction of striated muscle are the myofilaments which are the end effectors of EC-coupling (Bers, 1991). In both skeletal and cardiac muscle, the myofilaments are arranged in bundles and are composed of thick and thin filaments. The proteins which make up the thin filaments are troponin, tropomyosin and actin. The thick filaments are composed of myosin proteins which have two regions, a tail region and a globular head region. During relaxation, association of troponin and tropomyosin with actin prevents actin from interacting with the thick myosin filaments. Binding of Ca^{2+} to troponin results in a conformational change in the troponin-tropomyosin complex which allows actin to interact with the globular head regions of the myosin filaments. Interaction of these two proteins promotes hydrolysis of ATP which results in rotation of the myosin head and sliding of the actin filament. This process, known as the sliding filament theory, is what ultimately causes the cell to shorten or contract (Huxley, 1969; Huxley & Simmons, 1971; Bers, 1991).

Mitochondria:

The high energy demands of the heart are met by the numerous mitochondria that lie just under the SL and between adjacent myofibrils (Fawcett & McNutt, 1969; Bers, 1991). By comparison, skeletal muscle is capable of anaerobic metabolism and can build up a substantial oxygen debt, therefore the mitochondria are smaller and less numerous in this tissue. In both tissues, this organelle is responsible for production of ATP via oxidative phosphorylation. Early studies with isolated mitochondria indicated that this organelle was capable of accumulating large concentrations of Ca^{2+} (Lehninger, Carafoli

& Rossi, 1967; Carafoli & Lehninger, 1971). This lead to the suggestion that the mitochondria functioned as a storage site for activator Ca^{2+} (Lehninger, 1974; Carafoli, 1975). However, it was later discovered that, under physiological conditions, the rate of Ca^{2+} removal by the mitochondria is slow in comparison to that of the SR Ca^{2+} ATPase and SL $NaCa_{EX}$ (Bers & Bridge, 1989). Thus, it is unlikely that this organelle is involved in beat-to-beat regulation of $[Ca^{2+}]_i$. However, under pathological conditions it is possible that the mitochondria provide a Ca^{2+} sink to protect the cell from the detrimental effects of Ca^{2+} overload (Reimer & Jennings, 1986).

CARDIAC CALCIUM HOMEOSTASIS:

Conduction of an AP along the SL membrane and the T-system results in an increase in $[Ca^{2+}]_i$ via opening of RyRs. The exact mechanism by which the electrical stimulus causes opening of RyRs in the junctional SR is not known although two main hypotheses exist. One mechanism begins with transsarcolemmal influx of Ca^{2+} through voltage-gated Ca^{2+} channels. This influx of Ca^{2+} is thought to act as a trigger for SR release by binding to RyRs, causing them to open and release stored Ca^{2+}. The second mechanism involves a direct coupling between the SL membrane and the RyRs such that stimulation of the SL directly opens RyRs to release stored Ca^{2+}. The released Ca^{2+} then binds to the myofilaments to initiate contraction. Although most of the released Ca^{2+} is taken up into the longitudinal SR by Ca^{2+} -ATPase during relaxation, some of it is lost to the extracellular space by the action of the SL Ca-ATPase and $NaCa_{EX}$. Once in the lumen of the longitudinal SR, Ca^{2+} diffuses to the junctional SR to be released again by

the next stimulus. Thus, Ca^{2+} is an essential part of cardiac EC-coupling and is rapidly recycled within the cell with each contraction-relaxation cycle (Chapman, 1983; Bers, 1991). A similar process is thought to exist in skeletal muscle whereby most of the Ca^{2+} is recycled within the cells (Melzer, Herrmann-Frank & Lüttgau, 1995).

Although the above outline introduces some of the major structures involved in cardiac EC-coupling it is by no means complete. It does, however, provide a framework for a more in-depth discussion of the theories of EC-coupling in cardiac muscle.

MECHANISMS OF EC-COUPLING:

The anatomical similarities between skeletal and cardiac muscle suggest that findings in one may be applicable to the other. Thus, many of the mechanisms proposed for cardiac EC-coupling have stemmed from studies conducted on skeletal muscle. For example, the predominant mechanism proposed for cardiac EC-coupling is CICR, a mechanism which was originally described for EC-coupling in skeletal muscle. In skeletal muscle, CICR has been replaced by DICR, which proposes a direct connection between the SL and SR Ca^{2+} release channels such that depolarization of the SL directly opens RyRs in the SR (Melzer, Herrmann-Frank & Lüttgau, 1995). A mechanism of CICR is still thought to exist in skeletal muscle, however, the contribution of this mechanism to EC-coupling is thought to be secondary to DICR in that CICR simply amplifies DICR.

When one considers the history of EC-coupling in striated muscle, it is not surprising that recent evidence suggests that a mechanism analogous to skeletal muscle

DICR also exists in cardiac muscle (VSRM). Because of the close similarity between skeletal and cardiac muscle, a discussion of cardiac EC-coupling would not be complete without a discussion of skeletal EC-coupling. Thus, the following sections contain a brief description of the proposed mechanisms for EC-coupling in striated muscle beginning with skeletal muscle DICR.

a) Skeletal muscle EC-coupling:

Early studies with skeletal muscle suggested that the mechanism for EC-coupling involved calcium-induced calcium release (CICR) whereby transsarcolemmal influx of Ca^{2+} stimulates SR release of Ca^{2+} (Ford & Podolsky, 1970; Endo, Tanaka & Ogawa, 1970). However, results from studies in which other divalent ions, such as Ba^{2+}, were substituted for extracellular Ca^{2+} illustrated contraction in the absence of inward Ca^{2+} current. This lead to the hypothesis that the Ca^{2+} channel was stimulating SR release, not actual current through this channel. Today it is believed that in skeletal muscle release of Ca^{2+} actually involves a direct connection between the t-tubule membrane and Ca^{2+} release channels of the SR (Block *et al.*, 1988). Voltage-gated Ca^{2+} channels located in the SL of t-tubules are believed to act as voltage sensors (Ríos & Brum, 1987; Agnew, 1988; Block *et al*, 1988; Wagenknecht *et al.*, 1989).

Striated myocytes have both a transient or T-type Ca^{2+} current and a long lasting or L-type Ca^{2+} current. The L-type Ca^{2+} channels, also known as dihydropyridine receptors (DHPR) because of their high affinity for dihydropyridines, are thought to be responsible for stimulating SR Ca^{2+} release in skeletal muscle. In this scheme,

depolarization of the SL causes movement of a charged segment which lines the pore of these channels. This charge movement in some way controls SR Ca^{2+} release, possibly via conformational changes of linked proteins. The result is opening of RyRs and release of stored Ca^{2+} (Ríos & Pizarró, 1991; Lamb & Stephenson, 1992; Melzer, Herrmann-Frank & Lüttgau, 1995) upon depolarization. This process has been called DICR because depolarization of the SL directly opens SR RyRs (Endo, 1977; Fabiato & Fabiato, 1977).

Aside from their role as voltage-sensors for DICR, skeletal muscle L-type Ca^{2+} channels also allow transsarcolemmal influx of extracellular Ca^{2+}. The concentration of Ca^{2+} that enters via these channels is not sufficient to directly activate contraction nor does it play a direct role in EC-coupling in skeletal muscle (Brum, Stefani & Ríos, 1986). Aside from Ca^{2+} influx, L-type Ca^{2+} channels also have an extracellular binding site for cations which must be occupied for DICR to function. It is believed that, under normal conditions, Ca^{2+} occupies this site although studies have shown that many other divalent, as well as monovalent ions, will substitute for Ca^{2+} binding (Brum *et al.*, 1987; Pizarró *et al.*, 1989).

The anatomical location of RyRs with respect to DHPRs in t-tubules illustrates that there is not a one-to-one relationship between these proteins. Thus, some Ca^{2+} channels in the SR are not aligned with voltage sensors in the SL (Block *et al*, 1988). Channels that aren't aligned are thought to be opened and closed by Ca^{2+} released from neighboring SR channels, via CICR (Ríos & Pizarró, 1988). Thus, depolarization of the SL would directly open RyRs which are physically linked to L-type Ca^{2+} channels (DICR). Some of the Ca^{2+} released from these RyRs would then bind to neighboring

RyRs causing them to open and release Ca^{2+} (CICR). In this way, CICR may act as an amplifier or multiplier for DICR (Ríos & Pizarró, 1988; Jacquemond *et al.*, 1991; Ríos & Pizarro, 1991; Györke & Palade, 1993; Melzer, Herrmann-Frank & Lüttgau, 1995; Berridge, 1997). SR Ca^{2+} released by either mechanism, CICR or DICR, causes a rise in $[Ca^{2+}]_i$ and this Ca^{2+} then binds to the myofilaments for contraction. Relaxation is achieved when the released Ca^{2+} is pumped back into the SR via the Ca-ATPase. Thus, in skeletal muscle most of the activating Ca^{2+} cycles intracellularly (Langer, 1968).

b) Cardiac EC-coupling:

In heart, as in skeletal muscle, stimulation of the extracellular membrane must induce a rise in $[Ca^{2+}]_i$ for contraction to occur. Although influx of Ca^{2+} through voltage-dependent Ca^{2+} channels can directly stimulate contraction in some species (Lewartowski *et al.*, 1990; Sham, Cleeman & Morad, 1992), in many other species Ca^{2+} influx is inadequate (only 10-30 % of the amount required) for direct activation of myofilaments (Frank, 1980; Sutko & Willerson, 1980; Morad & Cleeman, 1987). In general, the contribution of Ca^{2+} influx to the increase in $[Ca^{2+}]_i$ varies among different species and tissues (Mitchell *et al.*, 1987; Bers, 1991; Wang, Winka & Langer, 1993; Terracciano & MacLeod, 1997). In many animals, the rise in $[Ca^{2+}]_i$ that occurs in EC-coupling is mostly the result of SR release as opposed to transsarcolemmal influx. The question that remains is by what mechanism does an AP stimulate SR release of Ca^{2+}?

Identification of DICR as the mechanism for EC-coupling in skeletal muscle led researchers to hypothesize that a similar mechanism probably exists in the other striated

muscle (Fuchs, 1974; Ebashi, 1976). In agreement with the existence of a voltage dependent mechanism for cardiac EC-coupling, early studies demonstrated initiation of contractions at very positive potentials in the absence of inward Ca^{2+} current (Fozzard & Hellam, 1968; Beeler & Reuter, 1970; Ochi & Trautwein, 1971; Trautwein, McDonald & Tripathi, 1975; Isenberg *et al.*, 1985; Reiter, 1988). However, experiments conducted with mechanically skinned myocytes provided strong support for a mechanism of EC-coupling in which Ca^{2+} itself stimulated release of Ca^{2+} from the SR (Fabiato & Fabiato, 1977). These results led researchers to focus on CICR as the mechanism of EC-coupling in cardiac myocytes in the years that followed. Thus, a discussion of cardiac EC-coupling should begin with a review of CICR followed by consideration of the more recently described VSRM. This section includes a short discussion of possible reasons why earlier studies did not detect a VSRM in cardiac myocytes.

i) Calcium-induced calcium release (CICR) (figure 1-1):

In cardiac muscle CICR begins with influx of extracellular Ca^{2+} either through voltage-gated channels or $NaCa_{EX}$ pump in the SL. The Ca^{2+} that enters the myocyte then binds to and induces opening of RyRs within the SR membrane (Inui, Saito & Fleischer, 1987; Lai *et al.*, 1988; Rardon *et al.*, 1989; Lai & Meissner, 1989; Lindsay & Williams, 1991; Ashley & Williams, 1990; Callewart, 1992). This results in a flood of Ca^{2+} into the cytoplasmic space for activation of myofilaments. Ca^{2+} substitution studies carried out with mechanically skinned myocytes indicated that Ca^{2+} release could not be triggered by most other ions, including H^+, K^+, Na^+, and Mg^{2+} (Fabiato, 1985a). Thus, in contrast to skeletal muscle, cardiac muscle has a requirement for extracellular Ca^{2+} in order to

Fig. 1-1. Proposed mechanisms of cardiac EC-coupling. Transsarcolemmal Ca^{2+} influx through voltage gated Ca^{2+} channels (L and/or T-type Ca^{2+} channels) or by $NaCa_{EX}$ working in reverse-mode can stimulate opening of SR RyRs via CICR. As well, depolarization of the SL membrane also can cause SR RyRs to open in the absence of Ca^{2+} influx. This mechanism, which is referred to as a VSRM, is illustrated by the loop connecting a SL voltage sensor to a RyR in the SR. As illustrated by the '?', the exact nature of this mechanism is unknown. The released SR Ca^{2+} binds to the myofilaments to activate contraction. Removal of Ca^{2+} from the cytosol by SR reuptake or efflux to the extracellular space results in relaxation. (Modified from Howlett & Ferrier, 1997)

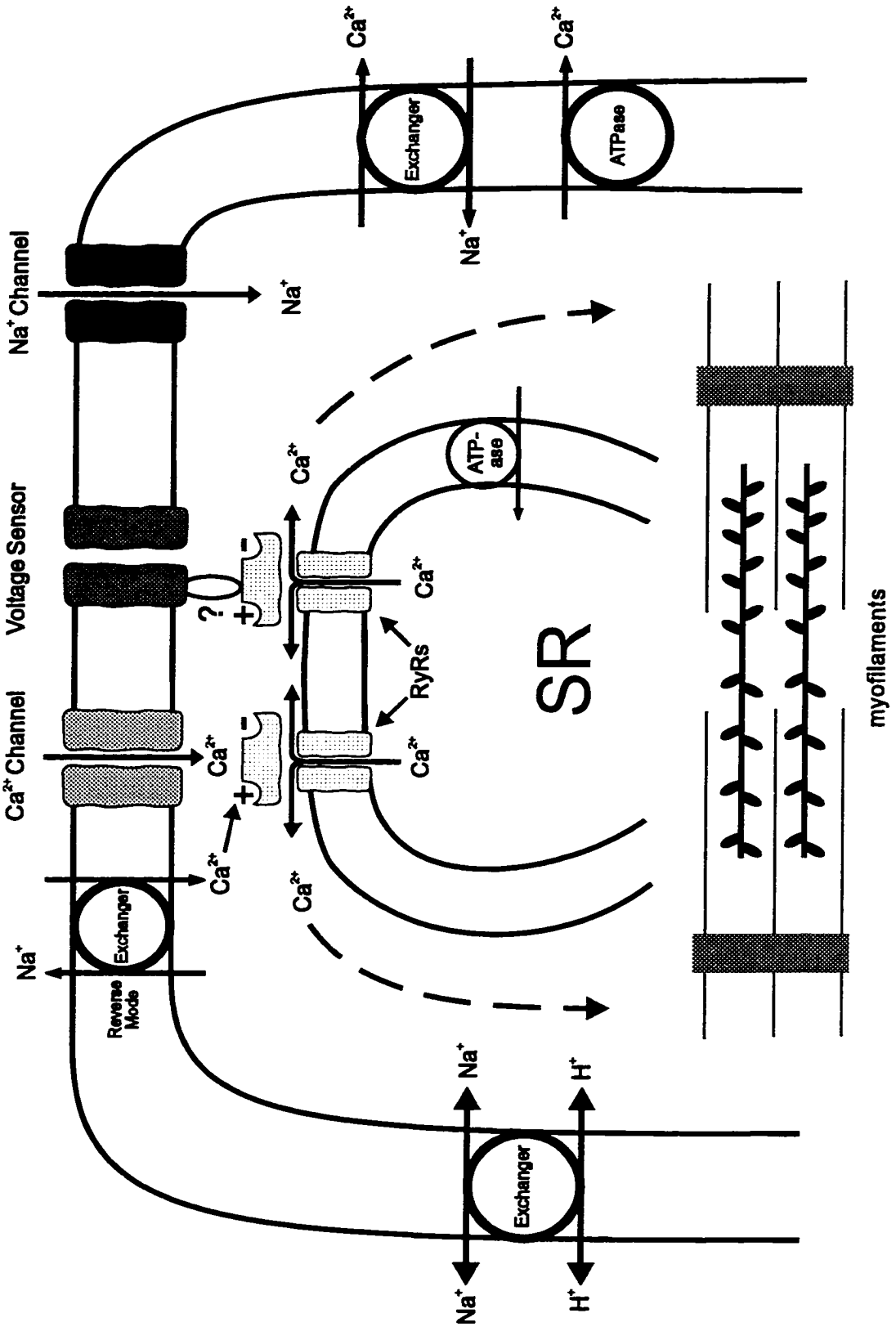

Ca2+
Ca2+
Exchanger
ATPase
Na+
Na+ Channel
Na+
Voltage Sensor
ATP-ase
Ca2+
Ca2+
?
+
-
RyRs
SR
Ca2+ Channel
Ca2+
Ca2+
Ca2+
Ca2+
myofilaments
Na+
Exchanger
Reverse Mode
Na+
H+
Exchanger
Na+
H+

Figure 1-1

contract. This has led to the hypothesis that Ca^{2+} itself is the trigger for further release of Ca^{2+} from the SR (Fabiato & Fabiato, 1975, 1977; Fabiato, 1985a, 1985b, 1985c). This mechanism, which is referred to as calcium-induced calcium release or CICR, is believed to be the primary mechanism by which cardiac contraction is elicited (London & Krueger, 1986; Barcenas-Ruiz & Wier, 1987; Beuckelman & Wier, 1988; duBell & Houser, 1989; Näubauer *et al.*, 1989; Valdeolmillos *et al.*, 1989; Niggli & Lederer, 1990; Bers, 1991; Cleeman & Morad, 1991; Stern & Lakatta, 1992; Niggli & Lipp, 1995).

Given the importance of extracellular Ca^{2+} in cardiac contraction, it is not surprising that cardiac cells have developed numerous mechanisms by which Ca^{2+} entry can occur. The SL of cardiac cells contain two types of voltage-gated Ca^{2+} channels which provide long lasting (L-type) and transient (T-type) currents (Bean, 1985; Mitra & Morad, 1986), either of which might initiate CICR. As well, under certain conditions, Ca^{2+} can enter a cell via reverse-mode $NaCa_{EX}$. Each of these Ca^{2+} influx pathways will be discussed in the following paragraphs in relation to a mechanism of CICR.

a) $I_{Ca\text{-}L}$ -induced calcium release:

Studies in intact cardiac myocytes indicated that substitution of extracellular Ca^{2+} with various other ions prevented activation of contraction (Näubauer *et al.*, 1989). These results combined with similar experiments carried out in skinned cardiac myocytes led to the conclusion that Ca^{2+} current was the trigger for SR Ca^{2+} release. Today most concepts of EC-coupling in the heart have focused on CICR whereby Ca^{2+} entry through L-type Ca^{2+} channels stimulates release of Ca^{2+} from the SR (Wier, 1991; Fabiato, 1985). The magnitude and voltage dependence of the L-type Ca^{2+} current correlate well with the

magnitude and voltage dependence of cardiac contractions and transients (London & Krueger, 1986; Barcenas-Ruiz, Beuckelmann & Wier, 1987; Cannell, Berlin & Lederer, 1987; Beuckelmann & Wier, 1988; Callewaert, Cleeman & Morad, 1988; duBell & Houser, 1989; Arreola *et al.*, 1991; Cleeman & Morad, 1991; Callewaert, 1992). For example, measurement of $[Ca^{2+}]_i$ transients in guinea-pig and rat myocytes produced a bell-shaped voltage dependence on membrane potential which reflected that of inward Ca^{2+} current ($I_{Ca\text{-}L}$) (Beuckelmann & Wier, 1988; Callewaert, Cleeman & Morad, 1988; Cannell, Berlin & Lederer, 1987). At sufficiently negative or positive membrane potentials, in the absence of inward $I_{Ca\text{-}L}$, the cytosolic Ca^{2+} transient was abolished. Similarly, depolarization of the cell membrane beyond the reversal potential of $I_{Ca\text{-}L}$ did not produce a Ca^{2+} transient, while repolarization from this potential, which produces an inward Ca^{2+} -tail current, did (Barcenas-Ruiz & Wier, 1987). As well, exposure of electrically stimulated cells to agents which depleted SR Ca^{2+} stores or blocked L-type Ca^{2+} channels resulted in inhibition of contraction. All of these results are in agreement with a mechanism of CICR triggered by $I_{Ca\text{-}L}$.

It appeared from these studies that the mechanism of cardiac EC-coupling had been identified as CICR triggered by $I_{Ca\text{-}L}$. However, there are several paradoxes inherent in this mechanism, the most obvious being that a high gain process of CICR should exhibit marked positive feedback. The concentration of Ca^{2+} released from the SR is far greater than that entering the cell through L-type Ca^{2+} channels, thus one would assume that such a process would produce a regenerative release of SR Ca^{2+} until all stores are depleted or some other process stops the release. In an attempt to explain this paradox,

Niggli and Lederer (1990) described a 'local control' theory for CICR in which the open probability of cardiac RyRs is regulated by Ca^{2+} influx through SL Ca^{2+} channels located nearby. In agreement with a 'local control' theory, L-type Ca^{2+} channels were found to be concentrated mainly in the junctional structures close to the Ca^{2+} release pathway (Wibo & Godraind, 1991). In this scheme, activation of RyRs would be regulated by Ca^{2+} influx through SL Ca^{2+} channels in the immediate vicinity of the RyR rather than by mean changes in $[Ca^{2+}]_i$ (Niggli & Lederer, 1990; Lopez-Lopez *et al.*, 1995; Lederer, Niggli & Hadley, 1990; Cheng, Lederer & Cannell, 1993; Cannell, Cheng & Lederer, 1995; Santana *et al.*, 1996).

The recent discovery of microscopic elementary Ca^{2+} release events, referred to as 'calcium sparks', gives some insight into the gating of RyRs, as well as support for a local control theory for CICR (Cheng, Lederer & Cannell, 1993). 'Calcium sparks' are thought to result from the activation of one or a small number of RyRs acting in concert to produce a small flux of Ca^{2+} from the SR to the cytosol. These release events can occur spontaneously in quiescent myocytes or by activation of SL $I_{Ca\text{-}L}$. Under both conditions, the opening of a single SL Ca^{2+} channel is believed to be sufficient for activation of one or a cluster of RyRs which are responsible for the 'calcium spark' (Santana *et al.*, 1996). The time course of 'calcium spark' activity was found to be independent of the duration of $I_{Ca\text{-}L}$ suggesting intrinsic gating of RyRs independent of $[Ca^{2+}]_i$ which would allow for the stability of CICR (Cannell, Cheng & Lederer, 1995).

The observation of spatial non-uniformities in $[Ca^{2+}]_i$ during cardiac EC-coupling provided further insight into 'calcium spark' activity and regulation of CICR (Cannell,

Cheng & Lederer, 1994). Calcium sparks observed randomly throughout a cell are responsible for the initial increase in $[Ca^{2+}]_i$ measured with fluorescent dyes. As the number of Ca^{2+} sparks increases the overall $[Ca^{2+}]_i$ increases which produces the peak of the transient. Thus, a Ca^{2+} transient is generated by several 'calcium sparks' occurring at the same time. This spatial non-uniformity of Ca^{2+} transients fits with the idea of a local control theory for EC-coupling and helps to explain the stability of this process, as SR Ca^{2+} release is graded by single SL Ca^{2+}-channel openings rather than by bulk changes in $[Ca^{2+}]_i$. As well, these studies provide an explanation for the observation that Ca^{2+} currents are not all equally effective in eliciting SR release (variable gain) as release is dictated by local changes in Ca^{2+} not by macroscopic Ca^{2+} current (Cannell, Berlin & Lederer, 1987; Niggli & Lederer, 1990; Wier *et al.*, 1994).

Inactivation / adaptation of SR RyRs:

Studies with 'calcium sparks' indicate that CICR is not a regenerative cycle, but rather is stabilized by the intrinsic gating of SR RyR which appear to close over time in spite of the continued presence of Ca^{2+}. The exact mechanism by which this occurs is not well known although several hypotheses have been proposed. The two most popular hypotheses today involve mechanisms referred to as adaptation and inactivation of RyRs (Chamberlain, Volpe & Fleischer, 1984; Fabiato, 1985c; Meissner & Henderson, 1987; Györke & Fill, 1993; Yasui, Palade & Györke, 1994; Valdivia *et al.*, 1995; Velez *et al.*, 1997; Laver & Lamb, 1998; Sham *et al.*, 1998; Lukyanenko, Wiesner & Györke, 1998). Adaptation involves a process whereby RyRs adapt to an increase in $[Ca^{2+}]_i$ such that,

over time, the open probability of these channels decreases in spite of the $[Ca^{2+}]_i$ (Györke & Fill, 1993; Yasui, Palade & Györke, 1994; Valdivia *et al.*, 1995; Velez *et al.*, 1997). On the other hand, inactivation involves a process whereby the RyRs close and are not able to be opened again by a further increase in $[Ca^{2+}]_i$ (Chamberlain, Volpe & Fleischer, 1984; Meissner & Henderson, 1987; Laver & Lamb, 1998; Sham *et al.*, 1998).

b) $I_{Ca\text{-}T}$ -induced Ca^{2+} release:

Transsarcolemmal Ca^{2+} influx in cardiac myocytes can occur through voltage-dependent opening and closing of SL T-type Ca^{2+} channels (Vassort & Alvarez, 1994). Cardiac T-type Ca^{2+} current ($I_{Ca\text{-}T}$) is smaller in amplitude and available at more negative potentials compared to the L-type current ($I_{Ca\text{-}L}$) (Bean, 1985; Nilius *et al.*, 1985; Mitra & Morad, 1986; Hirano, Fozzard & January, 1989; Tseng & Boyden, 1989; Zhou & Lipsius, 1994). The distribution of $I_{Ca\text{-}T}$ in heart varies with cell type, with a higher density of these channels found in atrial and Purkinje cells compared to ventricular cells (Hagiwara, Irisawa & Kameyama, 1988; Tseng & Boyden, 1989; Zhou & Lipsius, 1994). However, this distribution has been found to change under certain pathological conditions whereby the density in ventricular myocytes is increased compared to normal (Nuss & Houser, 1993; Sen & Smith, 1994). As well, during development, T-type Ca^{2+} channels tend to be more widely distributed throughout the heart (Xu & Best, 1992). Thus, it seems likely that Ca^{2+} influx through T-type Ca^{2+} channels could play a role in EC-coupling in early development and in diseased hearts but might not contribute to CICR under normal conditions. However, studies in guinea-pig ventricular cells (Sipido, Carmeliet & Van de

Werf, 1998) as well as canine Purkinje cells (Zhou & January, 1998) have demonstrated that $I_{Ca\text{-}T}$ can, in fact, induce contraction by way of CICR under normal conditions. A comparison of the ability of Ca^{2+} influx through both T- and L-type Ca^{2+} channels to trigger SR release indicated that Ca^{2+} entering via the T-type channel was less efficient at transducing the signal to the RyRs (Sipido, Carmeliet & Van de Werf, 1998). In the absence of $I_{Ca\text{-}L}$, Ca^{2+} influx through T-type channels produced smaller amplitude contractions, with slower kinetics and greater delay in activation (Zhou & January, 1998). Thus, although influx of Ca^{2+} through T-type Ca^{2+} channels can initiate contraction by CICR, this mechanism likely plays a minor role in cardiac EC-coupling under normal conditions (Sipido, Carmeliet & Van de Werf, 1998). It's possible, however, that CICR triggered by $I_{Ca\text{-}T}$ plays a more important role in EC-coupling during development (Xu & Best, 1992) and under pathological conditions where the density of $I_{Ca\text{-}T}$ is increased (Nuss & Houser, 1993). In these situations, $I_{Ca\text{-}T}$ may act as either a back-up mechanism or an amplifier for CICR triggered by $I_{Ca\text{-}L}$.

c) $NaCa_{EX}$ -induced calcium release:

The $NaCa_{EX}$ co-transporter was originally described by Reuter & Seitz (1968) and is now thought to be the major mechanism for cellular Ca^{2+} efflux (Fozzard, 1991). During contraction the $NaCa_{EX}$ uses the energy of the Na^+ and Ca^{2+} electrochemical gradients to pump Ca^{2+} out of the myocyte in exchange for entry of Na^+ (Sheu & Fozzard, 1982). Under certain physiological conditions $NaCa_{EX}$ works in reverse, that is, it pumps Ca^{2+} into the cell in exchange for Na^+ moving out (Mullins, 1979). In 1981, Mullins

illustrated that the ionic gradients during the plateau phase of an action potential favored Ca^{2+} entry via $NaCa_{EX}$ (Fozzard, 1991). This finding was confirmed by Barcenas-Ruiz *et al* and Brill *et al* (1987) who also showed that Ca^{2+} entry via reverse mode $NaCa_{EX}$ could occur during the plateau of the AP as well as with prolonged depolarizations (>300 msec) (Fozzard, 1991). The idea that Ca^{2+} influx via this mechanism could trigger SR Ca^{2+} release was first put forth by Berlin *et al* (1987). More recently it has been demonstrated that an increase in the subsarcolemmal Na^{+} concentration in the environment of the exchanger also can cause influx of Ca^{2+} via reverse mode $NaCa_{EX}$ (Leblanc & Hume, 1990).

Once it was determined that Ca^{2+} could enter a cell by way of the $NaCa_{EX}$ working in reverse-mode, researchers began to examine the implications this might have in cardiac EC-coupling. It was reported that, similar to Ca^{2+} influx through voltage-gated channels, Ca^{2+} entry via the $NaCa_{EX}$ also can initiate contraction by way of CICR (Terrar & White, 1989; Leblanc & Hume, 1990; Schuttler *et al.*, 1991; Nuss & Houser, 1992; Levi, Brooksby & Hancox, 1993; Levesque, Leblanc & Hume, 1994; Levi *et al.*, 1994; Lipp & Niggli, 1994; Vornanen, Shepherd & Isenberg, 1994; Hancox & Levi, 1995; Wasserstrom & Vites, 1996). In this scheme, influx of Ca^{2+} via reverse mode $NaCa_{EX}$ would stimulate the SR Ca^{2+} release channels to open resulting in release of stored Ca^{2+}. This Ca^{2+} would then bind to the myofilaments to elicit a contraction. However, aside from a mechanism of CICR, Ca^{2+} influx through reverse-mode $NaCa_{EX}$ also was reported to induce contraction via direct activation of myofilaments (Sham, Cleeman & Morad., 1992; Schuttler *et al.*, 1991). Evidence for a mechanism of direct activation came from

measurements of Ca^{2+} transients. Stimulation of Ca^{2+} transients via reverse mode $NaCa_{EX}$ produced slowly rising transients at positive membrane potentials (Barcenas-Ruiz & Wier, 1987; Barcenas-Ruiz, Beuckelmann & Wier, 1987) which were resistant to Ca^{2+} channel block by verapamil and SR release channel block by ryanodine. As well, Beuckelmann & Wier (1989) showed that these transients had an exponential voltage dependence and were sensitive to changes in intracellular Na^{+}, and Ni^{2+}, a blocker of $NaCa_{EX}$ current (Kimura, Miyamae & Noma, 1987). In agreement with Ca^{2+} transient studies, slow ramp-like contractions also were reported to be initiated in the presence of caffeine or ryanodine (Sham, Cleeman & Morad, 1992; Schuttler *et al.*, 1991). These results confirmed that, under some conditions, Ca^{2+} transients could be initiated by influx of Ca^{2+} through the $NaCa_{EX}$ working in reverse-mode.

By contrast, further studies examining the role of I_{Na} in reverse mode $NaCa_{EX}$ reported conflicting results compared to earlier work (Sham, Cleeman & Morad, 1992; Bouchard, Clark & Giles, 1993; Sipido, Carmeliet & Pappano, 1995; Cannell *et al.*, 1996). As well, others have reported that the contribution of the $NaCa_{EX}$ to EC-coupling is species and condition (membrane potential, $[Ca^{2+}]_i$, a^i_{Na}) dependent (Wier, 1991). The efficiency of reverse mode $NaCa_{EX}$ as a trigger for SR Ca^{2+} release has been reported to be lower than that for $I_{Ca\text{-}L}$ (Sham, Cleeman & Morad, 1995; Adachi Akahane *et al.*, 1997; Sipido, Maes & Van de Werf, 1997) which suggests that CICR via reverse mode $NaCa_{EX}$ is not a major contributor to cardiac EC-coupling. By contrast, Litwin *et al* (1998) have reported that $NaCa_{EX}$ can in fact induce SR Ca^{2+} release and that it may act as an amplifier for $I_{Ca\text{-}L}$ triggered SR release. Clearly, there have been many conflicting

results with regard to the ability of $NaCa_{EX}$ to induce SR Ca^{2+} release and contraction in cardiac myocytes. Thus, the physiological significance of this mechanism remains to be determined.

Results which do not support the idea of a single mechanism for cardiac EC-coupling:

Since Fabiato's work illustrating a mechanism of CICR in cardiac myocytes many studies have provided evidence in support of this mechanism. These studies have led scientists to believe that CICR is the primary mechanism for EC-coupling in the heart. Aside from experimental studies, evaluation of CICR as the primary mechanism for cardiac EC-coupling can be carried out by mathematical modeling. The basis for mathematical models comes from previous experimental findings as well as some assumptions on how the mechanism works. A model of CICR which mimics or predicts experimental findings would provide strong support in favor of this mechanism. However, studies have shown that it is difficult to model the graded Ca^{2+} release observed experimentally with only a CICR mechanism (Stern, 1992). In fact, one of the most highly developed models of CICR proposed by Hilgemann and Noble (1987) was not able to simulate the experimental results of Fabiato without including a system of direct voltage dependent activation (Stern, 1992). Thus, rather than providing evidence for CICR, these findings strongly suggest that this mechanism alone cannot adequately explain cardiac EC-coupling. As well, these models provide evidence in favor of a component of EC-coupling which is directly voltage dependent.

Many experimental findings also suggested that SR Ca^{2+} release might be directly

regulated by membrane potential. In 1987, Cannell *et al.* reported that the voltage threshold for the rise in $[Ca^{2+}]_i$ was actually more negative than that for Ca^{2+} current, suggesting that the former was not dependent on the latter. As well, results from this study illustrated that the maximal rise in $[Ca^{2+}]_i$ occurred at potentials where Ca^{2+} current was only half maximal and further increases in current did not produce greater increases in $[Ca^{2+}]_i$. Thus, the observed changes in $[Ca^{2+}]_i$ did not occur in tandem with transsarcolemmal Ca^{2+} influx. Their conclusions were two fold, one being that $I_{Ca\text{-}L}$ may normally be supramaximal for stimulating SR Ca^{2+} release, but secondly that it was possible that there was also some direct effect of membrane potential on initiation of contraction (Cannell, Berlin & Lederer, 1987). In agreement with these findings, studies measuring Ca^{2+} tail currents and the transients elicited by them also did not fit with the idea of CICR because the amplitude of the transients did not correlate with that of the Ca^{2+} -current tail (Beuckelmann & Wier, 1988; Cannell, Berlin & Lederer, 1987; Callewaert, Cleeman & Morad, 1988). If contraction occurred by way of CICR one would expect that, once triggered, the rise in $[Ca^{2+}]_i$ would continue independently of changes in $I_{Ca\text{-}L}$. However, studies which utilized short depolarizing steps found that early repolarization reduced the $[Ca^{2+}]_i$ (Cannell, Berlin & Lederer, 1987; Barcenas-Ruiz & Wier, 1987; Fabiato, 1985).

Aside from indirect evidence for a mechanism of cardiac EC-coupling which was regulated by membrane potential, some studies suggested a physical link between the SL Ca^{2+} channel and the SR RyR (Cohen & Lederer, 1988; Hyrshko, Stiffel & Bers, 1990; McCall *et al.*, 1996). For example, ryanodine, a plant alkaloid known to bind specifically

to the SR Ca^{2+} release channels, was found to cause a shift in the steady-state inactivation of $I_{Ca\text{-}L}$ by 13 mV positive (Cohen & Lederer, 1988). This effect was not blocked by buffering Ca^{2+} with EGTA or BAPTA suggesting it was a direct effect of ryanodine on the Ca^{2+} current rather than an indirect effect resulting from changes in $[Ca^{2+}]_i$. It was concluded that the ryanodine binding site had a physical connection to the SL L-type Ca^{2+} channels (Cohen & Lederer, 1988). As well, the L-type Ca^{2+} channel agonist Bay K 8644 was reported to stimulate ryanodine binding to SR RyRs in which SL-RyR junctions remained intact, but not after physical disruption, which suggests this effect requires a mechanical link between these two proteins (McCall *et al.*, 1996). However, there is no direct evidence that SL L-type Ca^{2+} channels are aligned with SR RyR in cardiac myocytes to allow for a direct connection. Further, Bers & Stiffel (1993) have reported that the number of RyR greatly exceeds the number of L-type Ca^{2+} channels (10:1) which challenges the idea of a direct connection between these two proteins. If there is a direct connection only one out of every 6 or 7 RyR would be stoichiometrically associated with a SL L-type Ca^{2+} channel (Bers & Stiffel, 1993).

Although evidence to date illustrates that a mechanism of CICR does exist for cardiac EC-coupling, there are also numerous findings that CICR alone can not explain. These studies indicate that the possibility of a direct effect of membrane potential on SR Ca^{2+} release cannot be ruled out. Evidence for the existence of both CICR and DICR in skeletal muscle EC-coupling strongly suggests that this also might be the case for cardiac muscle. Indeed, recent studies in ventricular myocytes provide new evidence in support of a second component of cardiac EC-coupling which involves direct regulation of

contraction by membrane potential. This mechanism is referred to as the voltage-sensitive release mechanism or VSRM.

ii) Voltage-sensitive release mechanism (VSRM) (figure 1-1):

Studies in isolated guinea-pig ventricular myocytes have provided evidence for a second mechanism of EC-coupling (Ferrier & Howlett, 1995) which may provide answers for some of the discrepancies noted above. This mechanism is believed to be a voltage-sensitive release mechanism, which couples SL depolarization to SR Ca^{2+} release (Ferrier & Howlett, 1995; Hobai *et al*, 1997; Howlett & Ferrier, 1997; Howlett, Zhu & Ferrier, 1998). Activation of the VSRM produces sigmoidal contraction-voltage (CV) relationships which reach a peak at approximately -10 mV and remain maximal at very positive potentials (+80 mV) (Howlett, Zhu & Ferrier, 1998; Ferrier *et al.*, 1998; Ferrier & Howlett, 1995). In contrast, contractions elicited in the absence of activation of the VSRM produce a bell-shaped CV relationship which reflects the current-voltage (IV) relationship of $I_{Ca\text{-}L}$, and thus represents CICR (Howlett, Zhu & Ferrier, 1998; Ferrier *et al.*, 1998; Ferrier & Howlett, 1995). Studies with verapamil, nifedipine (Ferrier & Howlett, 1995) and Cd^{2+} (Ferrier *et al.*, 1998; Pabbathi *et al.*, 1999), all of which block the L-type Ca^{2+} channel, indicate that the VSRM operates independently of transsarcolemmal Ca^{2+} influx through these channels. The shape of the CV relationship in the presence of the VSRM along with the lack of effect of Ca^{2+} channel blockers suggests that this mechanism is separate from CICR triggered by $I_{Ca\text{-}L}$.

As well, it is unlikely that the VSRM contractions are triggered by T-type Ca^{2+}

current, since these contractions can be elicited at very positive membrane potentials (Ferrier & Howlett, 1995; Howlett & Ferrier, 1997; Howlett, Zhu & Ferrier, 1998), whereas T-type Ca^{2+} current becomes negligible at positive potentials in cardiac myocytes well before the reversal potential for Ca^{2+} is reached (Nilius *et al.*, 1985; Mitra & Morad, 1986). Furthermore, VSRM contractions can be demonstrated in rat ventricular myocytes (Howlett, Zhu & Ferrier, 1998), in which T-type Ca^{2+} current is believed to be absent (Tytgat, Vereecke & Carmeliet, 1990). Nevertheless, activation and inactivation of the VSRM does occur at similar membrane potentials in rat myocytes to those at which T-type Ca^{2+} channels activate and inactivate (Howlett & Ferrier, 1997; Howlett, Zhu & Ferrier, 1998). As well, VSRM contractions are selectively inhibited by amiloride, a compound which blocks T-type Ca^{2+} channels (Howlett, Barry & Ferrier, 1999). Thus, it is possible that the VSRM is coupled to gating of T-type Ca^{2+} channels, or some other protein which activates and inactivates at similar potentials, rather than to T-type Ca^{2+} current (Howlett & Ferrier, 1997, Howlett, Zhu & Ferrier, 1998). However, if activation of the VSRM is coupled to gating of T-type Ca^{2+} channels, it becomes necessary to postulate that T-type channels are present in rat myocytes, but have lost the ability to carry detectable current.

The VSRM is not affected by blockade of $NaCa_{EX}$ with Ni^{2+} (Ferrier & Howlett, 1995; Hobai *et al.*, 1997) nor is it sensitive to Na^+ channel blockade with either lidocaine or tetrodotoxin (TTX) (Ferrier & Howlett, 1995). In fact, contractions elicited by the VSRM are not affected by changes in extracellular Na^+ concentrations (Ferrier & Howlett, 1996; Ferrier *et al.*, 1999), replacement of extracellular Na^+ with choline

chloride or sucrose (Ferrier & Howlett, 1995) or 0 mM intracellular Na^+ (Ferrier *et al.*, 1999). Together, these results indicate that contractions elicited by the VSRM cannot be attributed to Ca^{2+} influx via reverse- mode Na-Ca_{EX} (Ferrier & Howlett, 1995; Howlett & Ferrier, 1997; Howlett, Zhu & Ferrier, 1998).

Studies in guinea pig, rat, and rabbit ventricular myocytes have demonstrated that Ca^{2+} transients and contractions can be triggered by the VSRM in addition to CICR (Ferrier & Howlett, 1995; Hobai *et al*, 1997; Howlett, Zhu & Ferrier, 1998). Recent studies in human atrial myocytes have also illustrated a Ca^{2+} transient in the absence of inward current, characteristic of the VSRM component, along with a Ca^{2+} transient associated with $I_{Ca\text{-}L}$, characteristic of CICR (Van Wagoner *et al.*, 1999). Thus, it appears that similar to results in skeletal muscle, cardiac myocytes show two components of EC-coupling, one triggered by transsarcolemmal Ca^{2+} influx and the other regulated by membrane potential.

VSRM is not DICR:

The VSRM for cardiac EC-coupling may involve a direct connection between the SL membrane and SR release of Ca^{2+}. This idea is similar to that believed to exist in skeletal muscle. When one considers the redundancy of biological systems, it becomes attractive to draw comparisons between the VSRM in cardiac muscle and DICR in skeletal muscle. However, evidence to date indicates that the two are not identical. For example, in skeletal muscle extracellular Ca^{2+} can be substituted with other divalent ions for activation of DICR, indicating that this mechanism requires binding of a divalent ion

but does not require Ca^{2+}. In fact, the strongest evidence in support of a direct connection between the SL and the SR membranes in skeletal muscle came from experiments in which contractions were elicited in the complete absence of Ca^{2+}. Unfortunately this is not the case for the VSRM component of cardiac EC-coupling. Although contractions can be elicited by this mechanism in the absence of any inward current, they cannot be elicited in the absence of extracellular Ca^{2+}. Attempts to substitute other divalent ions such as Ba^{2+} for extracellular Ca^{2+} have been unsuccessful which suggests that there may be an extracellular binding site on the VSRM which is highly selective for Ca^{2+} (Ferrier *et al.*, 1998). Although these findings indicate that the VSRM is not completely analogous to skeletal DICR that does not mean that information from one cannot be used to the study the other. It is likely that either both mechanisms evolved from the same primitive mechanism or that one is the original and the other evolved from it. Either possibility would result in two highly specialized, tissue specific mechanisms with very similar general properties. It is the similarities in general properties that may allow one to utilize the same tools to study both skeletal and cardiac EC-coupling.

Inactivating and non-inactivating components of the VSRM:

Activation-deactivation and inactivation are properties of voltage-sensitive membrane proteins that determine the potential at which transitions between open and closed states of channels occur (Hille, 1992). These properties are characteristic of a particular channel and can be used as a fingerprint to differentiate between various classes and sub-classes of channels (Hille, 1992). In some cases the activation and inactivation

properties of membrane channels also describe the voltage-dependence of contractions that occur in response to inward current through these channels. For example, contractions elicited by CICR coupled to $I_{Ca\text{-}L}$ take on activation and inactivation properties of the L-type Ca^{2+} current. Therefore, the voltage dependence of contractions elicited by CICR coupled to $I_{Ca\text{-}L}$ is bell-shaped reflecting the voltage dependence of L-type Ca^{2+} current (Barcenas-Ruiz & Wier, 1987; Bers, 1991; Beuckelmann & Wier, 1988; Cleeman & Morad, 1991; duBell & Houser, 1989; London & Krueger, 1986).

Contractions elicited by the VSRM also show activation and inactivation properties although they are not dependent on inward current through voltage gated channels (Ferrier & Howlett, 1995; Zhu & Ferrier, 1996; Ferrier & Howlett, 1996; Ferrier *et al.*, 1998; Hobai *et al.*, 1997; Howlett & Ferrier, 1997; Howlett, Zhu & Ferrier, 1998). In fact, the activation and inactivation properties of VSRM contractions differ from CICR coupled to either $I_{Ca\text{-}L}$ or Na-Ca_{EX}. The threshold for activation of VSRM contractions is near -60 mV, which is more negative than that for CICR coupled to either $I_{Ca\text{-}L}$ or $NaCa_{EX}$. As well, unlike CICR contractions, the CV relationship for the VSRM is always sigmoidal. These properties of activation of VSRM contractions are useful for identification and differentiation of this mechanism from CICR coupled to $I_{Ca\text{-}L}$ or reverse mode $NaCa_{EX}$.

In contrast to contractions elicited by CICR coupled to reverse-mode $NaCa_{EX}$, VSRM contractions also can be characterized by properties of steady-state inactivation. Steady-state inactivation of the VSRM was demonstrated by initiating contractions with test steps from different post-conditioning potentials (V_{PC}) (Howlett & Ferrier, 1997;

Howlett, Zhu & Ferrier, 1998; Ferrier *et al.*, 1999; Zhu & Ferrier, 1996). This mechanism was reported to be fully available with steps from -65 mV and largely inactivated by potentials more positive than -40 mV. The steady-state inactivation curve was sigmoidal and could be fit with a Boltzman function which suggests that this mechanism operates by way of a voltage sensor in the SL membrane. The half-inactivation voltage of VSRM contractions was reported to be near -48 mV (Ferrier *et al.*, 1998; Howlett & Ferrier, 1997; Howlett, Zhu & Ferrier, 1998; Ferrier *et al.*, 1999) which is more negative than that for $I_{Ca\text{-}L}$ (-25 mV) (McDonald *et al.*, 1994) but similar to that for T-type Ca^{2+} current (near -50 mV) (Tseng & Boyden, 1989; Wu & Lipsius, 1990). Thus, it is possible that the voltage sensor for this mechanism is the T-type Ca^{2+} channel although the role of the L-type Ca^{2+} channel cannot be ruled out at this time. Clearly more studies need to be done before the voltage sensor for the VSRM can be identified.

Recent studies have demonstrated that aside from an inactivating component, VSRM contractions also demonstrate a non-inactivating sustained component (Ferrier *et al.*, 1999). Sustained Ca^{2+} transients and contractions were observed upon depolarization of a cardiac myocyte to potentials positive to the resting membrane potential (Ferrier *et al.*, 1999). The duration of sustained contractions was dictated by the duration of the depolarizing step with complete relaxation of a myocyte only occurring upon repolarization of the membrane to the resting potential. Sustained contractions were not affected by blockade of $I_{Ca\text{-}L}$ with Cd^{2+} and were maximal at potentials which inactivate $I_{Ca\text{-}L}$, therefore they could not be attributed to Ca^{2+} influx via L-type Ca^{2+} channels (Ferrier *et al.*, 1999). Sustained contractions also were not affected by changes in

extracellular Na^+ concentration or elimination of Na^+ from the patch pipette filling solution which suggests they are not generated by Ca^{2+} influx via reverse mode $NaCa_{EX}$ (Ferrier *et al.*, 1999). Activation of sustained contractions exhibited a sigmoidal voltage dependence which was identical to that of the VSRM. Together, these findings lead to the conclusion that sustained transients and contractions were generated by activation of the VSRM by depolarization.

The voltage dependence of the non-inactivating component of VSRM contractions was described by a Boltzman function which suggests that, similar to the inactivating component, the non-inactivating component also is regulated by charge movement in a voltage sensor. As well, the voltage dependence of this component was the same regardless of the direction of membrane potential change which suggests that it exhibits properties of activation and deactivation. Thus, the VSRM appears to link both contraction and relaxation of the heart to membrane potential (Ferrier *et al.*, 1999). This observation has important implications for current and future concepts on the mechanisms of SR Ca^{2+} release in cardiac myocytes.

Modulation of the VSRM by phosphorylation:

The autonomic nervous system modulates the activity of the heart in response to changes in demand through release of neurotransmitters from nerve endings (Hartzell, 1988). Binding of these neurotransmitters to cardiac cell surface receptors can lead to an increase in the intracellular concentrations of second messengers such as cyclic adenosine-3',5'-monophosphate (cAMP) or phosphatidylinositol-4,5-bisphosphate (PIP_2)

(Hartzell, 1988; Katzung, 1995). Stimulation of these second messenger signaling pathways ultimately results in phosphorylation of many intracellular proteins by way of activated protein kinases such as protein kinase A (PKA) and Ca^{2+}-calmodulin dependent protein kinase (CamK) (Hartzell, 1988). For example, SR Ca^{2+} release channels isolated in lipid bilayers (Takasago *et al.*, 1991; Witcher *et al.*, 1991; Lokuta *et al.*, 1995), as well as in intact cells (Li *et al.*, 1997; duBell, Lederer & Rogers, 1996) have been reported to be modulated by phosphorylation via PKA and/or CamK. It is not surprising then that mechanisms of EC-coupling that involve SR Ca^{2+} release also can be modulated by phosphorylation. However, the importance of phosphorylation in activation of CICR compared to the VSRM is quite different.

Studies carried out with low resistance patch electrodes illustrated that VSRM contractions were only transiently observed if cAMP or calmodulin were not added to the pipette filling solution. By contrast, VSRM contractions could be maintained for long periods of time in experiments carried out with high resistance electrodes. An important difference between these two experimental techniques is that the diameter of the patch pipette tip is much larger than that of the high resistance electrode. This observation suggested that loss of important signaling components, such as cAMP and calmodulin, through dialysis with the pipette filling solution (Isenberg & Wendt-Gallitelli, 1989) resulted in inhibition of contractions elicited by the VSRM (Ferrier *et al.*, 1998; Zhu & Ferrier, 1998). These results also suggested that activation of the VSRM must be facilitated by the cAMP-PKA (Ferrier *et al.*, 1998) and CamK (Zhu & Ferrier, 1998) pathways. The ability of PKA and CamK to restore VSRM contractions can be abolished

by selective PKA (Ferrier *et al.*, 1998) and CaM-K inhibitors (Zhu & Ferrier, 1998). These findings confirm that phosphorylation of the VSRM by these kinases can increase the availability of the VSRM. In undialyzed myocytes, each inhibitor by itself caused a 50 % decrease in contractions elicited by the VSRM, while complete inhibition was observed in the presence of both inhibitors. Thus, it appears that both phosphorylation pathways not only regulate activation of this mechanism but are essential for activation of it in undialyzed myocytes as well. By contrast, contractions initiated by CICR coupled to $I_{Ca\text{-}L}$, although reduced in magnitude, persisted even in the presence of inhibition of both PKA and CamK. Therefore, although both CICR and the VSRM can be modulated by phosphorylation, this process may actually be an essential part of activation of the VSRM. The phosphorylation site for activation of the VSRM is not known. However, one can speculate that it must exist on the release mechanism itself since $I_{Ca\text{-}L}$ is not involved in activation of the VSRM and SR release via CICR occurs in the absence of phosphorylation.

The VSRM component of contractions also can be stimulated through activation of the cAMP-PKA signaling pathway at various sites. Under normal circumstances, agonist binding to β-receptors results in stimulation of adenylate cyclase (AC) through activation of a G protein. Stimulation of AC leads to production of cAMP through breakdown of ATP, the cAMP then stimulates PKA resulting in phosphorylation of various proteins (see Appendix, page 224). Studies carried out with low resistance patch electrodes without addition of cAMP to the pipette solution demonstrated that VSRM contractions could be stimulated by activation of β-receptors with isoproterenol (Chartier

et al., 1999). As well, direct stimulation of AC by forskolin can also support VSRM contractions in the absence of cAMP in the pipette solution (Chartier *et al.*, 1999). These results suggest that it is not cAMP itself that is required to increase the availability of VSRM contractions but activation of PKA (see Appendix, page 224).

CONDITIONS WHICH PREVENT ACTIVATION OF THIS MECHANISM:

Early experiments demonstrating a VSRM component of cardiac EC-coupling were carried out with high resistance electrodes which minimize dialysis of the intracellular environment. When similar studies were repeated with low resistance electrodes, which have a larger diameter tip that allows dialysis, the VSRM was not activated. This study is a reminder of the many assumptions that are made when working with low resistance electrodes. The most important of these is the assumption that the solution in the electrode mimics the natural intracellular milieu. Although some of the essential components of the cytoplasm of a cell have been determined, studies on the VSRM have illustrated that there is still much to be learned. Although patch electrodes are extremely useful for adding compounds to the intracellular environment one can't forget that dialysis of endogenous compounds is occurring at the same time (Isenberg & Wendt-Gallitelli, 1989). The popularity of this electrophysiological technique may have delayed observation of the VSRM in cardiac myocytes.

Aside from use of low resistance patch electrodes, there are several other commonly used experimental conditions which have delayed observation of the VSRM (Howlett & Ferrier, 1997). For example, many electrophysiological studies have been

carried out with a holding potential of -40 mV. This protocol allows adequate activation of $I_{Ca\text{-}L}$ and the contractions associated with this current while at the same time inhibiting currents such as I_{Na} which can interfere with accurate measurement of $I_{Ca\text{-}L}$. Therefore, protocols with a holding potential of -40 mV are common in studies on CICR. However, the steady-state inactivation properties of the VSRM indicate that this mechanism would be inactivated by a -40 mV holding potential. By contrast, investigations of the VSRM component of EC-coupling were carried out with a holding potential of -80 mV, which is closer to the physiological holding potential. Under these conditions, the VSRM is fully available as determined by the fact that contraction-voltage relationships determined from a V_{PC} of -70 mV are sigmoidal while those determined from a V_{PC} of -40 mV are bell-shaped (Ferrier & Howlett, 1995; Howlett, Zhu & Ferrier 1998; Ferrier *et al.*, 1998).

Another experimental factor which likely prevented earlier discovery of the VSRM is temperature. Many electrophysiological experiments are carried out at room temperature (22-25 °C) because cells tended to last longer and are easier to work with at this temperature. However, VSRM contractions are inhibited at room temperature (Ferrier, 1996). Only experiments carried out near physiological body temperature (37 °C) allow activation of the VSRM component of cardiac EC-coupling. Overall, there are several commonly used experimental conditions which have hindered earlier detection of the VSRM in cardiac EC-coupling. The recent 'unveiling' of the VSRM in cardiac myocytes is a reminder of the fact that experimental results are greatly dependent on the techniques used and the conditions of the study.

PHYSIOLOGICAL SIGNIFICANCE OF A VSRM FOR CARDIAC EC-COUPLING:

Evidence to date indicates that the VSRM contributes substantially to cardiac contraction. This is supported by the fact that the voltage dependence of this mechanism suggests it is maximally activated over the range of potentials corresponding to the overshoot and plateau of the cardiac action potential. As well, the observation of a non-inactivating, sustained component of VSRM contractions suggests that this mechanism also plays an important role in relaxation of the heart. The contribution of this mechanism to cardiac functioning also has been illustrated in studies carried out with cardiomyopathic hamsters in which the VSRM component of contraction has been reported to be defective (Howlett & Mapplebeck, 1996; Howlett, Ferrier & Mapplebeck, 1997). Under these conditions, the VSRM was found to be suboptimally activated compared to normal hamsters, while contractions elicited by CICR were not affected. Similarly, VSRM Ca^{2+} transients could be elicited in normal human atrial myocytes but not in myocytes isolated from failing hearts (Van Wagoner *et al.*, 1999). These results suggest that the VSRM plays an integral role in normal cardiac functioning and defects in this mechanism may be the root of some cardiac pathologies.

USE OF DRUGS AS TOOLS TO STUDY PHYSIOLOGY:

Investigation of the relative contributions of the VSRM and CICR to overall cardiac EC-coupling would be beneficial in understanding normal cardiac functioning and possibly for determining the role of the VSRM in cellular mechanisms of heart

failure. The development and refinement of research techniques such as voltage clamp of single myocytes have allowed great advancements in the field of cardiac and skeletal muscle research. Along with electrophysiological techniques, many studies incorporated pharmacological techniques, in which drugs are used to inhibit or stimulate cellular processes in an attempt to determine the underlying physiology. For example, pharmacological agents selective for particular ion channels are useful not only for characterization of the currents carried by these channels but also for observation of the effects of inhibition of currents on overall functioning of a cell. Investigation of the contribution of the VSRM to cardiac contraction would be facilitated by development of pharmacological agents and protocols which would allow selective inhibition of either the VSRM or CICR. It is possible that agents which selectively affect the VSRM might be found among the agents which have been shown to inhibit EC-coupling in skeletal myocytes. For example, although all local anesthetics (LAs) block Na^+ channels, some have additional actions which made them very useful in investigations of EC-coupling in skeletal muscle.

LOCAL ANESTHETICS

The most widely used LAs today are lidocaine, bupivacaine and tetracaine. These compounds are very lipid soluble, therefore they are able to cross nerve sheaths and cell membranes to reach their site of action (Hille, 1992). LAs tend to be amine compounds with pKa values between 6 and 8.5. The uncharged base forms the lipid-soluble, readily diffusable, agent which is in equilibrium with the protonated form (τ= 300 μs at pH 7)

(Hille, 1992). The local anesthetic action of these agents occurs through block of Na^+ channels.

Inhibition of Na^+ current by local anesthetics:

The site of action of LAs is the cell membrane where they block the depolarization by Na^+ current (Ritchie & Greene, 1980). Inhibition of Na^+ influx occurs by a direct interaction of these agents with voltage-dependent Na^+ channels in the cell membrane (Hille, 1977). Action potential firing in nerve cells is Na^+ -dependent, the rapid transient influx of Na^+ produces the upstroke of an action potential. Thus, inhibition of Na^+ current (I_{Na}) in these cells increases the threshold for electrical excitability and decreases the rate of rise of an AP, and slows impulse propagation, all of which lead to nerve conduction failure (Ritchie & Greene, 1980). Studies have shown that LAs must cross the membrane in their uncharged form to reach their site of action on the inner surface of the channel in contrast to the neurotoxins, such as TTX, which bind within the mouth of the channel from the outside (Ritchie & Greene, 1980; Butterworth & Strichartz, 1990; Ritchie & Greengard, 1965; Hille, 1992). Once in the cytoplasm, however, it is the charged form that interacts with the Na^+ channel (Hille, 1992; Ritchie & Greengard, 1965; Butterworth & Strichartz, 1990). LAs show what are known as frequency- and use-dependent block of Na^+ channels (Ritchie & Greene, 1980; Hille, 1992). Thus, a resting nerve or myocyte is much less sensitive to LAs than a nerve that is being stimulated at a high frequency. These characteristics of block indicate that the channels must be in the open conformation in order for these agents to gain access to their

binding site (Ritchie & Greene, 1980; Hille, 1992). Also, LAs may bind more tightly to, and stabilize, the inactivated state of the Na^+ channels.

Effects of LAs on striated muscle EC-coupling:

Aside from effects on Na^+ channels, LAs have also been shown to interfere with EC-coupling in striated muscle by a mechanism independent of inhibition of I_{Na}. Early studies with frog sartorius muscle indicated that tetracaine and procaine inhibited caffeine contractures (Feinstein, 1963). Caffeine contractures result from stimulation of SR Ca^{2+} release, as well as block of Ca^{2+} reuptake by the SR. Thus, inhibition of caffeine contractures by LAs was thought to be mediated by interaction of these agents with SR release of Ca^{2+} (Feinstein, 1963). A few years later, studies by Johnson and Inesi (1969) found that the LAs tetracaine, procaine and lidocaine decreased Ca^{2+} accumulation of fragmented skeletal SR, with tetracaine providing the most effective block (Johnson & Inesi, 1969). As well, tetracaine was found to slightly decrease efflux of accumulated Ca^{2+} at low concentrations (<1 mM) whereas higher concentrations (>1 mM) increased efflux and reduced net Ca^{2+} accumulation (Johnson & Inesi, 1969). However, at concentrations ranging between 0.8 and 1 mM tetracaine inhibited Ca^{2+} efflux from the SR while at the same time reducing Ca^{2+} accumulation (Johnson & Inesi, 1969).

Subsequently, several investigations have been undertaken on the effects of LAs on skeletal and cardiac muscle SR activities. The results of these studies were somewhat conflicting in that many of them illustrated inhibition of Ca^{2+} release while others demonstrated stimulation of contraction and an increase in Ca^{2+} release by LAs

(Chapman & Miller, 1974; Hunter, Haworth & Berkoff, 1982; Chamberlain, Volpe & Fleischer, 1984; Stephenson & Wendt, 1986; Meissner & Henderson, 1987; Shoshan-Barmatz & Zchut, 1993; Xu, Jones & Meissner, 1993; Zahradnidova & Palade, 1993; O'Brien, Valdivia & Block, 1995; Györke, Lukyanenko & Györke, 1997). Thus, LAs were found to have complex effects on EC-coupling in skeletal and cardiac muscle. These agents were thought to act at the level of the ryanodine receptor (Almers & Best, 1976; Almers & Best, 1976b; Shoshan-Barmatz & Zchut, 1993) because they could block opening of isolated ryanodine receptors reconstituted in lipid bilayers (Volpe *et al.*, 1983). A two site model was proposed to explain the mechanism of interaction of LAs with RyRs (Shoshan-Barmatz & Zchut, 1993). One site was referred to as the inhibitory site, or 'B' site, while the other was the stimulatory site, or 'A' site. The distance between the two sites determined whether a particular LA would be inhibitory or stimulatory. Compounds which were long enough to interact with both sites, such as tetracaine, would be inhibitory while shorter compounds, such as lidocaine would interact with only the 'A' site resulting in stimulation of release by this agent (Shoshan-Barmatz & Zchut, 1993). This model provided an explanation for the conflicting results obtained with LAs on skeletal muscle EC-coupling. This model also may be useful for determining the effects of this agent on two components of cardiac EC-coupling.

Aside from effects on isolated RyRs, tetracaine also was found to selectively inhibit a component of contraction in skeletal muscle. In skeletal muscle EC-coupling, SR release channels which are not directly aligned with SL voltage sensors can be induced to open by the Ca^{2+} released from RyR which are attached to voltage sensors

(Ríos & Pizarró, 1988; Jacquemond *et al.*, 1991; Ríos & Pizarro, 1991; Györke & Palade, 1993; Melzer, Hermann-Frank & Lüttgau, 1995; Berridge, 1997). In frog skeletal muscle fibers, tetracaine was found to abolish the peak component of Ca^{2+} transients, which is thought to represent this CICR component, without affecting the steady-state component which represents DICR (Pizarró *et al.*, 1992).

The differential effects of tetracaine on two components of skeletal muscle contractions suggested that this agent may be useful in determining the relative contribution of the VSRM to cardiac EC-coupling. Previous studies in cardiac myocytes illustrated that tetracaine disrupted cardiac contraction (Lynch, 1991), however the VSRM was not available under the experimental conditions used in that study. As well, tetracaine was found to block opening of isolated cardiac ryanodine receptors similar to effects on isolated skeletal RyRs (Chamberlain, Volpe & Fleischer, 1984). Together, these results suggest that tetracaine might be a useful pharmacological tool to study cardiac EC-coupling.

During the course of the present study, Overend *et al* (1998) reported effects of tetracaine on cardiac EC-coupling in voltage-clamped ventricular myocytes. They reported that tetracaine caused a transient decrease in the amplitude of contractions and Ca^{2+} transients in rat ventricular myocytes at 22 °C. Since previous results have shown that the VSRM is inhibited at room temperature (Ferrier, 1996), their results likely correspond to effects on CICR in the absence of VSRM. It is not clear from their study what effect tetracaine would have on cardiac EC-coupling under conditions in which both mechanisms would be available. Therefore, one of the objectives of the present study

was to determine the effects of tetracaine on cardiac contraction in the presence of both VSRM and CICR mechanisms of EC-coupling.

RYANODINE:

Other agents which interact with RyRs may also be useful tools for investigation of the functioning of the VSRM, as well as the relative contribution of this component to EC-coupling. One such agent widely known for its effects on RyRs is ryanodine. Ryanodine is a neutral plant alkaloid that was originally used as an insecticide in the early 1950's. However, the majority of the research on ryanodine has focused on the profound effects of this agent on muscle from a variety of vertebrates and invertebrates (Jenden & Fairhurst, 1969). The effects of ryanodine are complex and vary depending on the muscle type, the calcium activity, and the pattern of stimulation (Jenden & Fairhurst, 1969; Penefsky, 1974; Sutko & Kenyon, 1983; Sutko, Ito & Kenyon, 1985). Ryanodine was initially believed to selectively inhibit SR Ca^{2+} release (Jenden & Fairhurst, 1969; Sutko & Kenyon, 1983) by binding with high selectivity and affinity to these channels in both skeletal and cardiac muscle cells (Pessah, Waterhouse & Casida, 1985, Fleischer *et al.*, 1985, Lattanzio *et al.*, 1987). It is now known that the effects of ryanodine on SR Ca^{2+} release channels range from fixing the channel in an open subconducting state (Fleischer *et al.*, 1985; Pessah, Waterhouse & Casida, 1985; Lattanzio *et al.*, 1987; Rousseau, Smith, & Meissner, 1987) to locking it in the closed conformation (Seiler *et al.*, 1984; Feher & Lipford, 1985; Meissner, 1986) depending on the experimental conditions and the concentration used (Meissner, 1986).

Because of the high specificity and selectivity of ryanodine binding to SR Ca^{2+} release channels in striated muscle, this agent is widely used as a pharmacological tool to investigate the processes of EC-coupling (Meissner, 1986; Alderson & Feher, 1987; McGrew *et al.*, 1989; Balke & Wier, 1991; Bers, 1991). Ryanodine is a useful tool for investigation of these processes because it disrupts Ca^{2+} release from the SR but entry pathways such as Ca^{2+} channels and $NaCa_{EX}$ are not inhibited (Sutko & Kenyon, 1983; Mitchell *et al.*, 1984; Marban & Wier, 1985).

A low concentration of ryanodine (30 nM), which results in depletion of SR stores, was used in initial investigations of the VSRM as a tool to determine whether contractions elicited by this mechanism were dependent on SR Ca^{2+} release. The original hypothesis was that the effects of this agent on SR Ca^{2+} stores would result in inhibition of both CICR and VSRM contractions. However, this study found a selective inhibition of contractions elicited by the VSRM with virtually no effect on contractions elicited by CICR (Ferrier & Howlett, 1995). These results raised many questions as to the mechanism of action of ryanodine which would result in a differential effect on these two components of contraction. Some possibilities are that 30 nM ryanodine has an effect on VSRM contractions which is independent of depletion of SR stores, VSRM contractions may be more sensitive to changes in SR stores or ryanodine may interfere with the coupling of charge movement to RyRs. Some of these possibilities will be explored in the present study to determine whether low concentration ryanodine can be used as a tool to investigate the properties of VSRM contractions.

HYPOTHESES AND OBJECTIVES

The overall objective of this study was to determine the effects of two pharmacological agents on cardiac EC-coupling mediated by both VSRM and CICR. These agents are tetracaine and ryanodine, both of which are known to act on SR Ca^{2+} release channels. Pharmacological tools have played an important role in elucidating the mechanisms of EC-coupling in striated muscle. For example, tetracaine has been reported to cause preferential inhibition of a component of EC-coupling in skeletal muscle. The recently described VSRM in cardiac muscle suggests that the processes of EC-coupling in this striated muscle also has more than one fundamental mechanism. Thus, **the major hypothesis of this study was that tetracaine and ryanodine will have similar effects on two mechanisms of cardiac EC-coupling as has been shown in skeletal muscle and will be useful pharmacological tools for evaluating the role of these two components in EC-coupling in cardiac muscle.**

The specific objectives of the first part of this study were to determine: 1) if tetracaine inhibits contraction in isolated guinea-pig ventricular myocytes at 37 °C with both CICR and the VSRM available; 2) whether tetracaine can selectively inhibit EC-coupling mediated by CICR or the VSRM; 3) if tetracaine can be used as a pharmacological tool to identify components of cardiac EC-coupling attributable to CICR or the VSRM; and 4) whether tetracaine can be used to evaluate the contribution of the VSRM to cardiac contraction.

Similarly, ryanodine is another well known pharmacological tool which has been used extensively in investigations of the mechanisms of EC-coupling in striated muscle.

This agent is commonly used for its ability to deplete SR Ca^{2+} stores through selective binding to RyRs. However, more recently, ryanodine has been reported to cause preferential inhibition of the VSRM component of cardiac contraction in spite of the fact that contractions elicited by both the VSRM and CICR result from SR Ca^{2+} release.

Therefore, the specific objectives of the second portion of this study were to determine: 1) whether ryanodine inhibition of the VSRM was related to depletion of SR Ca^{2+} stores or whether this agent selectively inhibits the VSRM by a mechanism independent of SR depletion; and 2) whether ryanodine can be used as a tool to investigate the contribution of the VSRM to cardiac contraction.

MATERIALS AND METHODS

I. ANIMALS:

All experiments were conducted on freshly isolated guinea-pig ventricular myocytes. Charles River (St. Constant, Quebec, Canada) albino male (approximately 90%) and female guinea-pigs, weighing 250-350g, were used randomly in this investigation. Studies were conducted within the guidelines published by the Canadian Council on Animal Care (CCAC; vol. 1, 1980; vol. 2, 1984) and approval for this investigation was obtained from the Dalhousie University Committee on Animal Care. Animals were housed in the Dalhousie Animal Care facility with food and water freely available. All animals were given an intraperitoneal injection of heparin (3.3 IU/g) approximately 10 minutes prior to removal of the heart to prevent blood coagulation. Two single cell isolation procedures were used randomly throughout this study, therefore two procedures will be described.

II) MYOCYTE ISOLATION:

a) Procedure #1: Perfusion Protocol:

Animals were sacrificed by stunning and exsanguination via the carotid vessels. Hearts were rapidly removed by a parasternal incision and placed in a 50 ml beaker of nominally Ca^{2+} free buffer solution of the following composition (mM): 120 NaCl, 4 KCl, 4 NaH_2PO_4, 22 $NaHCO_3$, 5.5 glucose, 1 $MgSO_4$. The heart was then transferred to a petri dish containing the same nominally Ca^{2+} free buffer which was bubbled with 95 % O_2/5 % CO_2 (Union Carbide, Canada Limited). Once in the petri dish, the heart was quickly trimmed of excess tissue and fat to expose the aorta. Hearts were then mounted

via the aorta on a cannula attached to a jacketed Liebig condenser (Langendorf column) and were perfused through the aorta with the nominally Ca^{2+} free buffer (10-12 ml/min). In a Langendorf column, the perfusion pressure is dependent on gravity, therefore, the column was kept at least half full throughout the perfusion to maintain pressure. A circulating water bath was used to preheat and maintain the temperature of the water circulating through the jacket of the condenser at approximately body temperature, 37 °C. Thus, any solution in the column was maintained at 37 °C.

Enzyme Digestion:

After approximately 7 minutes of perfusion, the heart was subjected to enzymatic digestion with the nominally Ca^{2+} -free solution supplemented with collagenase 1A (Sigma) and protease (Sigma type XIV). Protease (5 mg) and collagenase (50 mg) were dissolved in 50 ml of Ca^{2+} free buffer. At this time, the Ca^{2+} concentration of the buffer also was increased (4 μl of a 0.5 M stock solution of $CaCl_2$ were added to the 50 ml enzyme solution) to 15 μM. The enzyme solution was added to the column and the heart was perfused for approximately 3 minutes with this solution. Enzyme digestion was stopped when the outer surface of the heart appeared somewhat translucent upon visual inspection. The ventricles were then cut down into a beaker of K^+ and substrate enriched solution of the following composition (mM): 80 KOH, 50 glutamic acid, 30 KCl, 30 KH_2PO_4, 20 taurine, 10 HEPES, 10 glucose, 3 $MgSO_4$, 0.5 EGTA (pH 7.4 with KOH). The tissue was then minced in the beaker and gently swirled to wash away any residual enzyme and cellular debris away from the tissue. The supernatant was then decanted into a waste beaker and fresh K^+ and substrate enriched solution added to the beaker

containing the minced tissue. The K^+ solution was decanted and replaced by fresh solution 3 or 4 times with gentle swirling each time to ensure the tissue was adequately rinsed of residual enzyme solution and cell debris.

b) Procedure #2: Perfusion Protocol:

Animals were given an intraperitoneal coinjection of heparin to decrease blood clot formation and sodium pentobarbitol (160 mg/kg) to anesthetize them before opening the chest cavity by a parasternal incision. Once the chest cavity was opened, the aorta was incised and immediately cannulated *in situ*. The heart was perfused with a nominally Ca^{2+} free solution of the following composition (mM): 120 NaCl, 3.8 KCl, 1.2 KH_2PO_4, 1.2 $MgSO_4$, 10 HEPES, 11 glucose (pH 7.4 with NaOH) bubbled with 100 % O_2 (Union Carbide, Canada Limited). This solution was pumped through the aorta via a peristaltic pump (Cole-Parmer Instruments Co., Chicago, IL) at a constant rate of 10 ml/min for 7 minutes. The temperature of the buffer was maintained at approximately 37 °C by use of a heated water bath. After perfusion began, the heart was removed from the animal. The perfusion pressure was monitored by a pressure gauge. A drop in pressure indicated adequate dissociation of the tissue.

Enzyme Digestion:

Following perfusion, the heart was subjected to enzymatic digestion. A 50 ml volume of the nominally Ca^{2+} free solution was supplemented with 25 mg collagenase A (Boehringer Mannhein) and 4.8 mg protease (Sigma type XIV). The enzyme solution was bubbled with 100 % O_2 and kept in a 37 °C water bath to maintain the temperature. This solution was pumped through the heart at a rate of 10 ml/min for 5 minutes via a

peristaltic pump (Cole-Parmer Instruments Co., Chicago, IL). After digestion the ventricles were excised from the heart and placed in a petri dish where the tissue was minced in the same high K^+ substrate enriched solution described above. Cells were then placed in a beaker and washed several times with the high K^+ solution before being used.

In both procedures, the minced tissue was stored in the K^+ and substrate enriched solution at room temperature before use. Just before each experiment the tissue was rinsed again with K^+ and substrate enriched solution and the fresh cell suspension was decanted into a beaker and transferred to the experimental chamber with a Pasteur pipette.

c) Superfusion of myocytes:

Isolated myocytes were placed in a custom designed, acrylic, open perfusion chamber which was mounted on the stage of an inverted microscope (Olympus Model IMT-2, Tokyo, Japan). The bottom of the perfusion chamber was composed of a plastic coverslip the edges of which were glued to the acrylic chamber with a clear silicon cement. Myocytes were allowed to settle to the bottom of the perfusion chamber and remained undisturbed for approximately 10 minutes which was enough time for them to adhere to the coverslip before perfusion was started. Extracellular solutions were delivered to the experimental chamber from a reservoir by a peristaltic pump (either a Piper pump, type P20T, Dungey Incorporated or a high-flow, Gilson minipuls-3, serial no. 1006123) at a rate of 3 ml/min. Three different extracellular solutions were used throughout the course of this study; full Na^+ (145 mM), low Na^+ (45 mM) and a modified full Na^+ solution (140.4 mM). The modified full Na^+ solution was reported in previous studies in which reverse-mode $NaCa_{EX}$ contractions were elicited (Wasserstrom & Vites,

1996), therefore the same conditions were used in the present study to elicit contractions by this mechanism. The compositions of these three solutions are given in tables 1-3. In some experiments, 50 μM TTX also was added to the extracellular solution to block residual I_{Na} (see legends). The solution change over time, as determined by measuring myocyte membrane potential in response to a change in K^+ concentration, was approximately 90 seconds (Ferrier & Howlett, 1995). The depth of the bath was maintained by a simple gravity overflow system whereby solution pumped into the experimental chamber was allowed to overflow into a trough which drained into a waste reservoir. The temperature of the solution in the experimental chamber was maintained at 37 °C by a custom designed, heated, circulating water system. The temperature of the water in this system was maintained by an immersion circulator heater (model 1266-01; Cole Parmer Instruments Company, Chicago, IL).

Drugs were added to the experimental chamber either by switching the inlet to the pump from one buffer reservoir to another or through the use of a heated (37°C) rapid solution change device (Levi *et al.*, 1996; Ferrier *et al.*, 1998). Rapid solution changes were triggered by a computer so that they could be timed to the protocol. Thus, rapid solution changes were made after a train of conditioning pulses, 3 seconds before activation steps. This prevents possible effects of solution changes on loading of the SR during the trains of conditioning pulses. In some experiments, the rapid solution switching device was used to apply drugs continuously over a myocyte. In these experiments, the device was turned on and off manually rather than being triggered by a computer.

Table 1. Composition of '**full Na^+**' solution.

Compound	*Concentration (mM)*
NaCl	145
$CaCl_2$	2.5
KCl	4
$MgCl_2$	1
glucose	10
HEPES	10

pH 7.4 with NaOH (final $[Na^+] \cong 148$ mM)

Table 2. Composition of '**low Na^+**' extracellular solution.

Compound	*Concentration (mM)*
NaCl	45
choline Cl	100
$CaCl_2$	2.5
KCl	4
$MgCl_2$	1
glucose	10
HEPES	10
lidocaine	0.2

pH 7.4 with NaOH (final $[Na^+] \cong 48$ mM)

Table 3. Composition of '**modified full Na^+**' extracellular solution.

Compound	*Concentration (mM)*
NaCl	140
$CaCl_2$	1.8
KCl	5.4
$MgCl_2$	0.5
glucose	11
HEPES	5
NaH_2PO_4	0.4
lidocaine	0.2

pH 7.4 with NaOH (final $[Na^+] \cong 144$ mM)

III. RECORDING PROCEDURE:

a) Micromanipulators and electrodes:

High resistance electrodes:

Single cell recordings were performed using the whole cell voltage clamp method. Two types of recording electrodes were used: high resistance electrodes with small diameter tips and low resistance, patch electrodes with large diameter tips. High resistance electrodes were made from borosilicate glass tubing which contains a filament (Sutter Instruments Co., Novato, CA) and has an outer diameter of 1.2 mm and inner diameter of 0.69 mm. These high resistance electrodes were made with a P-87 Flaming/Brown micropipette puller (Model P-87, Sutter Instruments Co., Novato, CA). The final tip resistance was 15-25 MΩ (figure 2-1). A drop of filtered 2.7 M KCl solution was placed in the blunt end of the electrode to allow the tapered end to fill by capillary action. Once the tip of the electrode was filled, the remainder of the electrode was back-filled by injection of KCl through a fine hypodermic needle from a syringe. Once filled, the electrodes were inserted into a WPI microelectrode holder (World Precision Instruments, Sarasota, FL) containing a silver, silver-chloride (Ag/AgCl) pellet. The microelectrode holder was plugged directly into a pin jack on an amplifier headstage (Axoclamp 2A, Axon Instruments). The headstage has an internal resistor which determines the range and resolution of current passing capability of the amplifier through the microelectrode. The headstage is the first stage of microelectrode amplification and is connected to the main amplifier electronics by means of a cable. The headstage was mounted on a micromanipulator (Leitz Incorporated, Wetzlar, Germany) which allowed

positioning of the electrode in the experimental chamber. Once the electrode was immersed in the solution within the experimental chamber, an offset potential was generated by a variety of liquid and metal interfaces in the circuit between the amplifier input and the ground. The sum of these potentials must be cancelled in order to zero the amplifier so that only the voltage drop across the cell membrane is recorded. This offset potential was cancelled by manual adjustment of the input offset dial on the Axoclamp-2A amplifier which adds a potential of equal amplitude but opposite polarity so the amplifier input reads 0 mV. The electrode resistance was then measured by passing 1 nA of current through the electrode and measuring the potential. According to Ohm's Law, voltage is equal to the product of current and resistance (V=IR). Thus, the potential measured on the amplifier is the voltage drop across the microelectrode in response to the 1 nA current. When a 1 nA current is passed, the micropipette potential is 1 mV/MΩ of micropipette resistance. Thus, a 20 mV potential is equivalent to a 20 MΩ micropipette resistance. The bridge balance control on the Axoclamp-2A console was adjusted manually to exactly equal the resistance of the microelectrode thereby cancelling the voltage drop across the electrode. In experimental recordings, only the voltage drop across the cell membrane was observed during current passing.

The recording circuit was grounded by a silver wire coated with silver chloride (Ag/AgCl wire) which connected the recording amplifier to the experimental chamber. One end of the Ag/AgCl wire was immersed in a well containing 2.7 M KCl while the opposite end was plugged into the ground of the amplifier headstage. The Ag/AgCl wire was connected to the bath by way of an agar bridge. The agar bridge consisted of a length

of glass capillary tubing filled with a 1% agar solution in 2.7 M KCl. The glass capillary tubing was molded into a U-shape so one end could sit in the KCl well and the other in the bath. An advantage of using an agar bridge is that it creates an interface with the bath solution in which movement of ions into and out of the agar is small. Thus, changing the experimental solution does not result in errors due to changes in junction potentials.

Low resistance, patch pipettes:

The low resistance, patch electrodes were made from Kovar seal borosilicate glass rods (model 7052) with an outer diameter of 1.65 mm and an inner diameter of 1.2 mm (A-M Systems Inc., Carlsborg, WA) (figure 2-2). These electrodes also were pulled on a Flaming/Brown micropipette puller, however the final tip resistance of 1-3 MΩ was obtained by fire polishing with an MF-83 Narishige fire polisher (Narishige Scientific Instruments Lab., Tokyo, Japan). Two different types of solutions were used to fill the pipettes, the specific type of filling solution was dictated by the experimental objectives. Previous studies have shown that VSRM-induced contractions occurred only transiently in experiments carried out with patch pipettes filled with conventional intracellular solution (Ferrier *et al.*, 1998). Thus, to support VSRM contractions, patch pipettes were filled with a solution containing 50 μM 8-Bromo-cAMP (Ferrier *et al.*, 1998). A detailed composition of this solution can be found in table 4. In one set of experiments, contractions elicited by reverse-mode $NaCa_{EX}$ were desired. Therefore, the patch pipettes were filled with a solution which has been previously demonstrated to elicit reverse-mode $NaCa_{EX}$ contractions (Wasserstrom & Vites, 1996). The complete composition of this solution is presented in table 5. In order to fill the pipette tip, the blunt end was inserted

into a small plastic connector which connected the pipette to an empty syringe. The tip of the pipette was submerged in a small volume of intracellular solution. The syringe plunger was then withdrawn slowly from the syringe creating a negative pressure inside the syringe and pipette. This negative pressure forced intracellular solution into the tip of the pipette. Once the tip was filled a fine hypodermic needle attached to a syringe filled with intracellular solution was used to inject enough solution into the pipette to fill it half-way. A pipette containing intracellular solution was then placed in a microelectrode holder (HL-2-17, Axon Instruments) containing a AgCl-coated Ag wire. The holder was then plugged into an amplifier headstage which was connected to the main amplifier by a cable. The headstage was mounted on a micromanipulator which allowed positioning of the microelectrode.

Table 4. Composition of **8-Bromo-cAMP** intracellular solution.

Compound	*Concentration (mM)*
K-aspartate	70
8-Br-cAMP	0.05
$CaCl_2$	0.12
KCl	60
NaCl	8
$MgCl_2$	1
EGTA	0.50
HEPES	10
KH_2PO_4	2.5
Mg-ATP	4

pH 7.2 with KOH
(free $[Ca^{2+}] = 8 \times 10^{-9}$ M; free $[Mg^{2+}] = 0.6 \times 10^{-3}$ M)

Table 5. Composition of intracellular solution promoting **reverse-mode $NaCa_{EX}$**.

Compound	*Concentration (mM)*
Na_2ATP	3
EGTA	0.05
K-aspartate	120
KCl	30
$MgCl_2$	1.5
HEPES	20

pH 7.2 with KOH
(free $[Ca^{2+}] = 3.2 \times 10^{-8}$ M; free $[Mg^{2+}] = 8 \times 10^{-5}$ M)

Positive or negative pressure could be applied to the patch electrode either by forcing air in or drawing it out of the electrode holder through a small piece of tubing. The tubing was connected to a stop-cock which was used to maintain the pressure in the tubing. Positive pressure was applied to position the electrode over the cell as this prevented any debris in the experimental chamber from attaching to the tip. The difference in the ionic composition of the solution in the pipette and the solution in the bath creates a potential which is referred to as the liquid junction potential. The sum of this potential and any other small potentials generated by liquid and metal interfaces would be added to the voltage drop across the cell membrane if they were not cancelled prior to recording. Thus, the total value of these potentials was cancelled by applying an equal potential of opposite polarity to the amplifier. Once the amplifier potential was

balanced with the total potential of the experimental chamber, the resistance of the electrode was determined by passing 1 nA of current through it. The voltage drop across the microelectrode in response to the 1 nA current pulse determined the electrode resistance.

Liquid junction potential measurement:

The liquid junction potential, which is the potential between the extracellular solution in the bath and the intracellular microelectrode solution, was set in the amplifier before the pipette was positioned on the cell membrane to avoid a voltage shift artifact. The value of the liquid junction potential was measured in a separate experiment. To measure this potential, the tip of a filled microelectrode was placed in a well filled with the same solution as that used to fill the microelectrode and the offset potential on the amplifier was balanced to read 0 mV. Next, the same electrode was immersed in a well containing extracellular solution the same as that used in the experiments. The difference in ion concentrations between the intracellular and extracellular solutions creates the liquid junction potential. The value of the liquid junction potential was set on the amplifier input just before the electrode was put into contact with a myocyte. The edge of the pipette tip forms a seal with the cell membrane once the two are in contact. Gentle application of pressure caused the membrane surrounded by the edge of the tip to rupture so that the solution inside the pipette became continuous with the intracellular solution which was of similar composition. Thus, the liquid junction potential would disappear at this point. The value of the liquid junction potential must be determined for each combination of intracellular and extracellular solution used in experiments.

Seal resistance measurement:

Once the patch electrode was pressed lightly against the sarcolemma of a ventricular myocyte negative, pressure was applied to promote sealing of the membrane to the electrode. Formation of a seal between the membrane and the electrode isolates the interior of the electrode from the extracellular solution. The seal resistance was calculated using Ohm's Law of electricity which states that the potential difference is equal to the product of the current (I) and resistance (R) (V=IR). Thus, in some cells, a 0.1 nA current pulse was applied to monitor the seal resistance. According to Ohm's Law, injection of 0.1 nA current should result in a 100 mV increase in the potential of the membrane surrounded by the electrode tip once a 1 GΩ seal is formed;

$$V = (0.1 \times 10^{-9})\ (1 \times 10^{9}) = 0.1\ V = 100\ mV$$

A high-resistance seal (1 GΩ) was desired to minimize recording error due to leakage of current passing through the membrane/pipette interface and to minimize noise in the recordings. Once a seal was formed, gentle suction was applied to break through this patch of membrane to obtain the whole cell patch clamp configuration.

b) Electrophysiological recordings (figures 2-1 and 2-2):

Electrophysiological recordings were carried out using the voltage clamp technique whereby the membrane potential was held constant ('clamped') while the current flowing through the membrane was measured using an Axoclamp 2A voltage clamp amplifier (Axon Instruments Inc., Foster City, CA). During voltage clamping, a feedback circuit containing a differential amplifier is used to hold the membrane potential

constant by injecting current across the membrane (Hall, 1992). One input to the amplifier is the measured membrane potential and the other is a command potential set by the experimenter. A difference in voltage between these two inputs is amplified several thousand times. Current is then passed through the amplifier output in an attempt to eliminate the difference (Hall, 1992). Because the membrane potential is clamped, the output of the amplifier is a measure of the membrane current in response to a change in potential. Thus, any current flow through the membrane is opposed by an opposite current generated by the feedback amplifier in the voltage clamp circuit.

Membrane potential recordings and current injections were accomplished with discontinuous single-electrode voltage-clamp (dSEVC) technique (sample rate 10-16 kHz). In dSEVC, the tasks of voltage recording and current passing are allocated to the same micropipette. Membrane potential was monitored with a second independent electrode in a previous study and confirmed that the voltage measured by the current-passing electrode provided an accurate measure of the membrane potential during discontinuous voltage clamp (Ferrier & Howlett, 1995). With dSEVC, the Axoclamp-2A amplifier cycles between voltage recording and current passing modes. A sample-and-hold circuit samples the membrane potential and holds the recorded value until the end of a cycle. The recorded membrane potential is compared with a command voltage. The microelectrode then switches into a current passing mode to inject a current directly proportional to the voltage difference between the command and recorded potentials.

Fig. 2-1. Voltage clamp feedback circuit illustrating use of high resistance electrodes. The differential amplifier illustrated by the triangle has two inputs, the command potential (V_c) and the measured membrane potential (V_m). A flow of current is sent through the amplifier output to minimize the difference between these two inputs. In this way, the membrane potential is said to be 'clamped' or held at the command potential. Current is passed from the amplifier to the cell membrane via a high resistance electrode.

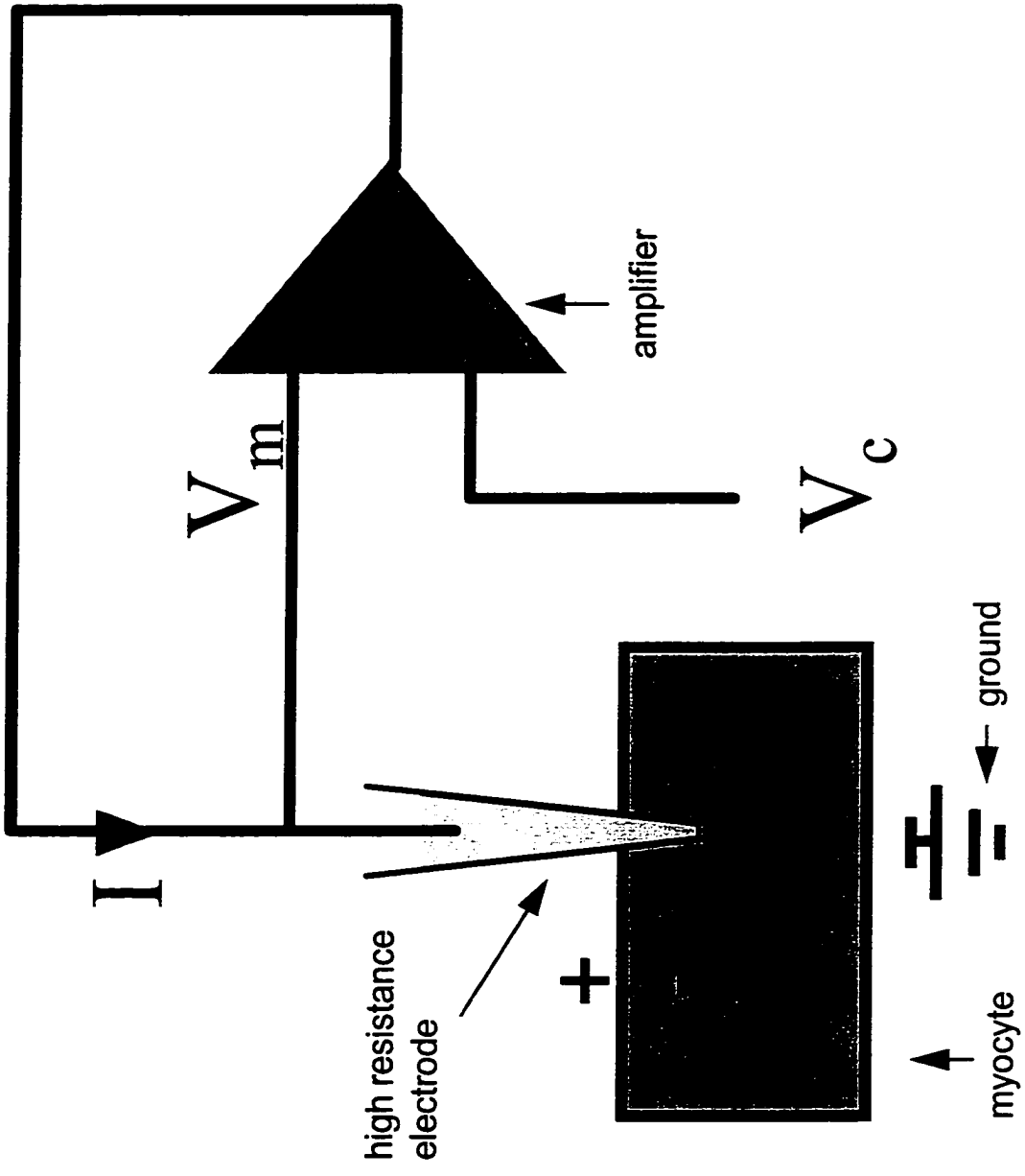

amplifier
V_m
V_c
I
high resistance
electrode
ground
myocyte

Figure 2-1

Fig. 2-2. Voltage-clamp feedback circuit illustrating use of patch electrodes. The feedback circuit is the same as that shown in figure 2-1 except that current is passed from the amplifier to the cell membrane via a low resistance, patch electrode. The large diameter tip of patch electrodes allow dialysis of the intracellular solution with the patch electrode filling solution.

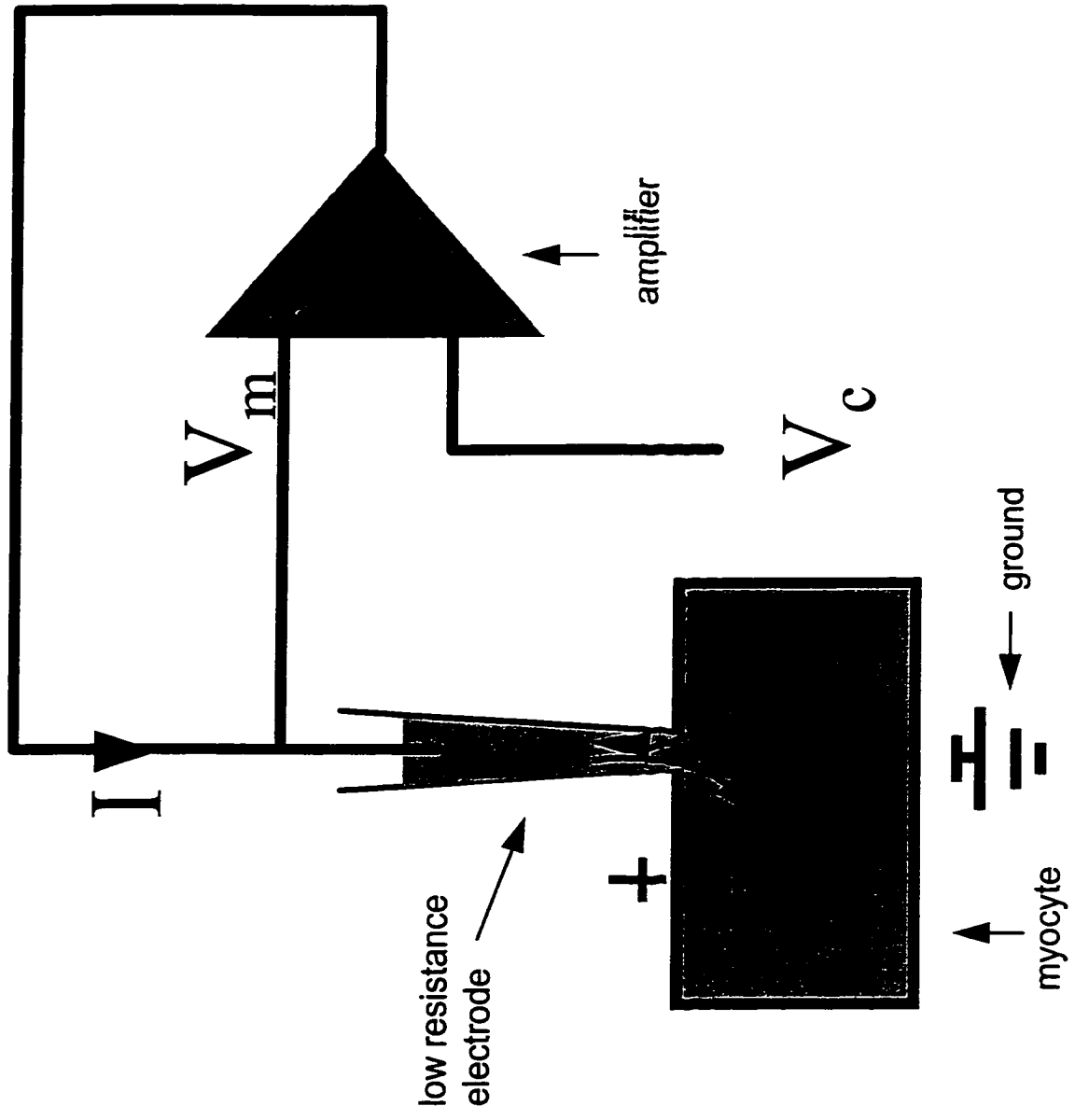


Figure 2-2

Injection of current causes an increase in the voltage recorded. Most of the voltage recorded at this point consists of the voltage drop across the electrode due to passage of current through the pipette resistance. At the end of the current pulse, the microelectrode switches to voltage recording mode again. At this point, the recorded potential decays asymptotically towards the membrane potential because the electrode resistance is no longer in series with the cell. Sufficient time must be allowed for the recorded potential to reach within a millivolt or less of the membrane potential. At the end of the voltage recording period, a new sample of membrane potential is taken and a new cycle begins. The output of this switching circuit was continuously monitored during dSEVC to ensure adequate settling time of the microelectrode for accurate voltage measurement of the cell membrane and that optimal accuracy of voltage control was maintained. This method of voltage clamp minimizes errors produced by the microelectrode resistance in series with the cell. This method has been described in detail (Finkel & Redman, 1984).

The output of the amplifier is an analog signal which provides a measure of the cell's membrane current. Analog-to-digital conversion was undertaken to store the analog data on a microcomputer. Therefore, the output of the amplifier was sent to an Acquisition Logic board (16 channel analog-to-digital (A/D) conversion board, Axon Instruments) which recorded the analog signal at uniform time intervals resulting in an array of data which described the variation in membrane current with changes in membrane potential. Thus, current, voltage and contractions were digitized with the Labmaster A/D interface (TL1-125, Axon Instruments Inc.) at sampling rates up to 50 kHz. The resulting digital signal was sent to a microcomputer which was used for data

acquisition and storage. pClamp software (version 6) was used to generate voltage clamp protocols, and to acquire and analyze data on a computer.

c) Cell shortening measurements:

Cell images were monitored with a closed-circuit television camera with interlace defeat and partial scan capability (Panasonic, Model 1-GP-CD60) and displayed on a television monitor (Hitachi Densi, Model VM-1220C). Unloaded cell shortening was measured with a video edge detector (Cresent Electronics, Sandy, UT, USA) coupled at 120 Hz to the television camera. The output of the video edge detector was sent to an oscilloscope and to the A/D board which digitized the data for storage on a microcomputer. Thus, the cell shortening traces were displayed simultaneously on the microcomputer and on the oscilloscope.

d) Calcium transient measurements:

Intracellular calcium transients were measured using the fluorescent dye fura-2. A 2 ml aliquot of cell suspension was placed in a plastic culture tube and centrifuged in a CL-table top centrifuge (International Clinical Centrifuge, International Equipment Co., Needham, Hts., Mass.) at 5X G for approximately 30 seconds. The supernatant was poured off and the remaining pellet resuspended in 1 ml of extracellular buffer. At this point, the acetoxymethyl ester form of fura-2 (fura-2 AM) was added to the 1 ml cell suspension (2 μl of a 1 μM stock solution). The AM form of the dye is hydrophobic, allowing it to easily cross the cell membrane. Once fura-2-AM was added, cells were set

aside for 20 minutes at room temperature to allow time for the dye to cross the sarcolemma. Inside the cytoplasm, endogenous enzymes cleave the AM ester from the dye, thereby trapping the hydrophilic form of this compound inside the cell. Cells were then placed in an experimental chamber on the stage of an inverted microscope (Olympus Model IMT-2, Tokyo, Japan) for measurement. Any extracellular dye was eliminated by superfusion of the cells in the experimental chamber with extracellular buffer. Cell fluorescence was measured with a Photon Technology International DeltaRAM system (Brunswick, NJ). Cells were excited at 340 nm and fluorescence emitted by the cell was recorded at 510 nm. The emission field was limited to the size of a single myocyte with an adjustable window. Background fluorescence was not subtracted. Changes in intracellular Ca^{2+} were expressed as the ratio of peak fluorescence transient (F) over baseline fluorescence (F_o).

IV) EXPERIMENTAL PROTOCOL:

Once the high resistance electrode tip (microelectrode or patch electrode) was placed near the cell, the resistance was measured. After the resistance was measured, the Axoclamp-2A amplifier was set in discontinuous current clamp (DCC) mode and a 1 nA current applied to the microelectrode. The oscilloscope was set at a fast time base so that the settling time of the microelectrode at the end of current injection could be observed. This waveform was optimally adjusted using the capacitance neutralization dial so that the wave form decayed to baseline before the next current injection cycle. Capacitance neutralization is used to compensate for effects of microelectrode capacitance which can

compromise the performance of the micropipette amplifier. The electrode tip must be placed near the cell for capacitance adjustment as the depth of the micropipette in solution affects its capacitance. The switching rate for current passing and membrane potential recording was set to approximately 11 kHz to allow time for complete decay of the micropipette voltage artifact. This rate was increased to approximately 19 kHz in experiments carried out with low resistance, patch electrodes. The micropipette decay was monitored on an oscilloscope and the capacitance neutralization control adjusted. After these adjustments, the Axoclamp-2A amplifier was set in bridge mode and the injected current removed. The next step was to place the pipette tip on the cell membrane and to penetrate to the inside. Penetration was observed as a drop in potential at the input of the amplifier to near the resting potential of the cell, approximately -90 mV. If current injection greater than 0.5 nA was required to maintain a potential of approximately -80 mV before voltage-clamp, then the impalement was considered unsuccessful. Similarly, if current injection greater than 0.5 nA was required to clamp the membrane at the command potential, then the impalement was considered unsuccessful. This same protocol was followed for patch electrodes as well.

Voltage clamp recordings were made in a successfully impaled cell. The voltage clamp protocols were carried out with a holding potential of -80 mV. The activation steps were preceded by trains of conditioning pulses, usually to 0 mV, to activate L-type Ca^{2+} repetitively and provide a consistent history of activation. The stimulation frequency was 2 Hz. The last conditioning pulse and the activation steps were separated by a repolarization step to a post-conditioning potential (V_{PC}). The duration and voltage

of the V_{PC} is described in the results section for specific protocols. The duration of the activation steps is also described in the results section for specific protocols. Control and drug treated recordings were obtained from each myocyte, thus all drug treated data were compared to their respective control values. In most experiments, 0.2 mM lidocaine was present throughout the experiment to block sodium current. All experiments were conducted at 37 °C.

The peak amplitude of contractions were measured with reference to the baseline cell length just before the activation step. The peak $I_{Ca\text{-}L}$ was measured with reference to the steady-state current at the end of an activation step. Previous studies showed that current measured in this way was blocked by 2 μM verapamil, an established L-type Ca^{2+} channel blocker. The difference current derived by subtracting the current trace in the presence of verapamil from the control trace was identical to the measured $I_{Ca\text{-}L}$, therefore this measurement is an accurate representation of $I_{Ca\text{-}L}$. The accuracy of the measured $I_{Ca\text{-}L}$ also was confirmed with rapid application of 100 μM Cd^{2+} and 0 mM Ca^{2+}. Potassium (K^+) currents were not blocked because the effects of K^+ channel blockers on contractions initiated by the VSRM are not known. As well, previous studies have shown that some K^+ channel blockers (i.e. Cs^{2+}) alter EC-coupling substantially (Wasserstrom & Vites, 1996; Levi *et al.*, 1996b). The rapid kinetics and large amplitude of I_{Na} make it impossible to maintain voltage control when this current is activated. Thus, the curves illustrating the effects of tetracaine on I_{Na} do not give an accurate value for the maximum current, when I_{Na} was large. The dose-response curves for tetracaine become accurate as I_{Na} approaches complete block and the quality of the voltage clamp improves.

V. PHYSIOLOGICAL BUFFERS:

During the isolation procedure, hearts were perfused with nominally Ca^{2+} -free solution. There were two different protocols used for this procedure and each had a specific nominally Ca^{2+} -free solution. The composition of each solution is listed on page 54 and 56. Freshly isolated myocytes were stored at room temperature in a high K^{+} solution listed on page 55. Three different extracellular solutions were used throughout the course of this study, they are listed in tables 1-3 on pages 59 and 60. Two different intracellular patch microelectrode solutions were used throughout this study, they are listed in tables 4 and 5 on pages 64 and 65, respectively.

VI. DRUGS AND REAGENTS:

The drugs used in this study include: lidocaine hydrochloride, tetracaine hydrochloride, nifedipine and cadmium chloride all of which were purchased from Sigma Chemical Co. (Oakville, Ontario, Canada). Ryanodine was purchased from Calbiochem (San Diego, CA, USA) and choline chloride purchased from Fisher Scientific (Fairlawn, NJ, USA). Tetrodotoxin was purchased from Alomone Labs (Jerusalem, Israel). All drugs were dissolved in deionized water except nifedipine which was dissolved in 95 % ethanol.

VII. STATISTICAL ANALYSIS:

All data are presented as mean ± S.E.M. No more than two cells per heart were included in a data set. Statistical differences between two experimental groups were analyzed with a two way repeated measures ANOVA. Multiple comparisons versus control were performed with a Student-Newman-Keuls test. Statistical differences between control and drug treated with a single comparison were analyzed with a paired Student's t-test. A p level less than 0.05 was considered significant for all comparisons. All statistical analyses were performed using SigmaStat software (Jandel Scientific).

RESULTS

Effect of tetracaine on transmembrane currents and contractions. Although tetracaine has been used as a pharmacological tool to characterize E-C coupling in skeletal muscle, it also is a local anesthetic and therefore blocks I_{Na}. The first series of experiments was conducted to determine the effects of tetracaine on I_{Na} and $I_{Ca\text{-}L}$, elicited by a voltage clamp protocol with two sequential activation steps to -40 and 0 mV. Previous studies have shown that in the presence of Na^+ channel blockade, this protocol also sequentially activates contractions triggered by the VSRM and L-type Ca^{2+} current (Ferrier & Howlett, 1995; Howlett, Zhu & Ferrier, 1998). Figure 3-1A shows representative recordings of contractions and currents elicited by this protocol. Under control conditions steps to -40 and 0 mV, from a V_{PC} of -65 mV, activated I_{Na} and $I_{Ca\text{-}L}$, respectively. Both currents were accompanied by rapid, phasic contractions.

Figure 3-1B shows representative traces recorded during exposure of the myocyte to 30 μM tetracaine. This concentration of tetracaine blocked I_{Na}, but $I_{Ca\text{-}L}$ was not affected. The contraction accompanying I_{Na} was abolished whereas, the contraction accompanying $I_{Ca\text{-}L}$ was only slightly reduced in amplitude. Mean data for the effects of increasing concentrations of tetracaine on I_{Na} and $I_{Ca\text{-}L}$ are shown in figure 3-2. The values for peak I_{Na} in the absence of tetracaine underestimate the magnitude of I_{Na}, when I_{Na} is large because of loss of voltage control. However, when the magnitude of peak current is reduced by tetracaine, voltage control improves and measurement of peak I_{Na} becomes accurate. This permitted the concentration of tetracaine required to block I_{Na} to be determined. Tetracaine inhibition of I_{Na} was concentration dependent and complete

Figure 3-1. Effects of tetracaine on contractions and currents in a guinea pig ventricular myocyte. Contractions and currents were initiated by sequential steps to -40 and 0 mV, from a post-conditioning voltage (V_{PC}) of -65 mV (top trace). Panel A shows that the steps to -40 and 0 mV activated I_{Na} and $I_{Ca\text{-}L}$ respectively before exposure to tetracaine. Both currents were accompanied by phasic contractions. Panel B shows contractions and currents elicited during continuous exposure to 30 μM tetracaine. Tetracaine blocked I_{Na} but not $I_{Ca\text{-}L}$. As well, the contraction accompanying I_{Na} was abolished, however the contraction accompanying $I_{Ca\text{-}L}$ was only partially reduced in amplitude.

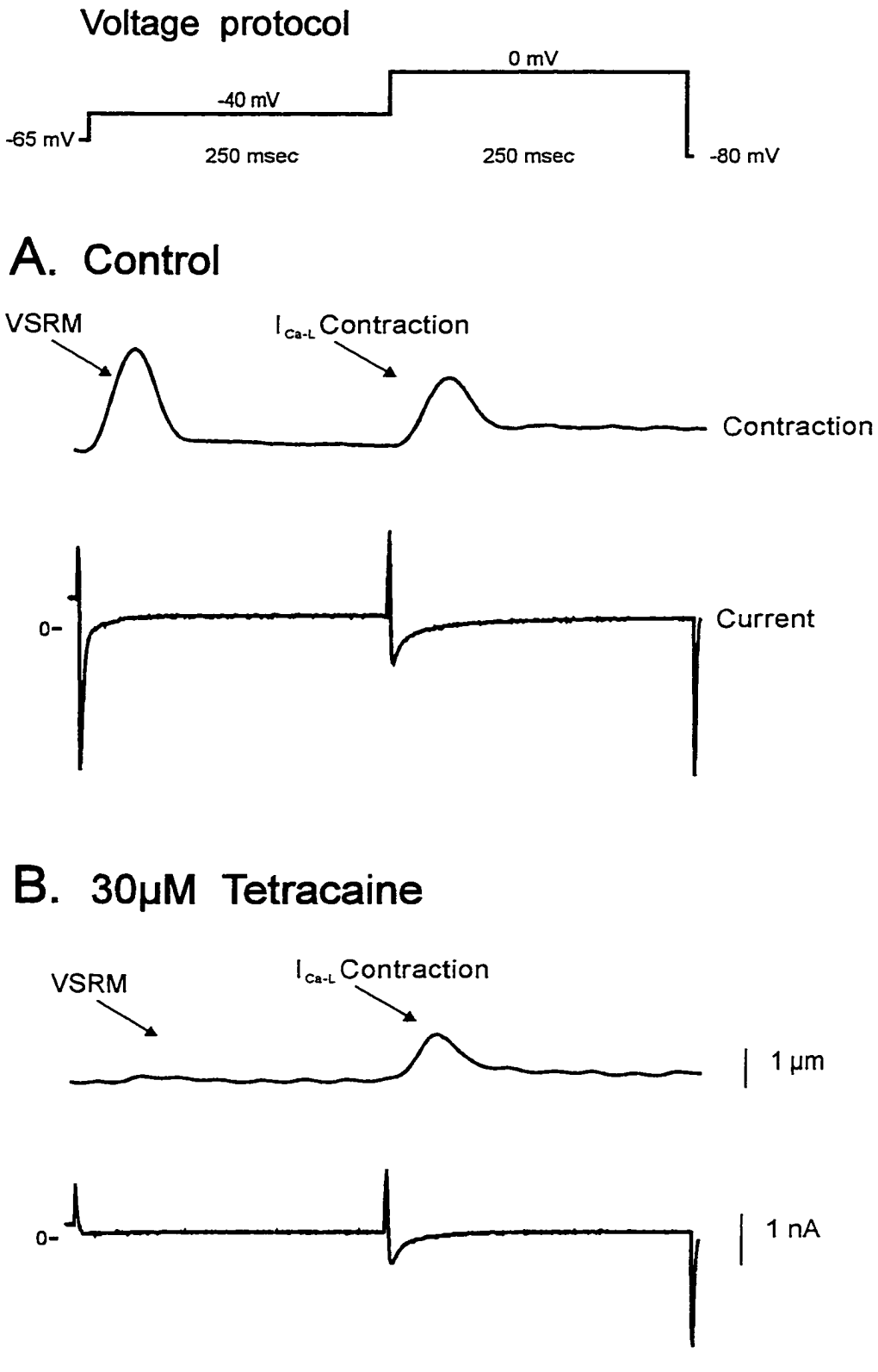

Voltage protocol
0 mV
-40 mV
-65 mV
250 msec
250 msec
-80 mV
A. Control
VSRM
I_{Ca-L} Contraction
Contraction
0-
Current
B. 30μM Tetracaine
VSRM
I_{Ca-L} Contraction
1 μm
0-
1 nA

Figure 3-1

inhibition was observed with a concentration of 30 μM (figure 3-2A). $I_{Ca\text{-}L}$, on the other hand, was virtually unaffected by tetracaine over this same concentration range (figure 3-2B).

Figure 3-2 also shows mean data for effects of tetracaine on contractions from the same cells. The first contraction, elicited by a step to -40 mV, decreased with increasing concentrations of tetracaine and was almost completely inhibited at 30 μM (figure 3-2D). The second contraction, initiated by the step to 0 mV, was virtually unaffected by tetracaine (figure 3-2E). Histograms illustrating the mean effects of 30 μM tetracaine on currents and on both contractions are presented in figures 3-2C and F. Tetracaine blocked current elicited by the step to -40 mV almost completely, whereas the effect on current elicited by the step to 0 mV was not significant. The mean amplitude of contractions elicited by the step to -40 mV also were markedly decreased by 30 μM tetracaine. In contrast, the mean amplitude of contractions elicited by the step to 0 mV was not significantly different from control. These results suggest that tetracaine selectively blocks I_{Na} and the component of contraction activated by the same step.

Is inhibition of the first contraction by tetracaine related to block of I_{Na}? It is not clear whether inhibition of the first contraction by tetracaine is linked to block of I_{Na}. According to Leblanc and Hume (1990) local increases in $[Na^+]_i$ in the vicinity of the Na-Ca_{EX} can cause this pump to work in reverse mode, pumping Ca^{2+} in and Na^+ out of the cell. This influx of Ca^{2+} could then trigger release of stored SR Ca^{2+} for contraction. Thus, inhibition of contraction by tetracaine might be explained by block of I_{Na} if the contraction associated with the step to -40 mV in the present study is caused by reverse

Figure 3-2. Concentration dependence of effects of tetracaine on contractions and currents. The voltage clamp protocol was identical to that in figure 3-1. Tetracaine was pumped continuously through the experimental chamber. Panel A shows that inhibition of I_{Na} was concentration dependent with near complete inhibition at 30 μM tetracaine. Panel B demonstrates that $I_{Ca\text{-}L}$ (elicited by the step to 0 mV) was not significantly affected by tetracaine at these concentrations. The mean peak current amplitudes measured before and during exposure to 30 μM tetracaine are shown in panel C. Inhibition of I_{Na} (elicited by the step to -40 mV) by 30 μM tetracaine was significant (* denotes $p< 0.01$). Panels D-F show the differential effects of tetracaine on the two phasic contractions elicited with each step. Panel D shows the first contraction, at -40 mV (VSRM) decreased with increasing tetracaine concentration and was almost completely inhibited at 30 μM. Panel E shows the second contraction, initiated at 0 mV (CICR) was virtually unaffected by tetracaine. Panel F shows the mean effects of 30 μM tetracaine on both contractions (* denotes $p< 0.05$).

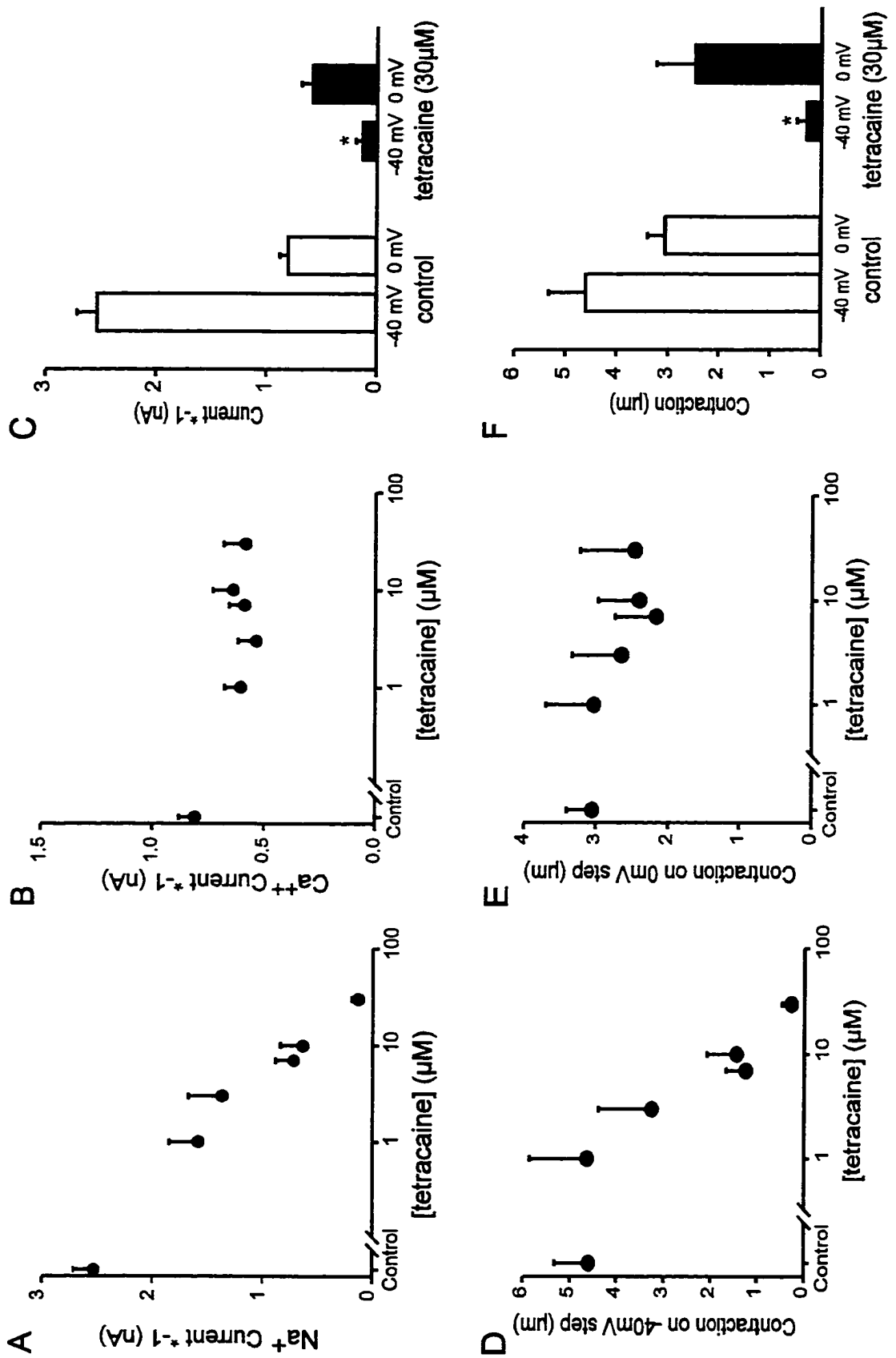

A
Na+ Current *-1 (nA)
[tetracaine] (μM)
Control
B
Ca++ Current *-1 (nA)
[tetracaine] (μM)
Control
C
Current *-1 (nA)
-40 mV
0 mV
control
-40 mV
0 mV
tetracaine (30μM)
D
Contraction on -40mV step (μm)
[tetracaine] (μM)
Control
E
Contraction on 0mV step (μm)
[tetracaine] (μM)
Control
F
Contraction (μm)
-40 mV
0 mV
control
-40 mV
0 mV
tetracaine (30μM)

Figure 3-2

mode Na-Ca_{EX} as described by Leblanc and Hume (Leblanc & Hume, 1990). On the other hand, the contraction associated with the step to -40 mV might be initiated by the VSRM (Ferrier & Howlett, 1995), in which case inhibition by tetracaine would be independent of I_{Na} block. In order to differentiate between these two possibilities, another series of experiments was carried out in the presence of Na^+ channel blockade with lidocaine (0.2 mM) to eliminate CICR in response to Na influx. Continuous exposure of cardiac muscle to high concentrations of local anesthetics results in a negative inotropic effect (Wilson *et al.*, 1993). Therefore, these experiments were conducted with a reduced level of extracellular Na (45 mM NaCl) to counter this negative inotropic effect.

Figure 3-3 shows representative recordings of currents and contractions elicited in the presence of lidocaine. The voltage-clamp protocol was similar to that shown in figure 3-1. Lidocaine eliminated I_{Na}, however, the contraction elicited by the step to -40 mV was still present (figure 3-3A) in agreement with previous results (Ferrier & Howlett, 1995; Howlett, Zhu & Ferrier, 1998). Similar results were observed in all myocytes exposed to lidocaine including the 8 myocytes in this data set. Thus, the contraction elicited by this step cannot be attributed to CICR secondary to rapid Na^+ influx via I_{Na}, and likely represents activation of the VSRM. $I_{Ca\text{-}L}$ and $I_{Ca\text{-}L}$-induced contractions were elicited by the step to 0 mV in the presence of lidocaine. Figure 3-3B shows the effects of 100 μM tetracaine in the presence of lidocaine. Tetracaine strongly inhibited the VSRM contraction, while $I_{Ca\text{-}L}$ and the $I_{Ca\text{-}L}$ -induced contraction were virtually unaffected. Figure 3-3C shows partial recovery of the VSRM contraction after return to solution containing lidocaine but not tetracaine, for approximately 10 minutes. Mean data for the

Figure 3-3. Inhibition of the VSRM contraction by tetracaine is not caused by block of I_{Na}. The voltage clamp protocol was the same as in figure 3-1. Panel A was recorded in the presence of 200 μM lidocaine. Lidocaine blocked I_{Na} but not the VSRM contraction. $I_{Ca\text{-}L}$ and $I_{Ca\text{-}L}$-induced contractions also remained. Panel B demonstrates that continuous exposure (through the experimental chamber) to 100 μM tetracaine strongly inhibited the VSRM contraction, while $I_{Ca\text{-}L}$ and $I_{Ca\text{-}L}$-induced contractions were unaffected. Panel C shows that the effects of tetracaine on VSRM contractions reverses with washout of tetracaine. Panel D shows the mean contraction amplitudes before and during exposure to 100 μM tetracaine in the presence of lidocaine (n=8 myocytes). The results demonstrate that the VSRM contractions were significantly inhibited (* denotes $p< 0.01$) while the $I_{Ca\text{-}L}$-induced contractions were not significantly affected.

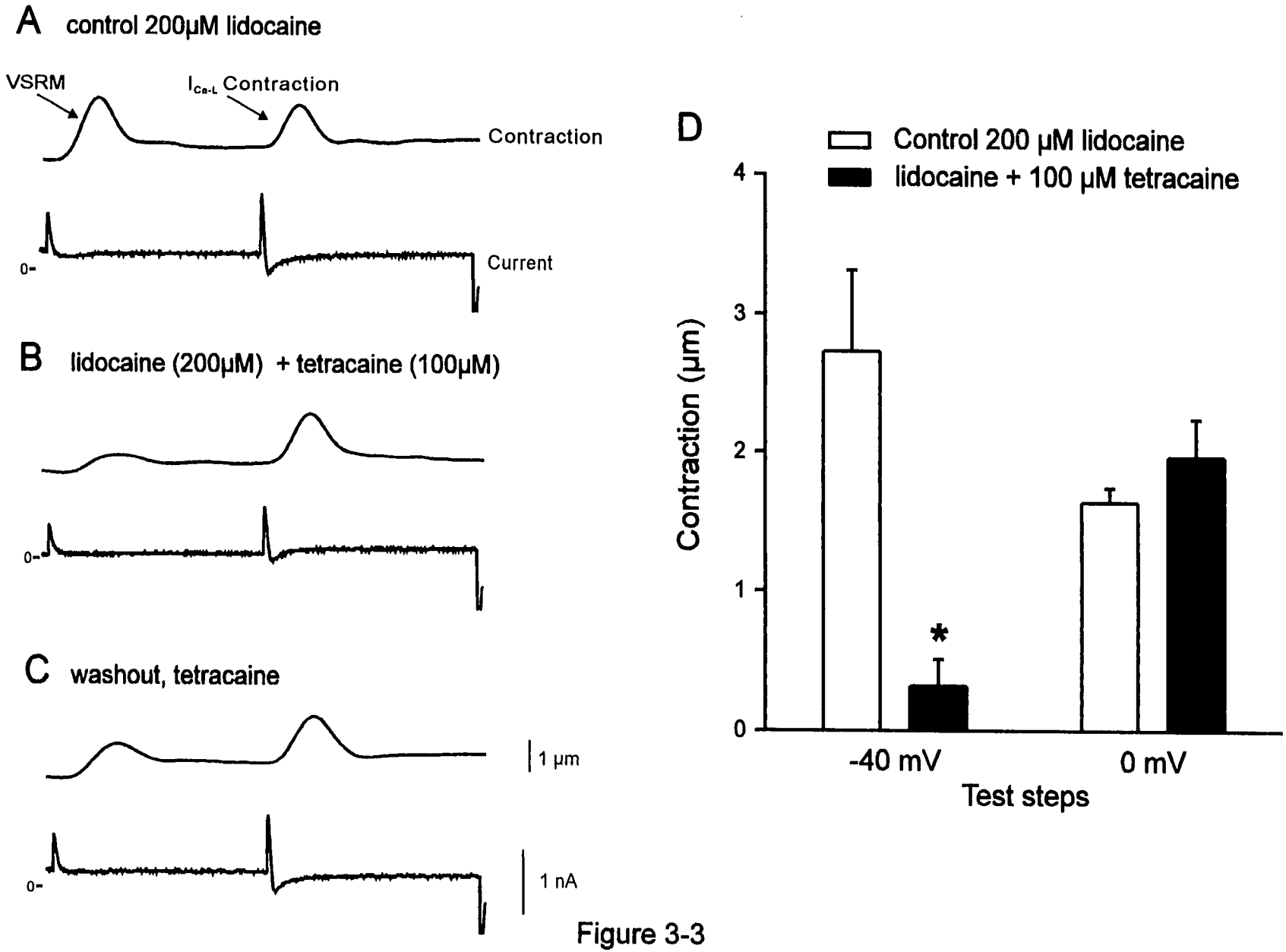

A control 200μM lidocaine
VSRM
I_{Ca-L} Contraction
Contraction
Current
0-
B lidocaine (200μM) + tetracaine (100μM)
0-
C washout, tetracaine
1 μm
1 nA
0-
D
Control 200 μM lidocaine
lidocaine + 100 μM tetracaine
Contraction (μm)
4
3
2
1
0
*
-40 mV
0 mV
Test steps

Figure 3-3

effects of tetracaine in the presence of lidocaine are presented in figure 3-3D. The VSRM contraction elicited by the step to -40 mV was strongly and significantly inhibited by tetracaine, while the $I_{Ca\text{-}L}$-induced contraction initiated by the step to 0 mV was not significantly different from control. Thus, these results demonstrate that tetracaine inhibits the VSRM component of contraction by an action independent of I_{Na} blockade.

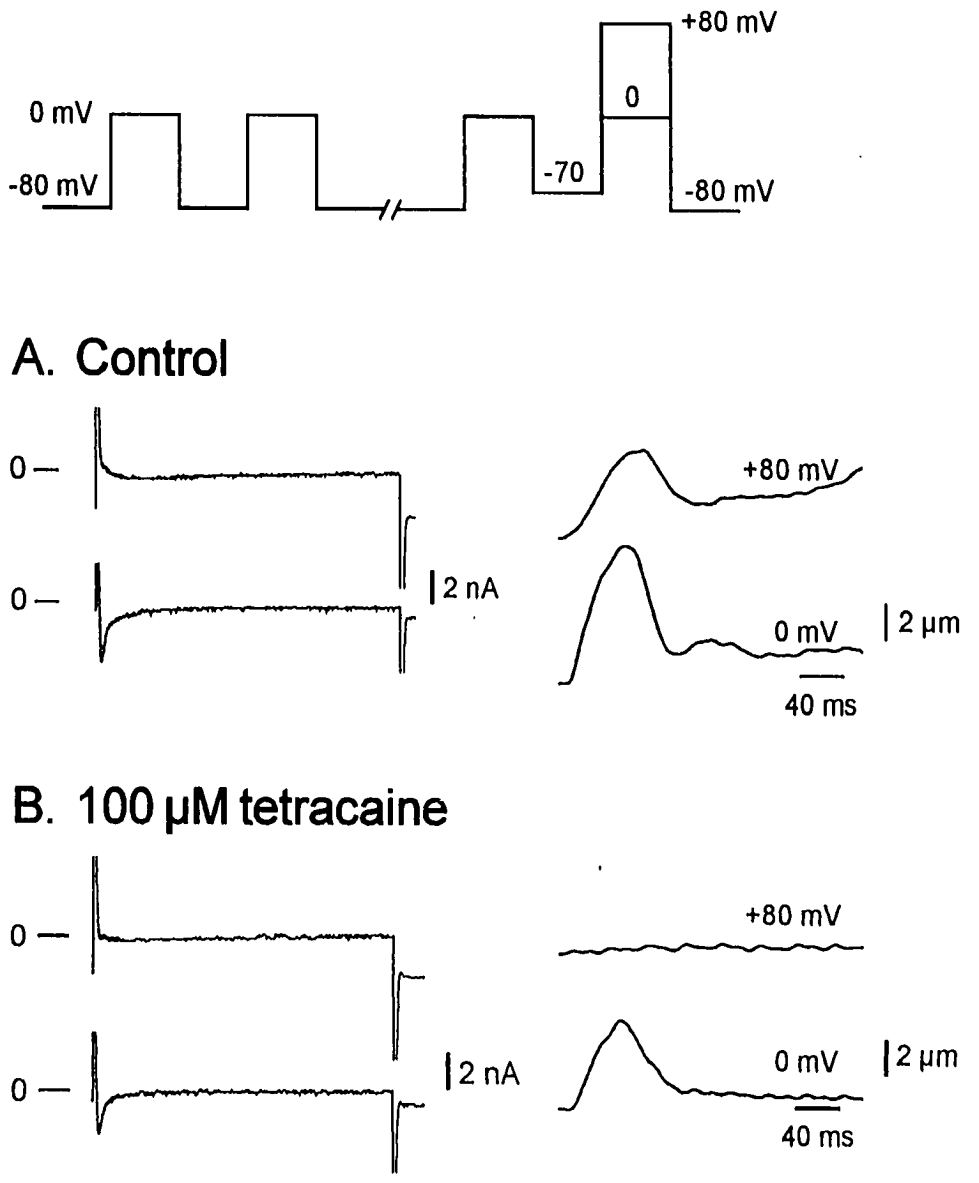
Effects of tetracaine on contraction-voltage (CV) and current-voltage (IV) relationships. CV and IV curves were determined in 13 experiments carried out under the same conditions as the experiment illustrated in figure 3-3. The voltage clamp protocol is illustrated at the top left of figure 3-4. Contractions and currents were initiated by test steps from a V_{PC} of -70 mV which allows activation of both VSRM and CICR (Howlett, Zhu & Ferrier, 1998). With each repetition of the voltage clamp protocol, the test step was made progressively more positive in 10 mV increments. As well, each test step was preceded by a train of 10 conditioning pulses.

Figure 3-4A shows representative traces of contractions and currents elicited with the steps to 0 mV and +80 mV under control conditions. Contractions were elicited by steps to each of these potentials, however, inward current was present only with the step to 0 mV as the step to +80 mV is beyond the reversal potential for $I_{Ca\text{-}L}$. Thus, the contraction accompanying the step to +80 mV was independent of inward current. Figure 3-4B shows representative traces of contractions and currents occurring with steps to 0 mV and +80 mV in the presence of 100 μM tetracaine. There was both a contraction and an inward current elicited by the step to 0 mV in the presence of tetracaine, similar to the control trace. However, the contraction elicited by the step to +80 mV in control was

abolished by tetracaine. There was no inward current present with the step to +80 mV, as in the control trace. Figure 3-4C shows mean CV curves before and after treatment with tetracaine. Under control conditions the CV relation was sigmoidal with contractions appearing at -60 mV and reaching a plateau near -20 mV. In the presence of tetracaine (100 μM) the CV relation became bell-shaped and the threshold for activation was shifted 10 mV to the right (-50 mV). Although the CV curve was bell-shaped the maximum amplitude of contraction was similar before and after tetracaine treatment. Figure 3-4D shows the mean IV relationships for the same myocytes. Under control conditions the IV relation was bell-shaped with a peak at -10 mV. In the presence of tetracaine the initial portion of the IV relation was shifted 10 mV to the right. Otherwise the IV relationships were similar before and after treatment with tetracaine.

The observation that the CV relationship in the presence of tetracaine became bell-shaped reflecting the IV relationship for $I_{Ca\text{-}L}$ suggests that tetracaine-insensitive contractions represent CICR coupled to $I_{Ca\text{-}L}$. These results also suggest that inhibition of contraction by tetracaine cannot be explained by inhibition of $I_{Ca\text{-}L}$ as there was very little change in the IV relationship in the presence of tetracaine. In fact, tetracaine-sensitive contractions were elicited in the absence of measurable inward current which is characteristic of activation of the VSRM (Ferrier & Howlett, 1995; Ferrier *et al.*, 1998; Hobai *et al.*, 1997; Howlett & Ferrier, 1997; Howlett, Zhu & Ferrier, 1998). Together these results suggest that tetracaine causes inhibition of the VSRM component of contraction with little effect on inward current or CICR contractions.

Figure 3-4. Effect of tetracaine on contraction-voltage (CV) and current-voltage (IV) relations. The voltage clamp protocol is shown at the top of panel A. Contractions and currents were initiated by test steps that increased in 10 mV increments to +80 mV from a V_{PC} of -70 mV. Panel A shows representative traces of currents and contractions elicited by test steps to 0 mV and +80 mV under control conditions. The step to 0 mV elicited an inward current as well as a contraction. The step to +80 mV also elicited a contraction, however, inward current was absent as this step is beyond the reversal potential for $I_{Ca\text{-}L}$. Panel B shows representative traces of currents and contractions elicited in the presence of 100 μM tetracaine applied continuously through the experimental chamber. The step to 0 mV again elicited an inward current as well as a contraction. In the presence of tetracaine, the step to +80 mV no longer elicited a contraction. Panel C shows mean contraction-voltage curves. Under control conditions, in the presence of 200 μM lidocaine, the CV relation was sigmoidal. Contractions first appeared at -60 mV and reached a plateau near -20 mV. In the presence of tetracaine (100 μM), the CV relation became bell-shaped and proportional to $I_{Ca\text{-}L}$. Panel D shows that the IV relations in control and in the presence of tetracaine were bell-shaped (* denotes $p < 0.05$ with respect to controls). (n=13 myocytes; mean length of tetracaine exposure was 14 minutes).


+80 mV
0 mV
0
-80 mV
-70
-80 mV
A. Control
0
0
2 nA
+80 mV
0 mV
2 µm
40 ms
B. 100 µM tetracaine
0
0
2 nA
+80 mV
0 mV
2 µm
40 ms

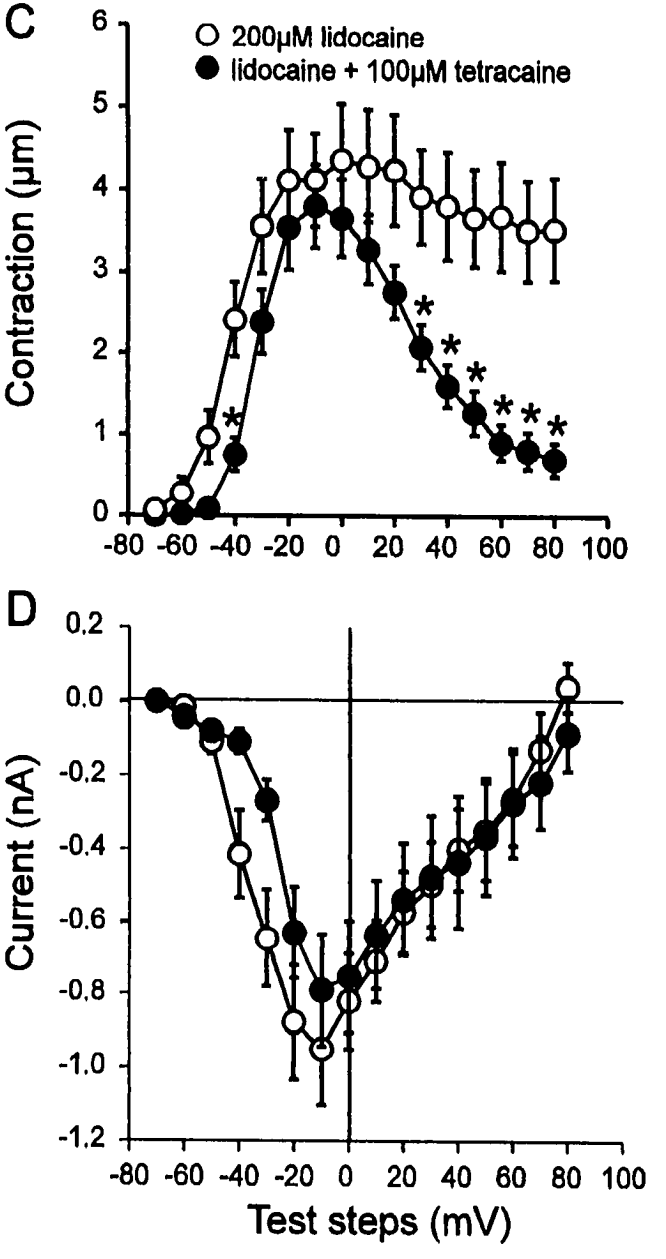
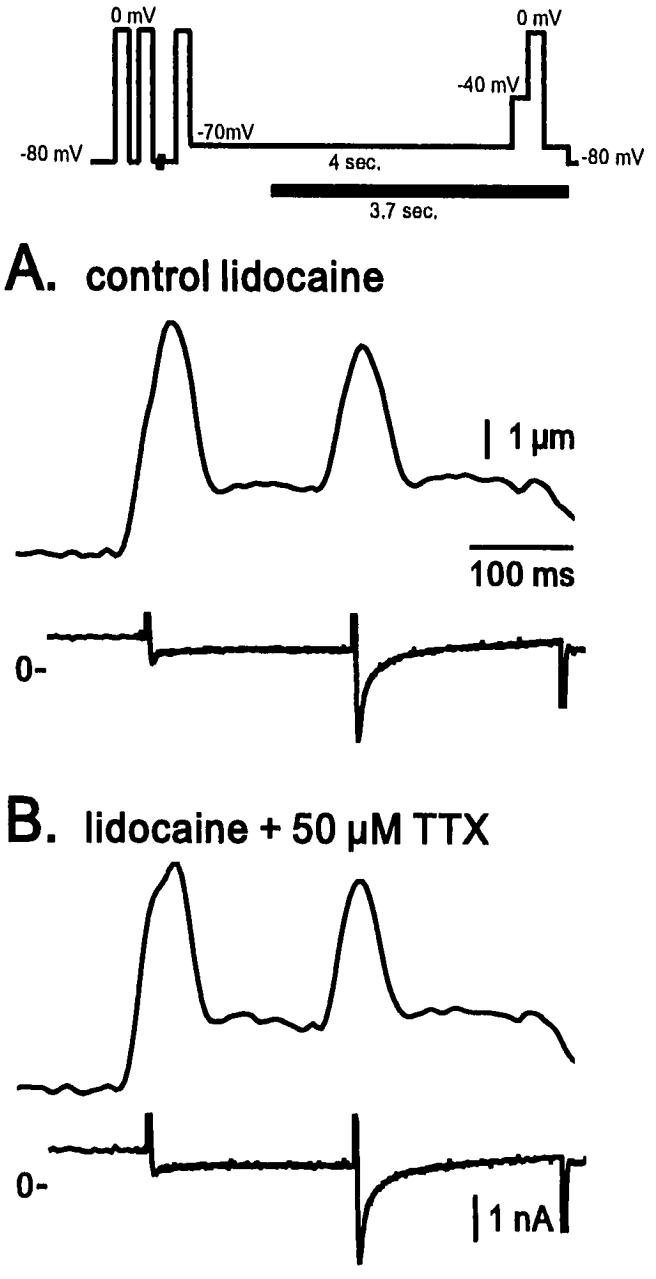

C
200µM lidocaine
lidocaine + 100µM tetracaine
Contraction (µm)
6
5
4
3
2
1
0
-80 -60 -40 -20 0 20 40 60 80 100
D
Current (nA)
0.2
0.0
-0.2
-0.4
-0.6
-0.8
-1.0
-1.2
-80 -60 -40 -20 0 20 40 60 80 100
Test steps (mV)

Figure 3-4

Will TTX, substituted for tetracaine, inhibit the VSRM? In the preceding experiments the effects of tetracaine were observed in the presence of lidocaine. However, inhibition of Na^+ channels with lidocaine is both use and voltage dependent. If block of I_{Na} with lidocaine was less than complete, it still might be possible that the contraction elicited by the step to -40 mV was initiated by CICR coupled to stimulation of reverse Na-Ca_{EX} by Na^+ influx, as described by Leblanc and Hume (1990). As well, recent studies have shown that under some conditions selectivity of Na^+ channels can be altered in such a way as to allow Ca^{2+} entry (Santana *et al.*, 1998). Therefore, incomplete block of Na^+ channels by lidocaine in the present study might result in CICR contractions induced by Ca^{2+} influx through open Na^+ channels. In both of these situations, tetracaine might block the contraction through additional blockade of I_{Na}. Therefore, the effects of adding 50 μM tetrodotoxin (TTX) (Cohen *et al.*, 1981), instead of tetracaine, in the presence of lidocaine were evaluated. The TTX solution was applied with a rapid solution changer. With this device solution containing 50 μM TTX could be applied to a myocyte just before the activation steps. The voltage clamp protocol used in these experiments is shown at the top of figure 3-5. The protocol utilized trains of conditioning pulses followed by sequential test steps to -40 and 0 mV as in earlier experiments however, the V_{PC} was lengthened to 4 sec. The switch to 50 μM TTX was made 3 sec in advance of the test step to -40 mV, to allow time for complete switch of solutions (approximately 300 ms, assessed with solutions with different concentrations of K^+, not illustrated) and equilibration of t-tubules (approximately 1.5 seconds) (Blatter & Niggli, 1997).

Figure 3-5. The VSRM contraction persists in the presence of Na^+ channel blockade with lidocaine plus tetrodotoxin. The voltage clamp protocol is illustrated above panel A. Panel A. In the presence of 200 μM lidocaine a step to –40 mV elicited a VSRM contraction but little current. The $I_{Ca\text{-}L}$ and $I_{Ca\text{-}L}$-induced contraction were observed with the step to 0 mV. Panel B demonstrates that rapid application of 50 μM TTX (3 seconds before and during test steps) in addition to lidocaine, had no effect on the VSRM contraction. $I_{Ca\text{-}L}$-induced contractions and $I_{Ca\text{-}L}$ were unaffected as well. Panel C shows the mean contraction and current amplitudes in the presence and absence of TTX (n=28 myocytes). TTX caused significant inhibition of residual I_{Na} with the step to -40 mV (* denotes $p< 0.001$), however, there was no significant change in the amplitude of VSRM or $I_{Ca\text{-}L}$-induced contractions, or $I_{Ca\text{-}L}$.


0 mV
0 mV
-40 mV
-70mV
-80 mV
4 sec.
-80 mV
3.7 sec.
A. control lidocaine
1 µm
100 ms
0-
B. lidocaine + 50 µM TTX
0-
1 nA

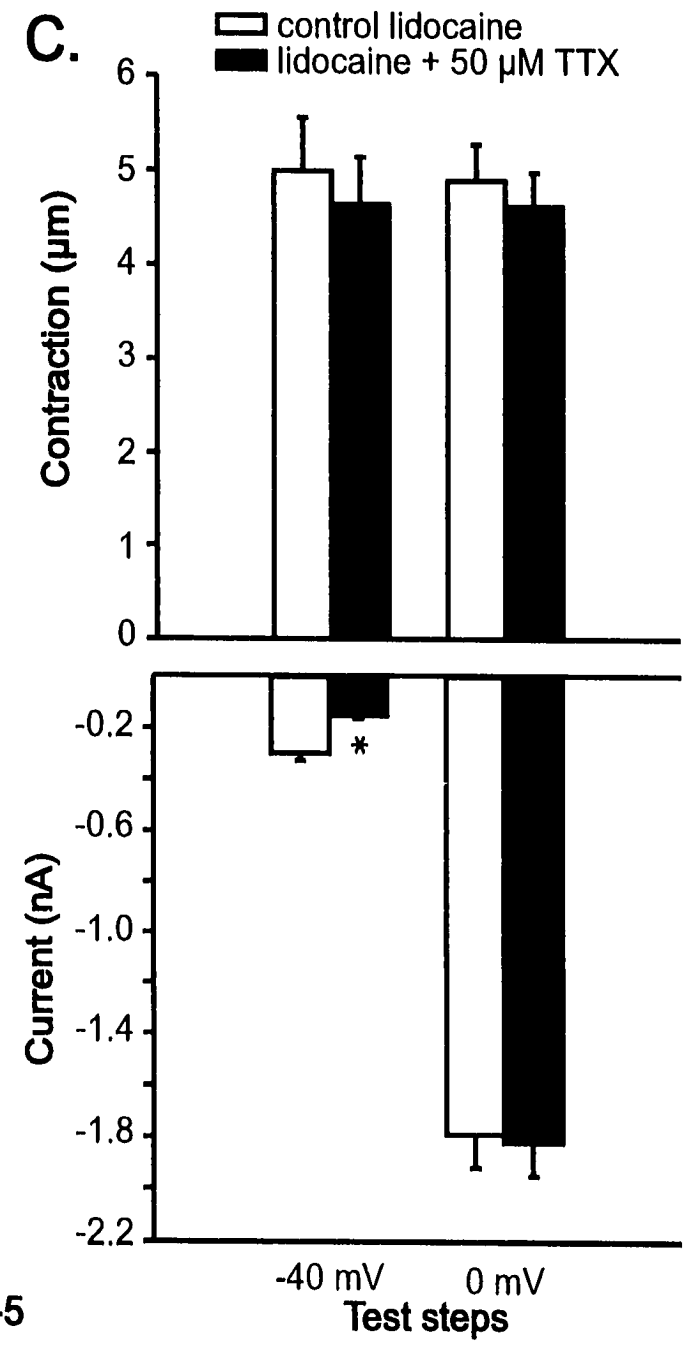

C.
control lidocaine
lidocaine + 50 µM TTX
Contraction (µm)
6
5
4
3
2
1
0
Current (nA)
-0.2
-0.6
-1.0
-1.4
-1.8
-2.2
*
-40 mV
0 mV
Test steps

Figure 3-5

Figure 3-5A shows contractions and currents elicited by the steps to -40 and 0 mV in the presence of 200 μM lidocaine. Inward $I_{Ca\text{-}L}$ was observed with the step to 0 mV. Figure 3-5B shows that addition of TTX (50 μM) to the lidocaine solution had no effect on the contraction associated with the step to -40 mV. There was also no significant change in the $I_{Ca\text{-}L}$ or $I_{Ca\text{-}L}$-induced contractions. The addition of TTX did block some residual inward current associated with the step to -40 mV, suggesting that this agent does provide a more effective block of Na channels compared to lidocaine. Similar results were observed in 28 myocytes in which I_{Na} was blocked with lidocaine plus TTX. Mean data presented in figure 3-5C show that TTX caused a small but significant decrease in current elicited by the step to -40 mV, but did not affect contractions. Thus, it is unlikely that the contractions initiated by the steps to -40 mV are related to incomplete block of I_{Na} by lidocaine.

Figure 3-6 illustrates CV and IV relationships obtained in the presence and absence of TTX. Here TTX was applied continuously throughout test steps and conditioning pulses through the rapid switcher rather than just before and during test steps. Lidocaine was present throughout. The voltage clamp protocol was the same as shown at the top of figure 3-4. Figure 3-6A shows mean CV relations in the presence and absence of TTX. Under control conditions, the CV relation was sigmoidal with contractions appearing at -60 mV and reaching a plateau at -20 mV. In the presence of TTX, the CV relationship was identical to the control. Figure 3-6B shows mean IV relations in the presence and absence of TTX. Under control conditions, the IV relation was bell-shaped and reached a peak at -10 mV and declined to 0 at +80 mV. In the

Figure 3-6. Effects of 50 μM TTX on contraction-voltage and current-voltage relations. The voltage clamp protocol was identical to that shown in figure 3-2. Lidocaine was present throughout. Panel A shows that there was no change in the CV relationship in the presence of TTX compared to control. TTX was applied continuously throughout conditioning pluses and test steps. The CV relation remained sigmoidal with contractions appearing at –60 mV and reaching a plateau at –20 mV. Panel B shows that, in the presence of TTX, the IV relation remained bell-shaped with the same maximum amplitude. TTX caused a 10 mV shift in the IV relationship at negative potentials suggesting that the initial portion of this curve represents residual I_{Na}. (n=16 myocytes) (* denotes $p<0.05$)

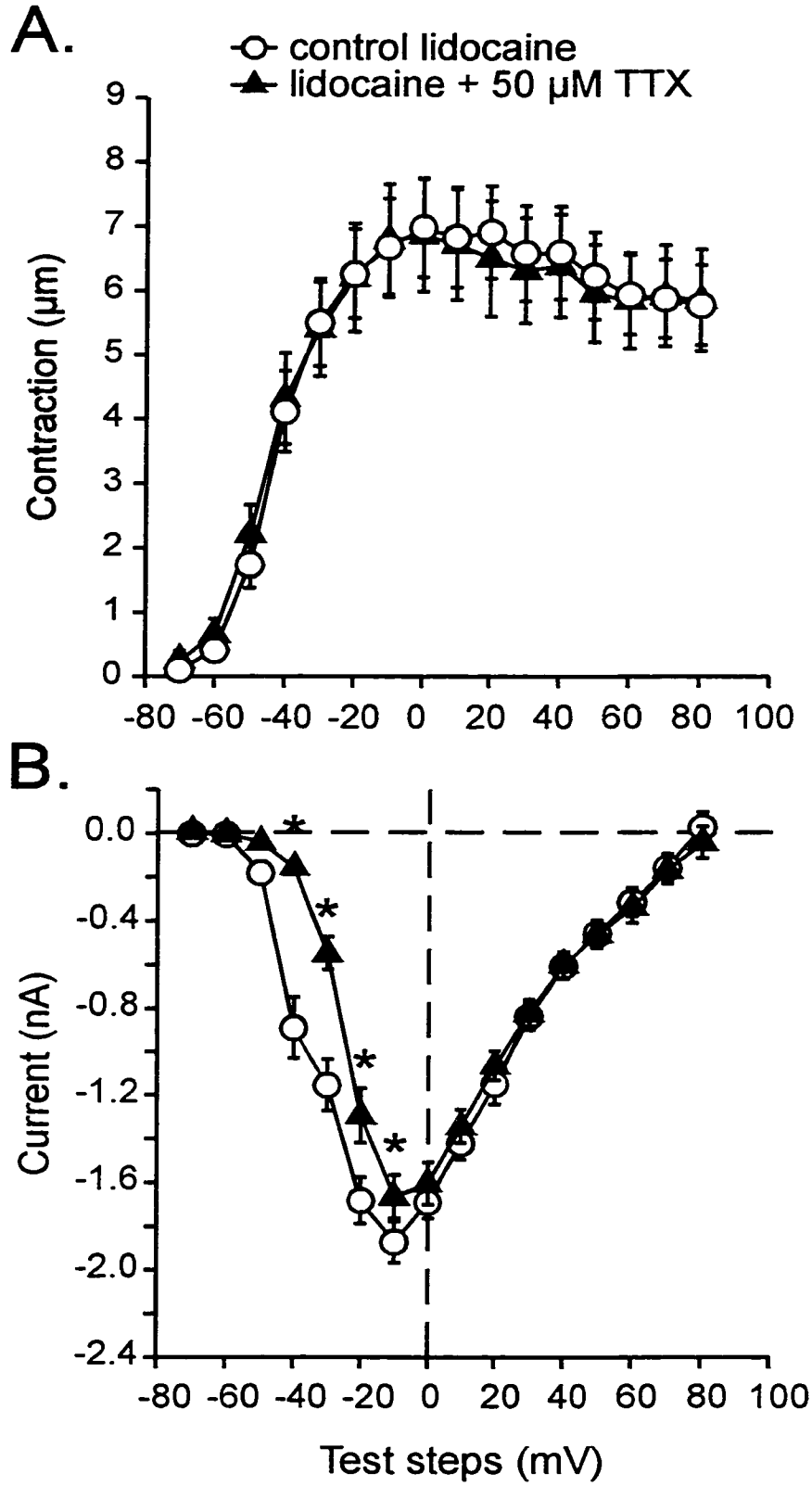

A.
control lidocaine
lidocaine + 50 µM TTX
Contraction (µm)
9
8
7
6
5
4
3
2
1
0
-80 -60 -40 -20 0 20 40 60 80 100
B.
0.0
-0.4
-0.8
-1.2
-1.6
-2.0
-2.4
Current (nA)
*
*
*
*
-80 -60 -40 -20 0 20 40 60 80 100
Test steps (mV)

Figure 3-6

presence of TTX, the IV relation was similar to control except that the initial portion of the IV relationship was shifted approximately +10 mV. This shift was similar to that observed with tetracaine (figure 3-4D). These results suggest that TTX and tetracaine may inhibit a residual I_{Na} activated at negative membrane potentials. However, inhibition of this residual current by TTX had no effect on the CV relationship. This finding with TTX contrasts sharply with the marked effects of tetracaine on the CV relationship (figure 3-4C). Thus, these observations demonstrate that TTX cannot be substituted for tetracaine to inhibit the VSRM.

Are the effects of tetracaine on the VSRM component of contraction related to blockade of residual I_{Na}? The results shown in figures 3-4 and 3-5 indicate that TTX does block some residual I_{Na} in the presence of lidocaine. Tetracaine also was found to cause a similar 10 mV shift in the IV relationship (figure 3-4) in the presence of lidocaine. This shift also may represent blockade of residual I_{Na} by tetracaine. Thus, experiments were conducted to determine whether blockade of residual I_{Na} contributed to the effects of tetracaine on the VSRM component of contraction. To accomplish this CV and IV relations were determined in the presence and absence of tetracaine (300 μM) with lidocaine and TTX present throughout. Solutions were applied continuously with the rapid switcher for the duration of the protocol rather than just before the test steps. The concentration of tetracaine was increased to 300 μM because 100 μM did not result in the CV relation returning to baseline at very positive test steps (figure 3-4C). This might indicate that the VSRM is not completely inhibited by 100 μM tetracaine.

Figure 3-7A shows that 300 μM tetracaine caused the CV relation to become bell shaped. In the presence of tetracaine, contraction reached the same maximum amplitude as in control, but approached baseline at positive potentials. Tetracaine, also shifted the threshold for contraction approximately +10mV. Figure 3-7B shows that in the presence of lidocaine plus TTX, tetracaine no longer shifted the IV relation at negative membrane potentials. This suggests that the shift in the absence of TTX, illustrated in figure 3-4, represented inhibition of residual I_{Na}. Thus, our observations with TTX (figure 3-6) and with TTX plus tetracaine (figure 3-7) demonstrate that the effects of tetracaine on the VSRM component of contraction are independent of inhibition of Na^+ current by this agent.

In an earlier study it was shown that the amplitude of contraction is not proportional to $I_{Ca\text{-}L}$ when the VSRM is available for activation (Ferrier & Howlett, 1995; Howlett, Zhu & Ferrier, 1998). However, contraction becomes proportional to $I_{Ca\text{-}L}$ when the VSRM is inactivated through its steady-state inactivation properties (Ferrier & Howlett, 1995). A similar difference might be expected when the VSRM is inhibited with tetracaine. The effects of tetracaine on the relationship between contraction and inward current for the experiments illustrated in figures 3-7A and B are shown in figure 3-7C. In the presence of lidocaine and TTX contraction first appeared before any current was detected. Then, with greater depolarizations, contraction increased progressively as the inward current increased to a maximum. However, for test steps positive to the peak of the IV relation, current decreased progressively but contractions remained large. This resulted in a marked hysteresis in the control contraction-current relationship. For points

Figure 3-7. Effects of 300 μM tetracaine, in the presence of lidocaine plus TTX, on the relation between contraction and current. The voltage clamp protocol was identical to that shown in figure 3-2. Lidocaine and 50 μM TTX were present throughout. Panel A shows that 300 μM tetracaine, applied continuously, changed the shape of the CV relationship from sigmoidal to bell-shaped (* denotes $p< 0.05$ with respect to controls). The threshold for activation of contraction shifted 10 mV more positive in the presence of tetracaine. Panel B shows that there was no significant change in the IV relation in the presence or absence of tetracaine. Panel C illustrates the relationship between contraction and current under control (lidocaine plus TTX) and tetracaine treated conditions. Under control conditions, the amplitude of contractions was not closely related to amplitude of currents. In the presence of tetracaine, contractions were proportional to inward current. (n=11 myocytes; mean duration of exposure to tetracaine 2 minutes)

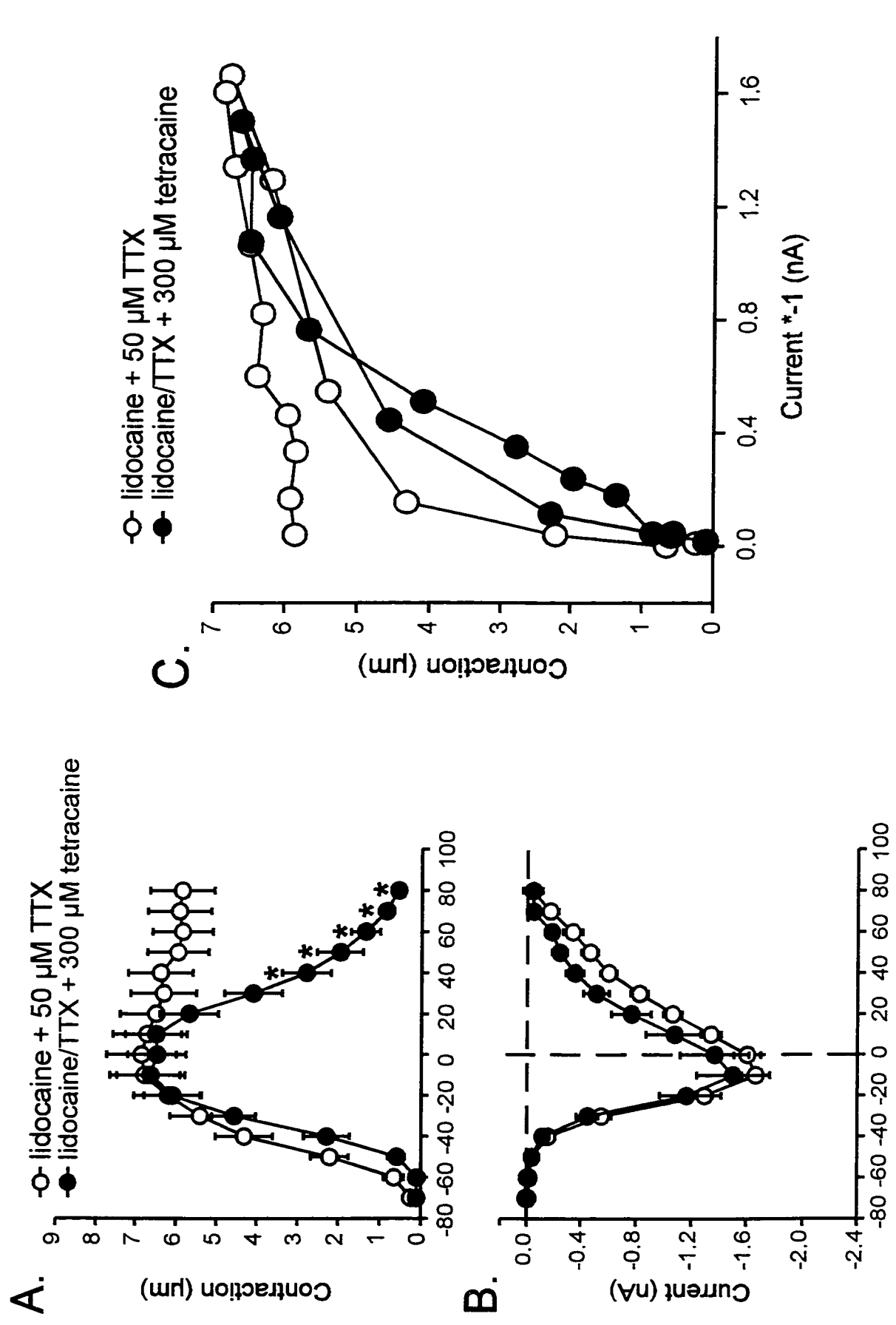

A.
lidocaine + 50 µM TTX
lidocaine/TTX + 300 µM tetracaine
Contraction (µm)
Test steps (mV)
B.
Current (nA)
Test steps (mV)
C.
lidocaine + 50 µM TTX
lidocaine/TTX + 300 µM tetracaine
Contraction (µm)
Current *-1 (nA)

Figure 3-7

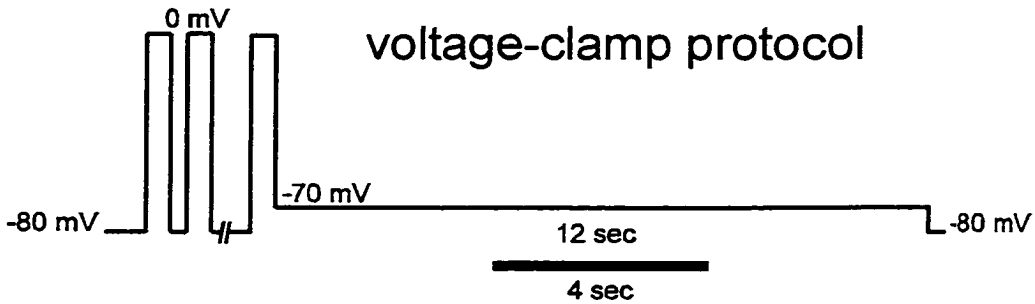
corresponding to the most positive steps, current again was negligible. When the myocytes were exposed to 300 μM tetracaine, in addition to lidocaine and TTX, contractions only appeared when measurable current was activated. The contraction-current curve after tetracaine exhibited little hysteresis and closely reflected back on itself as the magnitude of $I_{Ca\text{-}L}$ reached maximum and then decreased. These plots show that contraction became proportional to current in the presence of tetracaine. Thus, tetracaine inhibited the component of contraction which was not proportional to $I_{Ca\text{-}L}$.

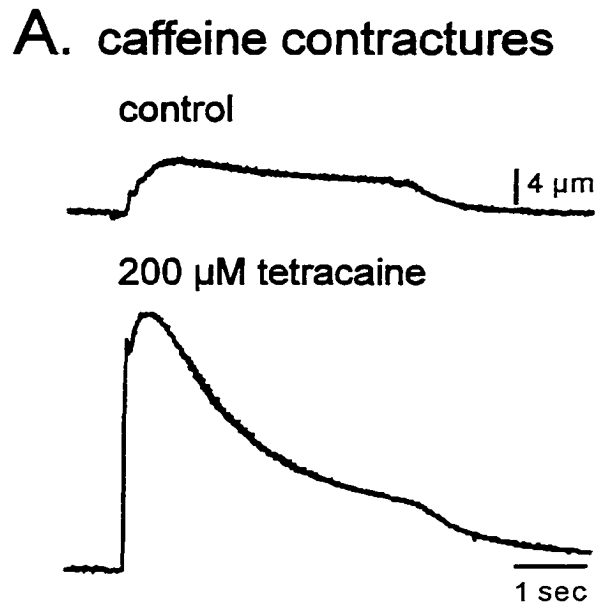
Are the effects of tetracaine accompanied by changes in SR Ca^{2+} load? One might propose that the effects of tetracaine on VSRM contractions and CV curves might reflect depletion of SR Ca^{2+} stores by tetracaine. However, studies by Overend *et al* (Overend, Eisner & O'Neill, 1997; Overend, O'Neill, Eisner, 1998) demonstrated that tetracaine actually increased SR Ca^{2+} stores in rat myocytes at 22 °C. In the present study an increase in SR stores by tetracaine would be opposite to what one would predict on the basis of the effects of this agent on contractions. This discrepancy might be explained if tetracaine did not have the same effects on SR stores in guinea-pig myocytes at 37 °C as was observed in rat myocytes at 22 °C. Therefore, the effects of this agent on SR Ca^{2+} stores under the present experimental conditions were investigated. Figure 3-8 shows the effects of tetracaine on SR Ca^{2+} stores, assessed with rapid application of 10 mM caffeine (Rousseau & Meissner, 1989; O'Neill, Donoso & Eisner, 1990; Bers, 1991) before and during continuous exposure of myocytes to tetracaine. At this concentration caffeine is known to cause opening of SR Ca^{2+} release channels and efflux of stored Ca^{2+} which produces a large caffeine contracture (Rousseau, & Meissner, 1989; O'Neill, Donoso &

Eisner, 1990; Bers, 1991). The amplitude of this contracture can be used as an index of SR Ca^{2+} content (Endo, 1975; Fabiato, 1985c; Fabiato & Fabiato, 1975, 1978; Bers, 1987; O'Neill, Donoso & Eisner, 1990). The protocol for caffeine application is illustrated at the top of figure 3-8. A train of conditioning pulses to 0 mV was followed by a 12 second step to -70 mV. During the step -70 mV, caffeine was applied through the rapid solution switcher for a period of 4 seconds. Under control conditions caffeine caused a contracture that reached a peak then fell to a steady-state, before declining to baseline at the end of the caffeine application (panel A, top). When caffeine was applied in the presence of tetracaine (mean length of exposure 5 minutes), the peak of the caffeine contracture was approximately 4 times larger than control (panel A, bottom). Mean data are presented in figure 3-8B. These results indicate that tetracaine did not deplete SR Ca^{2+} stores, but rather caused a significant increase, similar to that reported by Overend *et al* (1998). Similar results were observed in two additional experiments with 300 μM tetracaine (not illustrated). Therefore, a mechanism of depletion of SR Ca^{2+} stores cannot be used to explain inhibition of the VSRM by tetracaine.

Does the bell-shaped CV relation remaining in the presence of tetracaine represent CICR coupled to $I_{Ca\text{-}L}$? The foregoing observations suggest that tetracaine inhibits the VSRM and increases SR Ca^{2+} stores. Thus, although the peak amplitude of the CV relation was not reduced, the CV relation remaining in the presence of tetracaine may be generated entirely by contractions initiated by CICR. We therefore, determined experimentally whether contractions in the presence of tetracaine were caused by SR Ca^{2+} release triggered by $I_{Ca\text{-}L}$. To test involvement of SR release we used ryanodine. At low

Figure 3-8. Inhibition of the VSRM by tetracaine is accompanied by elevation of SR Ca^{2+} stores. The voltage clamp protocol is shown at the top. The V_{PC} was held at -70 mV for 12 seconds before returning to the holding potential of -80 mV. During the V_{PC} 10 mM caffeine was applied for a period of 4 seconds. The resulting contracture was used as an index of SR Ca^{2+} stores. Panel A, top, shows the amplitude of the caffeine contracture under control conditions. Panel A, bottom, demonstrates a four fold increase in the amplitude of the caffeine contracture in the presence of 200 μM tetracaine applied continuously. Panel B presents mean data which shows that tetracaine caused a significant increase in the maximum amplitudes of caffeine contractures (* denotes $p<0.001$). (n=9 myocytes)


0 mV
voltage-clamp protocol
-70 mV
-80 mV
-80 mV
12 sec
4 sec
10 mM caffeine


A. caffeine contractures
control
4 µm
200 µM tetracaine
1 sec

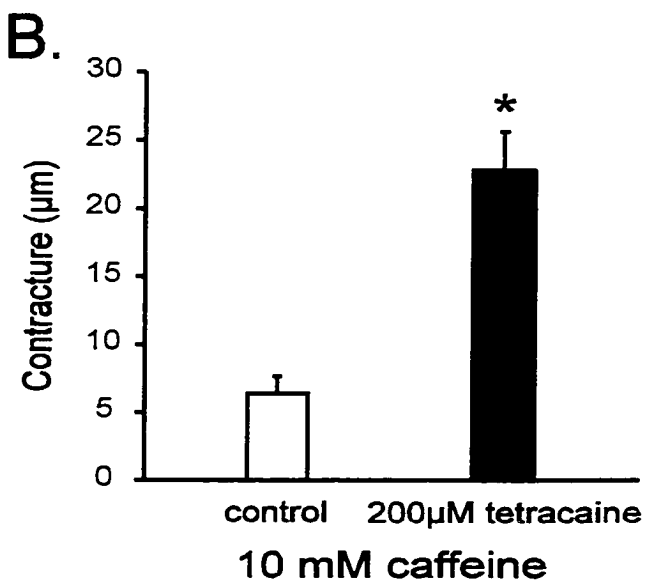

B.
Contracture (µm)
30
25
20
15
10
5
0
*
control
200µM tetracaine
10 mM caffeine

Figure 3-8

concentrations, ryanodine binds to SR RyRs and leaves them in an open sub-conducting state (Rousseau, Smith & Meissner, 1987). This allows Ca^{2+} to leak out, thereby depleting SR stores. Figure 3-9A shows the voltage clamp protocol (top) and representative recordings of contractions and currents in the presence of 200 μM lidocaine and 300 μM tetracaine. In the presence of tetracaine steps to –40 mV did not elicit a VSRM contraction. However, steps to 0 mV still elicited a phasic contraction and $I_{Ca\text{-}L}$. Figure 3-9B shows that 0.1μM ryanodine abolished the tetracaine-insensitive contraction initiated by the step to 0 mV, although $I_{Ca\text{-}L}$ was still present. Similar effects were observed in 5 of 5 myocytes studied with this protocol.

We also examined the effects of ryanodine on CV and IV relations in myocytes exposed to 300 μM tetracaine. Figures 3-9C and D show the mean CV and IV relations for experiments carried out using the same voltage clamp protocol as in figure 3-4. In the presence of 300 μM tetracaine, the CV relation was bell-shaped. Addition of ryanodine strongly inhibited the tetracaine-insensitive contractions. However, the IV relation demonstrated only a small non-significant decrease in amplitude with little change in configuration of the relationship. The results shown in figure 3-9 indicate that the contractions remaining in the presence of tetracaine are mediated primarily by SR release of Ca^{2+}.

Although the forgoing experiments show that tetracaine-insensitive contractions depend on SR release of Ca^{2+}, they do not determine whether $I_{Ca\text{-}L}$ is the trigger for tetracaine-insensitive contractions. We therefore examined the effects of Ca^{2+} channel blockade on tetracaine-insensitive contractions. Figure 3-10A shows that under control

Figure 3-9. Is the tetracaine-insensitive contraction dependent on SR Ca release? The voltage-clamp protocol is shown above panel A. Panel A shows representative recordings of currents and contractions elicited in the presence of 300 μM tetracaine applied continuously. The VSRM contraction was abolished, but the second phasic contraction and $I_{Ca\text{-}L}$ remained. Panel B shows that ryanodine (0.1 μM) virtually abolished the tetracaine-insensitive contraction, while $I_{Ca\text{-}L}$ was unaffected. Panels C and D show mean CV and IV relationships acquired with the protocol illustrated in figure 3-2. Panel C shows that, in the presence of 300 μM tetracaine, the CV relationship was bell-shaped. Ryanodine almost completely abolished the tetracaine-insensitive contractions. Panel D shows that the corresponding IV relationship was not significantly reduced in amplitude by ryanodine. These results indicate that contractions remaining with tetracaine are dependent on SR release of Ca^{2+}. (n=5 myocytes, mean length of exposure to ryanodine was 20 minutes) (* denotes $p<0.05$)

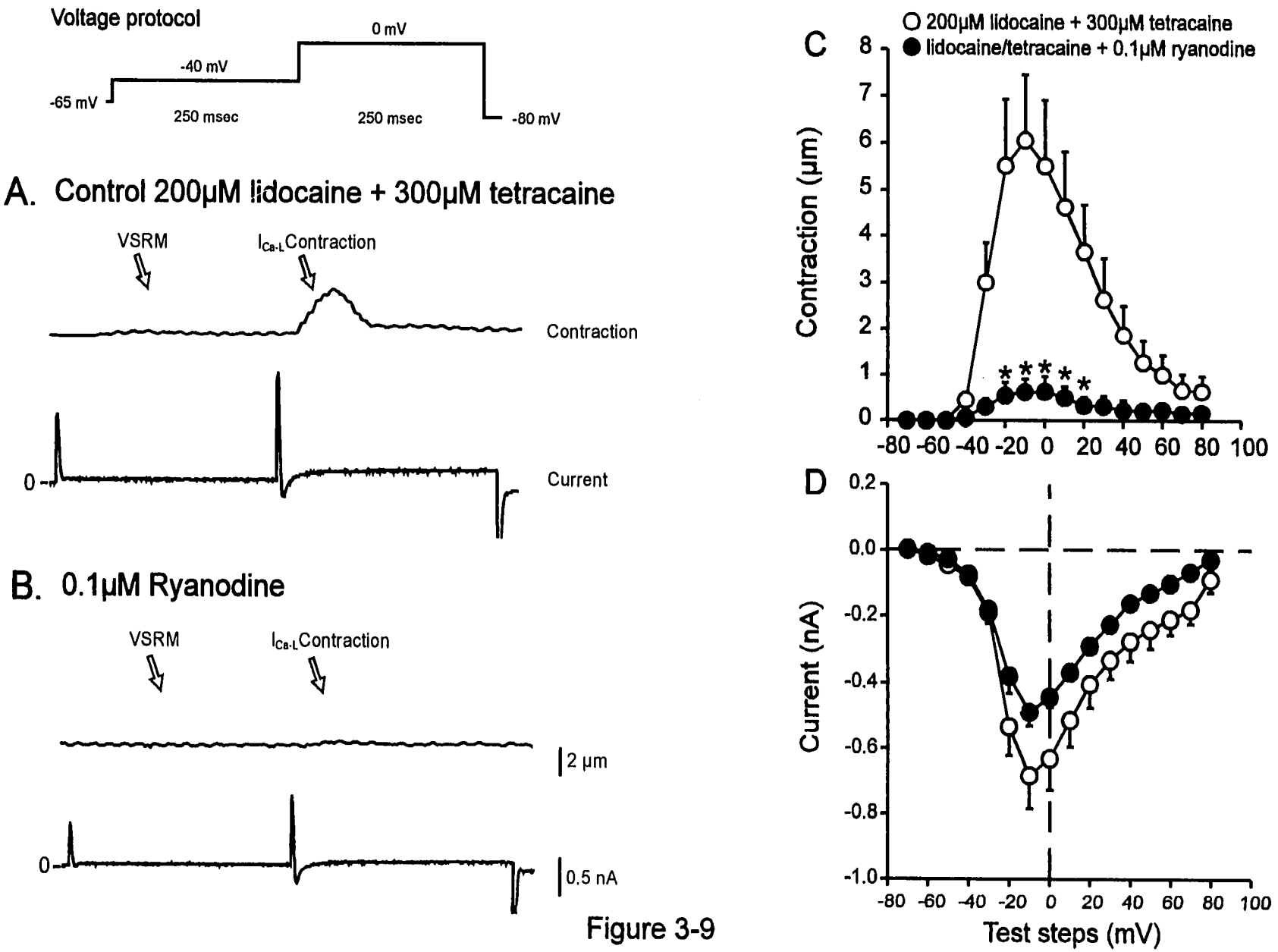

Voltage protocol
0 mV
-40 mV
-65 mV
250 msec
250 msec
-80 mV
A. Control 200µM lidocaine + 300µM tetracaine
VSRM
I_{Ca-L}Contraction
Contraction
0
Current
B. 0.1µM Ryanodine
VSRM
I_{Ca-L}Contraction
2 µm
0
0.5 nA
C
O 200µM lidocaine + 300µM tetracaine
● lidocaine/tetracaine + 0.1µM ryanodine
Contraction (µm)
8
7
6
5
4
3
2
1
0
-80 -60 -40 -20 0 20 40 60 80 100
D
Current (nA)
0.2
0.0
-0.2
-0.4
-0.6
-0.8
-1.0
-80 -60 -40 -20 0 20 40 60 80 100
Test steps (mV)

Figure 3-9

Figure 3-10. Is the tetracaine-insensitive contraction dependent on influx of Ca^{2+} via $I_{Ca\text{-}L}$? The voltage-clamp protocol was the same as in figure 3-4. Panel A shows that under control conditions, in the presence of a continuous application of 300 μM tetracaine the CV relationship was bell-shaped. Addition of 2.5 μM nifedipine to the tetracaine solution abolished all contractions. Panel B demonstrates that inward current elicited in the presence of tetracaine was significantly inhibited by nifedipine. (* denotes $p < 0.05$) (n= 4 myocytes).

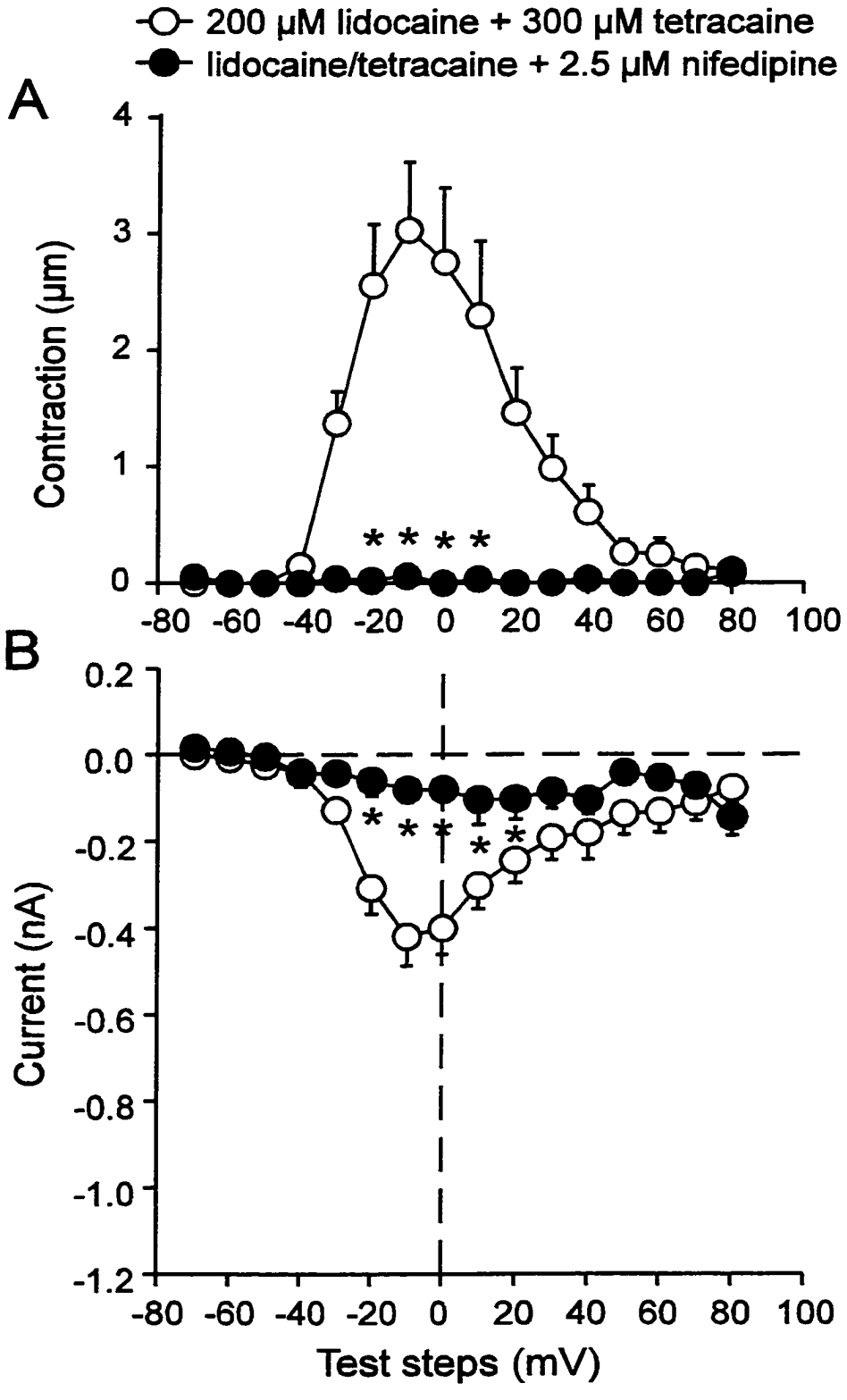

200 µM lidocaine + 300 µM tetracaine
lidocaine/tetracaine + 2.5 µM nifedipine
A
Contraction (µm)
4
3
2
1
0
-80 -60 -40 -20 0 20 40 60 80 100
B
Current (nA)
0.2
0.0
-0.2
-0.4
-0.6
-0.8
-1.0
-1.2
-80 -60 -40 -20 0 20 40 60 80 100
Test steps (mV)

Figure 3-10

conditions, in the presence of 300 μM tetracaine, the CV relation was bell-shaped and reflected the IV relationship. The similar shape and voltage-dependence of the CV and IV relationships suggests that contractions remaining in the presence of tetracaine may be triggered by $I_{Ca\text{-}L}$. When cells were superfused with 2.5 μM nifedipine in addition to 300 μM tetracaine, the tetracaine-insensitive contractions were abolished. Figure 3-10B shows that addition of nifedipine to the tetracaine solution resulted in a significant decrease in the peak amplitude of the IV relationship compared to the effects of tetracaine alone.

These results suggest that $I_{Ca\text{-}L}$ is required for the tetracaine-insensitive component of contraction to be elicited. However, continuous exposure to nifedipine during trains of conditioning pulses as well as test steps may deplete SR Ca^{2+} stores by abolishing Ca^{2+} influx through L-type Ca^{2+} channels. Therefore, the forgoing experiments demonstrate that $I_{Ca\text{-}L}$ is required for activation of the tetracaine-insensitive contractions, but do not demonstrate whether $I_{Ca\text{-}L}$ functions as a trigger to release Ca^{2+} from the SR. In order to answer this question we used a rapid solution switching device to switch to solution containing 100 μM Cd^{2+} just before the activation steps. Thus, Cd^{2+} was not present during the conditioning pulses and would not affect SR load. The voltage clamp protocol used in these experiments was the same as that illustrated at the top of figure 3-5. Figure 3-11A shows that under control conditions both test steps elicited phasic contractions. The test step to 0 mV also elicited $I_{Ca\text{-}L}$. Figure 3-11B shows that rapid application of 100 μM Cd^{2+} abolished $I_{Ca\text{-}L}$ and the phasic contraction induced by the step to 0 mV. However, the large phasic contraction induced by the step to -40 mV was virtually

Figure 3-11. Differential block of VSRM and $I_{Ca\text{-}L}$-induced contractions by rapid application of tetracaine and Cd^{2+}, respectively. Tetracaine or Cd^{2+} were applied 3 seconds before and during test steps but not during conditioning pulses, to avoid affects on loading of the SR. The voltage-clamp protocol used in this experiment is the same as that illustrated at the top of figure 3-5. Panel A shows both VSRM and $I_{Ca\text{-}L}$-induced contractions, and $I_{Ca\text{-}L}$ were present in control. Panel B demonstrates that 100 μM Cd^{2+} abolished $I_{Ca\text{-}L}$ and the second phasic contraction. The first phasic contraction was unaffected. In panel C, 200 μM tetracaine abolished the first phasic contraction, while $I_{Ca\text{-}L}$ and the $I_{Ca\text{-}L}$-induced contraction were unaffected. Panel D shows that when Cd^{2+} was added, in the presence of tetracaine, the second phasic contraction and $I_{Ca\text{-}L}$ were inhibited in addition to the VSRM contraction. These results demonstrate that the tetracaine-insensitive contraction is triggered by $I_{Ca\text{-}L}$, and that VSRM and CICR contractions can be separated by selective inhibition. Similar results were observed in 6 myocytes.

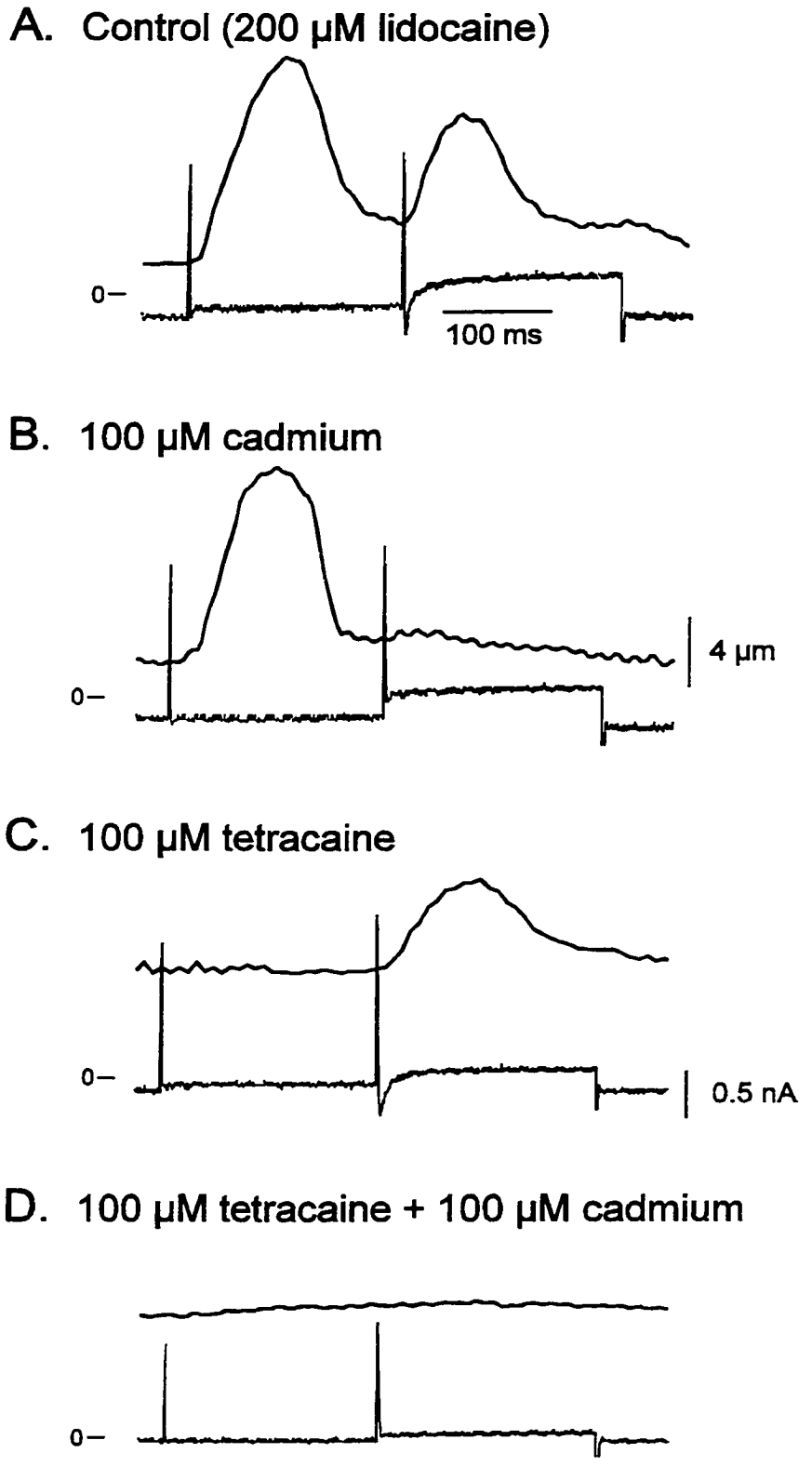

A. Control (200 µM lidocaine)
0–
100 ms
B. 100 µM cadmium
0–
4 µm
C. 100 µM tetracaine
0–
0.5 nA
D. 100 µM tetracaine + 100 µM cadmium
0–

Figure 3-11

unaffected. Figure 3-11C shows that 100 μM tetracaine, in the absence of Cd^{2+}, abolished the first phasic contraction triggered by the step to -40 mV, while the second phasic contraction and $I_{Ca\text{-}L}$ were unaffected. When a rapid switch was made to solution containing both Cd^{2+} and tetracaine, both phasic contractions and $I_{Ca\text{-}L}$ were inhibited. Similar results were observed in 6 myocytes from 5 hearts. These observations demonstrate that the tetracaine-insensitive contraction was triggered by $I_{Ca\text{-}L}$. These experiments also demonstrate that VSRM and $I_{Ca\text{-}L}$-induced contractions can be inhibited selectively by tetracaine and Cd^{2+} respectively.

Effects of rapid application of tetracaine on CV relations without effects on SR Ca^{2+} load. When myocytes were exposed to tetracaine continuously, CV relations became bell-shaped however the maximum peak contraction was not reduced (figures 3-4 and 3-7). By contrast, earlier studies demonstrated a decrease in the maximum peak contraction of CV curves when the contribution of the VSRM was removed by voltage-dependent inactivation (Ferrier & Howlett, 1995; Howlett, Zhu & Ferrier, 1998). It is possible that the peak of the CV relation in the presence of tetracaine is elevated because of increased SR Ca^{2+}. Therefore, we re-assessed the effects of tetracaine on CV relations with tetracaine applied only during and 3 seconds before test steps to minimize effects on SR Ca^{2+} load. Rapid application of tetracaine just before the test steps is unlikely to affect SR stores of Ca^{2+} since tetracaine would not be present during the conditioning pulse train. If rapid application of tetracaine does not affect SR Ca^{2+} load then this protocol should be useful for separating the effects on contractions from effects on SR Ca^{2+} load.

First determined was whether rapid application of tetracaine affected SR Ca^{2+} content as assessed with caffeine applications. The protocol used was similar to that used for figure 3-5 except that 10 mM caffeine was applied in place of test steps. Figure 3-12A illustrates mean data showing only a small, non-significant increase in caffeine contractures in response to a rapid 3 second application of tetracaine. Figures 3-12B and C illustrate the corresponding mean data for the effects of rapid application of 300 μM tetracaine on contractions and currents elicited by sequential steps to -40 and 0 mV, respectively. The voltage clamp protocol was the same as that shown at the top of figure 3-5. Lidocaine was present throughout to inhibit I_{Na}. The mean data show preferential inhibition of the VSRM component of contraction while CICR contractions were only slightly depressed (90 % inhibition of VSRM contractions versus 24 % inhibition of CICR contractions). Figure 3-12C shows that there was no significant effect of rapid application of tetracaine on the currents associated with each test step.

Since rapid application of tetracaine 3 seconds before test steps resulted in little change in SR Ca^{2+} stores, we then determined the effects of tetracaine on CV relations with this method of application. The voltage clamp protocol used in these experiments is illustrated at the top of figure 3-13. Tetracaine (300 μM) was rapidly applied 3 seconds before and during each test step. Figure 3-13A shows that rapid application of tetracaine changed the shape of the CV relationship from sigmoidal to bell-shaped and caused a 10 mV shift to more positive potentials in the activation of contractions as observed with continuous application of tetracaine. However, in contrast to the results shown in figures 3-4 and 3-7 a significant decrease in the maximum amplitude of contractions was

Figure 3-12. Effects of rapid application of 300 μM tetracaine on SR Ca^{2+} stores. The voltage clamp protocol is similar to that shown in figure 3-5, except that the sequential voltage steps to -40 and 0 mV were replaced by a 10 mM caffeine application. Tetracaine (300 μM) was rapidly applied 3 seconds before and during the caffeine pulse. Panel A. Rapid application of tetracaine 3 seconds before and during caffeine resulted in a slight, non-significant increase in the mean amplitude of caffeine contractures. (n= 4 myocytes). The results illustrated in panels B and C were obtained using the same voltage clamp protocol illustrated in figure 3-5 except with rapid application of 300 μM tetracaine 3 seconds before and during sequential test steps to -40 and 0 mV. Panel B shows that rapid application of tetracaine virtually abolished the contraction elicited at -40 mV (** denotes $p<0.001$). The contraction elicited at 0 mV also was significantly (*denotes $p<0.05$) decreased in the presence of tetracaine, however the magnitude of inhibition was much less than that at -40 mV (24 % of contraction at 0 mV compared to 90 % inhibition at -40 mV). Panel C shows that rapid applied tetracaine had no significant effect on the current associated with either step. (n=10 cells)

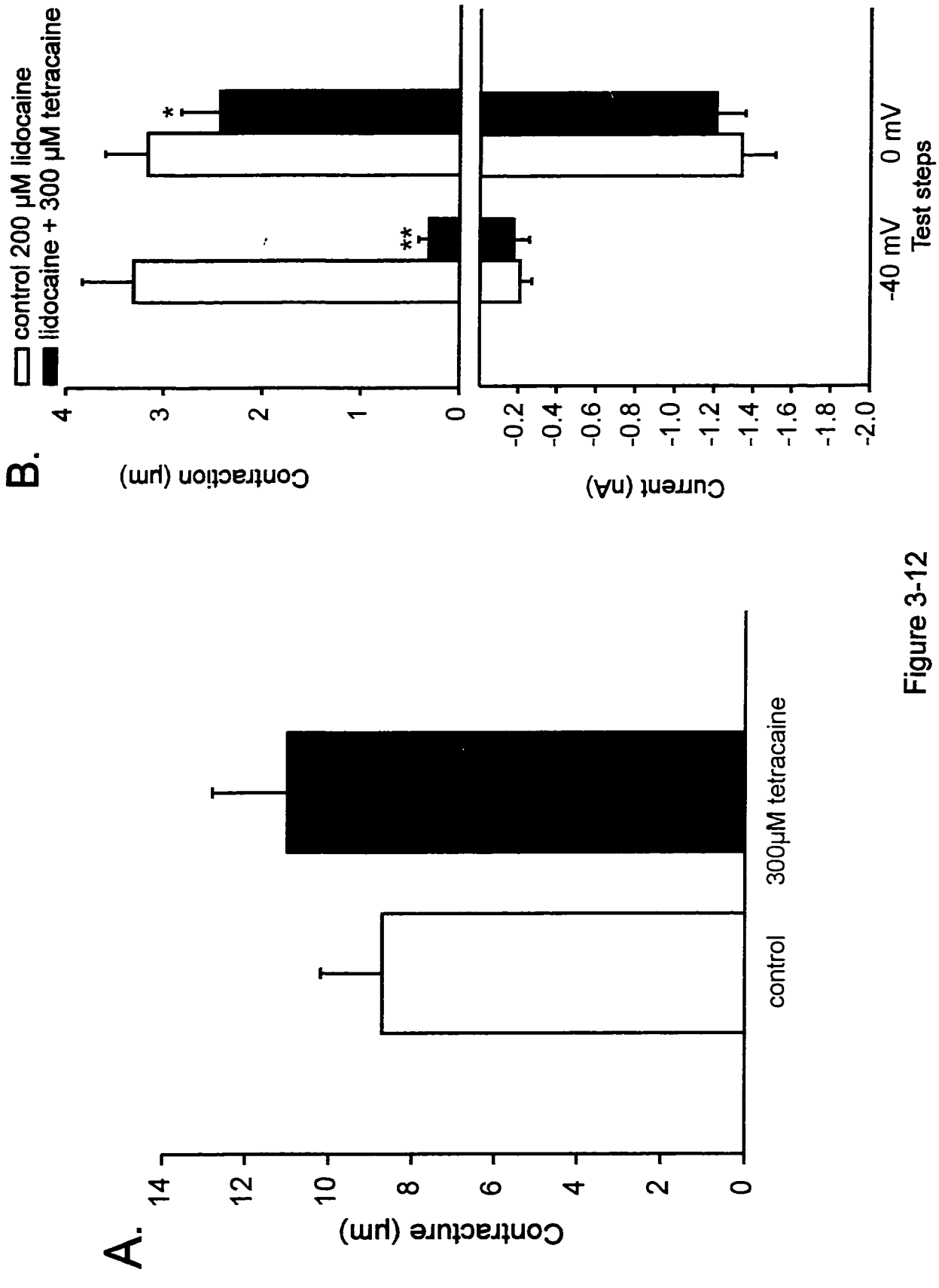

B.
control 200 µM lidocaine
lidocaine + 300 µM tetracaine
Contraction (µm)
4
3
2
1
0
**
*
Current (nA)
-0.2
-0.4
-0.6
-0.8
-1.0
-1.2
-1.4
-1.6
-1.8
-2.0
-40 mV
0 mV
Test steps
A.
Contracture (µm)
14
12
10
8
6
4
2
0
control
300µM tetracaine

Figure 3-12

Figure 3-13. Effects of rapid application of 300 μM tetracaine on CV and IV relationships. The voltage clamp protocol is shown at the top. Panel A shows that rapid application of 300 μM tetracaine (3 seconds before and during test steps) changed the CV relationship from sigmoidal to bell-shaped. The threshold for activation of contraction was shifted 10 mV more positive and the maximum amplitude of contractions was significantly reduced by tetracaine. Panel B shows a tetracaine caused little change in the configuration of the IV relation (n= 9 myocytes) (* denotes $p<0.05$ with respect to controls).

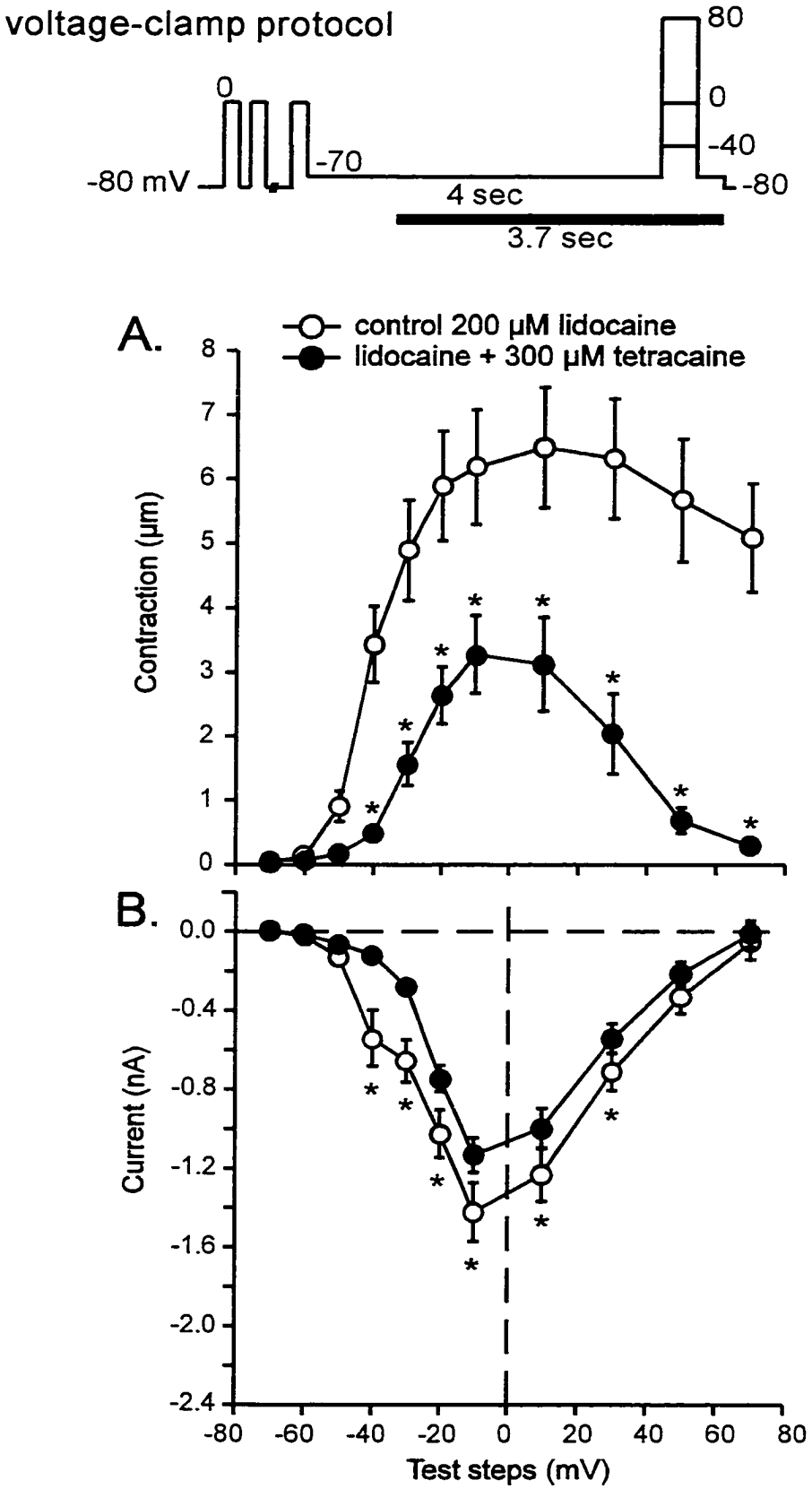

voltage-clamp protocol
80
0
-40
-80
0
-70
-80 mV
4 sec
3.7 sec
A.
control 200 µM lidocaine
lidocaine + 300 µM tetracaine
Contraction (µm)
B.
Current (nA)
Test steps (mV)

Figure 3-13

observed with rapid applications of tetracaine. Tetracaine also caused a small decrease in inward current with little change in the configuration of the IV relation (figure 3-13B). Results shown in figures 3-11 to 3-13 illustrate that inhibition of the VSRM with tetracaine is independent of changes in SR Ca^{2+} stores. Furthermore, inhibition of the VSRM with tetracaine causes changes in CV relations similar to those observed with voltage dependent inactivation of the VSRM, when effects on SR Ca^{2+} are eliminated.

Does tetracaine preferentially block VSRM contractions at higher concentrations?

The results presented so far indicate that tetracaine at concentrations up to 300 μM selectively blocks the VSRM. We also determined whether still higher concentrations of tetracaine would remain selective, or whether nonspecific effects would occur. Figure 3-14 shows the effects of 1 mM tetracaine on contractions and currents elicited by sequential steps to -40 and 0 mV, in a representative experiment. I_{Na} was inhibited by 200 μM lidocaine throughout the experiment. Figure 3-14A shows representative recordings before exposure of the myocyte to tetracaine. Both phasic contractions and $I_{Ca\text{-}L}$ were present. Figures 3-14B-C show the sequential development of the effects of 1 mM tetracaine. Tetracaine first abolished the VSRM contraction (14B) at a time when there was much less effect on $I_{Ca\text{-}L}$ and the $I_{Ca\text{-}L}$-induced contraction. However, within 10 min $I_{Ca\text{-}L}$ was almost completely inhibited and the $I_{Ca\text{-}L}$-induced contraction was abolished (figure 3-14C). Myocytes were then exposed to extracellular solution containing lidocaine but not tetracaine to determine whether the effects of 1 mM tetracaine were reversible. Figures 3-14D and E show that the reverse sequence was observed upon washout of tetracaine. First $I_{Ca\text{-}L}$ and $I_{Ca\text{-}L}$-induced contractions reappeared

Figure 3-14. Time course of effects of 1 mM tetracaine. The voltage-clamp protocol was as in figure 3-1. Panel A shows that both phasic contractions as well as $I_{Ca\text{-}L}$ were present under control conditions. Panels B and C demonstrate the development of effects of continuous application of 1 mM tetracaine. Tetracaine first abolished the VSRM contractions, then $I_{Ca\text{-}L}$ and $I_{Ca\text{-}L}$-induced contractions. Panels D and E demonstrate that the reverse sequence was observed upon washout of tetracaine. First $I_{Ca\text{-}L}$ and $I_{Ca\text{-}L}$ contractions reappeared, then the VSRM contractions returned. Times indicate time after complete change of solution in the experimental chamber.

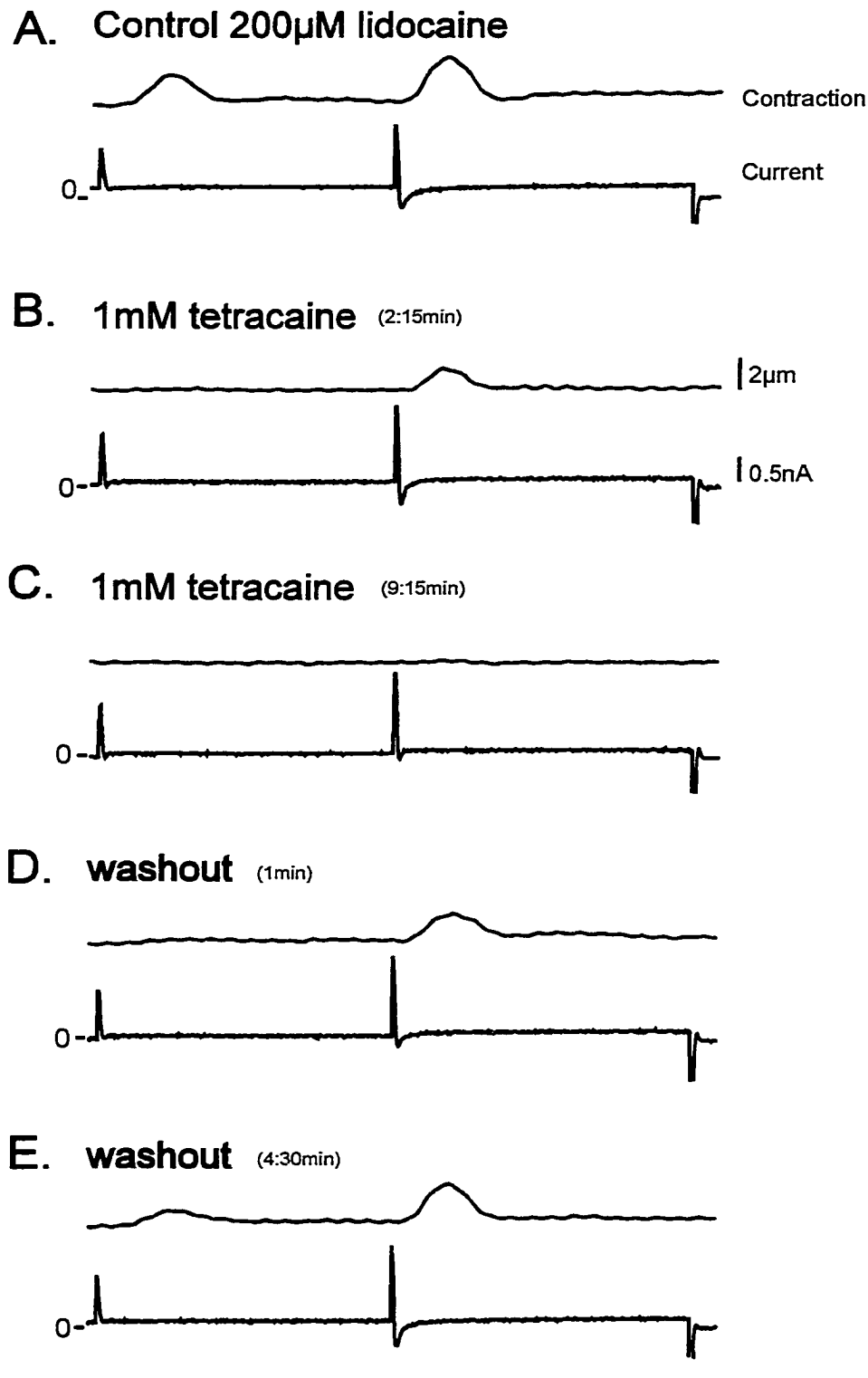

A. Control 200μM lidocaine
Contraction
Current
0
B. 1mM tetracaine (2:15min)
2μm
0.5nA
0
C. 1mM tetracaine (9:15min)
0
D. washout (1min)
0
E. washout (4:30min)
0

Figure 3-14

(figure 3-14D), then the VSRM contraction returned (figure 3-14E). Similar effects were observed in 4 of 4 experiments.

Figure 3-15 shows the mean CV and IV relations determined in experiments with 1 mM tetracaine. Under control conditions the CV relation was sigmoidal (figure 3-15A). Addition of 1mM tetracaine completely abolished contractions at all test potentials. Figure 3-15B shows the corresponding IV relations. The maximum amplitude of $I_{Ca\text{-}L}$ was decreased compared to control but not abolished. Figure 3-15 also shows that the effects of tetracaine on both CV and IV relations were completely reversible. These results demonstrate that tetracaine is a selective inhibitor of the VSRM component of contraction at concentrations up to 300 μM, but inhibits $I_{Ca\text{-}L}$ and contractions triggered by $I_{Ca\text{-}L}$ at higher concentrations (1 mM).

Is inhibition of CICR coupled to $I_{Ca\text{-}L}$ time-dependent or activation-dependent? The previous results indicate that inhibition of the VSRM by tetracaine occurs faster than inhibition of $I_{Ca\text{-}L}$ and $I_{Ca\text{-}L}$-induced contractions. This suggests that longer exposure of myocytes to lower concentrations of tetracaine may also lead to inhibition of $I_{Ca\text{-}L}$ and $I_{Ca\text{-}L}$-induced contractions. In order to determine this CV and IV curves were determined in myocytes exposed to 300 μM tetracaine for 10 min. The voltage-clamp protocol was the same as that illustrated in figure 3-4. Figure 3-16 shows the mean CV and IV relations measured under control and tetracaine treated conditions. Lidocaine and TTX were used throughout to ensure complete block of Na^+ channels. Figure 3-16A shows that under control conditions the CV relation was sigmoidal. After 10 mins exposure to 300 μM tetracaine CV and IV curves were determined again. With this protocol the CV

Figure 3-15. Effects of 1 mM tetracaine on CV and IV relationships. The voltage-clamp protocol was as in figure 3-4. Panel A shows that, under control conditions, the CV relationship was sigmoidal. Continuous application of 1mM tetracaine completely abolished all contractions. Panel B shows $I_{Ca\text{-}L}$ was decreased compared to control. Panels A and B also demonstrate that the effects of tetracaine on contractions and currents were completely reversible. (* denotes $p < 0.05$ with respect to pretreatment control). (n=4 myocytes, mean length of exposure to 1 mM tetracaine was 8 minutes)

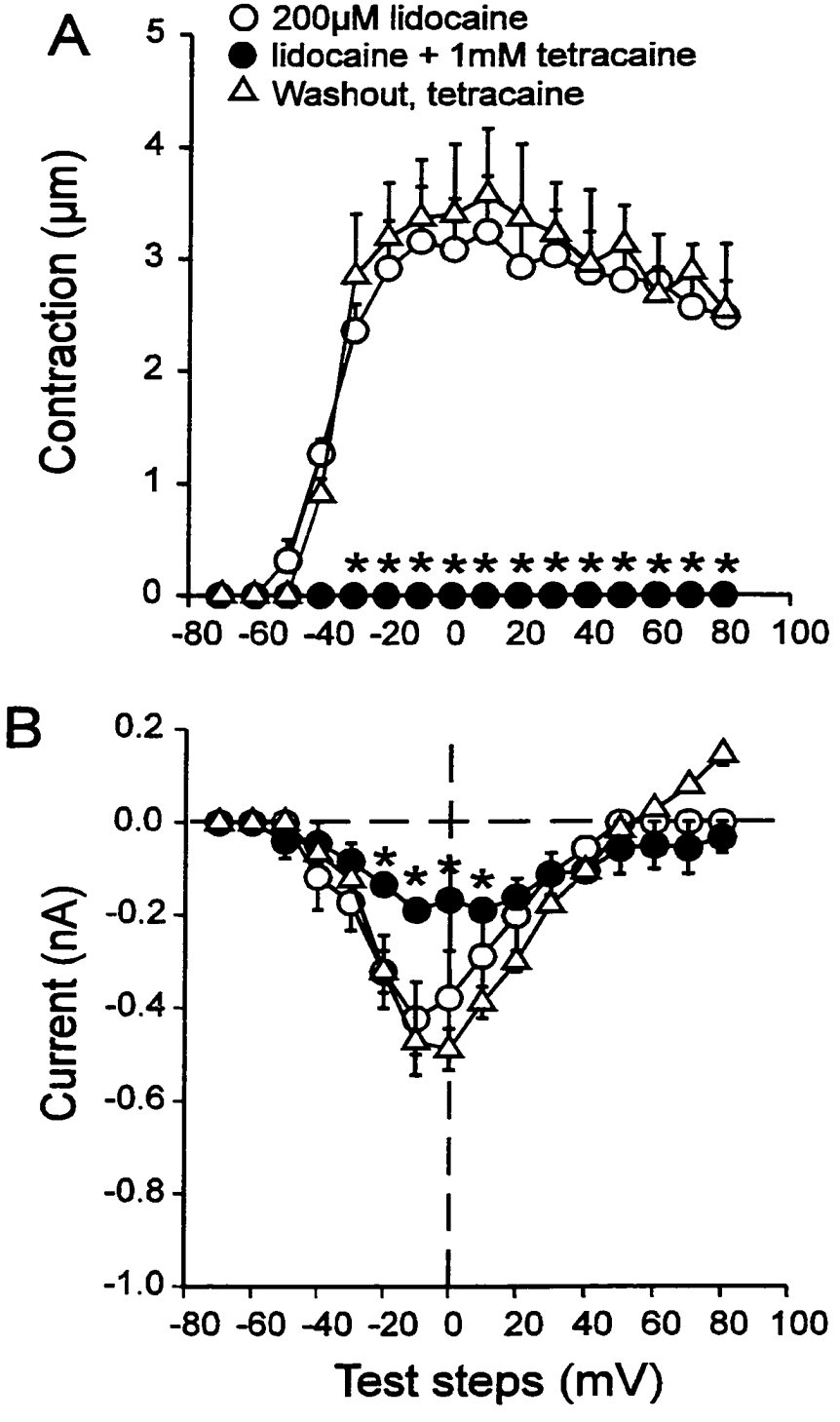

A
200μM lidocaine
lidocaine + 1mM tetracaine
Washout, tetracaine
Contraction (μm)
5
4
3
2
1
0
-80 -60 -40 -20 0 20 40 60 80 100
B
0.2
0.0
-0.2
-0.4
-0.6
-0.8
-1.0
Current (nA)
-80 -60 -40 -20 0 20 40 60 80 100
Test steps (mV)

Figure 3-15

relation became bell-shaped with a significant depression in the maximum amplitude of contractions. The strong decrease in the maximum amplitude of contractions contrasts with results shown in figure 3-7A where exposure to 300 μM tetracaine for approximately 2 minutes did not decrease the maximum amplitude of contractions. Figure 3-16B shows the corresponding IV relations. When IV relations were determined after exposure to 300 μM tetracaine for 10 minutes the maximum amplitude of $I_{Ca\text{-}L}$ was significantly depressed. These results suggest that the effect of tetracaine on VSRM contractions is both time and concentration dependent. Short-term exposure of myocytes to low concentrations of tetracaine results in preferential inhibition of the VSRM component of contraction. However, increases in concentration or duration of exposure to tetracaine may result in inhibition of $I_{Ca\text{-}L}$ and contractions coupled to $I_{Ca\text{-}L}$ in addition to inhibition of the VSRM.

Can lidocaine antagonize the inhibitory effects of tetracaine on the VSRM?

Lidocaine does not prevent activation of the VSRM in cardiac myocytes at concentrations up to 500 μM (Ferrier & Howlett, 1995). In skeletal muscle lidocaine is thought to bind to the same site on the ryanodine receptor as tetracaine. However, in skeletal muscle lidocaine does not inhibit SR release of Ca^{2+}, and in fact it may stimulate release at high concentrations (Shoshan-Barmatz & Zchut, 1993). If lidocaine binds to the same site as tetracaine in cardiac muscle, it might reverse the effects of tetracaine. To test this possibility we used a concentration of lidocaine twenty times greater than that of tetracaine. An excess of lidocaine was used to increase the probability that lidocaine would displace tetracaine if both drugs have a common binding site. The voltage clamp

Figure 3-16. Effects of long-term exposure to 300 μM tetracaine on CV and IV relationships. The voltage clamp protocol is shown on the top left of figure 3-4. Lidocaine and TTX were present throughout to ensure complete inhibition of Na^+ channels. In panel A, under control conditions the CV relation was sigmoidal. Exposure to 300 μM tetracaine for approximately 10 minutes resulted in a bell-shaped CV relation. There was a significant decrease in the maximum amplitude of contractions in the presence of tetracaine to less than half of the amplitude of control contractions. Panel B illustrates a similar decrease in the maximum amplitude of the IV relationship during long-term exposure to tetracaine (* denotes $p<0.05$) (n=5 myocytes).

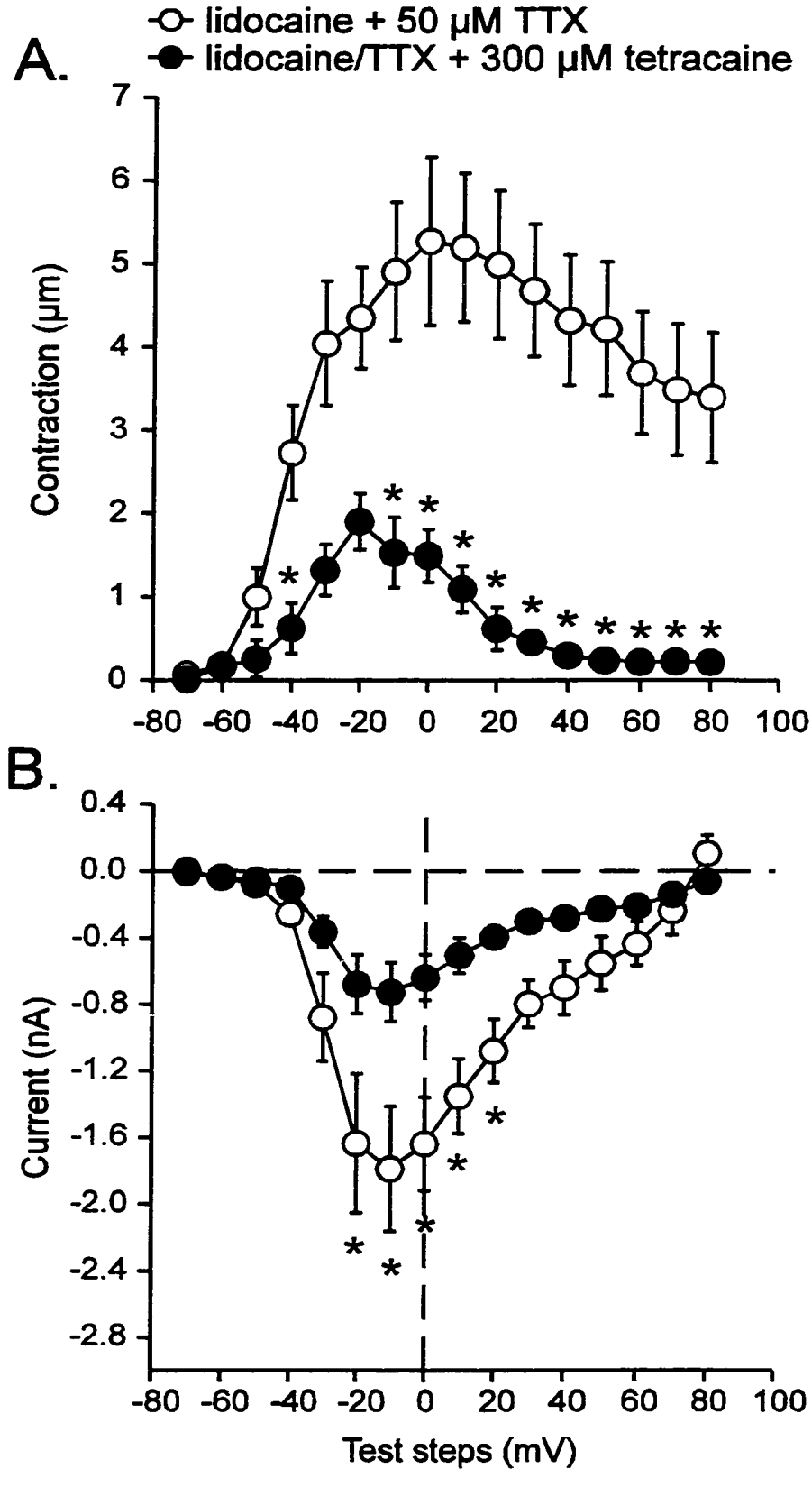

A.
lidocaine + 50 µM TTX
lidocaine/TTX + 300 µM tetracaine
Contraction (µm)
7
6
5
4
3
2
1
0
-80 -60 -40 -20 0 20 40 60 80 100
B.
Current (nA)
0.4
0.0
-0.4
-0.8
-1.2
-1.6
-2.0
-2.4
-2.8
-80 -60 -40 -20 0 20 40 60 80 100
Test steps (mV)

Figure 3-16

protocol was identical to that used in figure 3-4. Figure 3-17A shows that in the presence of 100 μM tetracaine, the CV relationship determined with steps from a V_{PC} of -70 mV was bell-shaped. Addition of 2 mM lidocaine did not reverse the effects of tetracaine, but further depressed contraction. The IV relationships shown in figure 3-17B demonstrate that 2 mM lidocaine also inhibited $I_{Ca\text{-}L}$. We have already shown that tetracaine-insensitive contractions are inhibited by Ca^{2+}-channel blockade, thus it is likely that the further depression of contraction by lidocaine was caused by its effect on $I_{Ca\text{-}L}$. Further, our results show that lidocaine does not reverse the effects of tetracaine in cardiac myocytes.

Effects of tetracaine on staircase phenomena. During the course of our experiments we observed effects of tetracaine on contractions accompanying the trains of conditioning pulses which preceded the test steps. Figure 3-18 illustrates recordings of the contractions elicited by the conditioning pulse train in the presence and absence of tetracaine (100 μM). Protocols were run repetitively with 7 sec pauses between runs. Under control conditions the first contraction (rest contraction) of the train was large. The second contraction was smaller, and subsequent contractions of the train showed a progressive increase in amplitude (positive staircase) (figure 3-18B). Figure 3-18C was recorded from the same myocyte after exposure to 100 μM tetracaine. Tetracaine did not affect the rest contraction, but caused a marked inhibition of the staircase. Figures 3-3 and 3-4 show that this concentration of tetracaine causes selective inhibition of the VSRM. Thus, inhibition of the VSRM component of contraction was accompanied by

Figure 3-17. Lidocaine does not reverse the effects of tetracaine on the VSRM. The voltage-clamp protocol was identical to that in figure 3-4. Panel A shows that in the presence of continuously applied 100 μM tetracaine the CV relationship was bell-shaped. Addition of 2 mM lidocaine did not reverse the effects of tetracaine, but further depressed contraction. In panel B the IV relationships show that high concentrations of lidocaine inhibit $I_{Ca\text{-}L}$. Thus, the further depression of contraction by lidocaine may be related to an effect on $I_{Ca\text{-}L}$ (* denotes $p < 0.05$ with respect to pretreatment control) (n=5 myocytes).

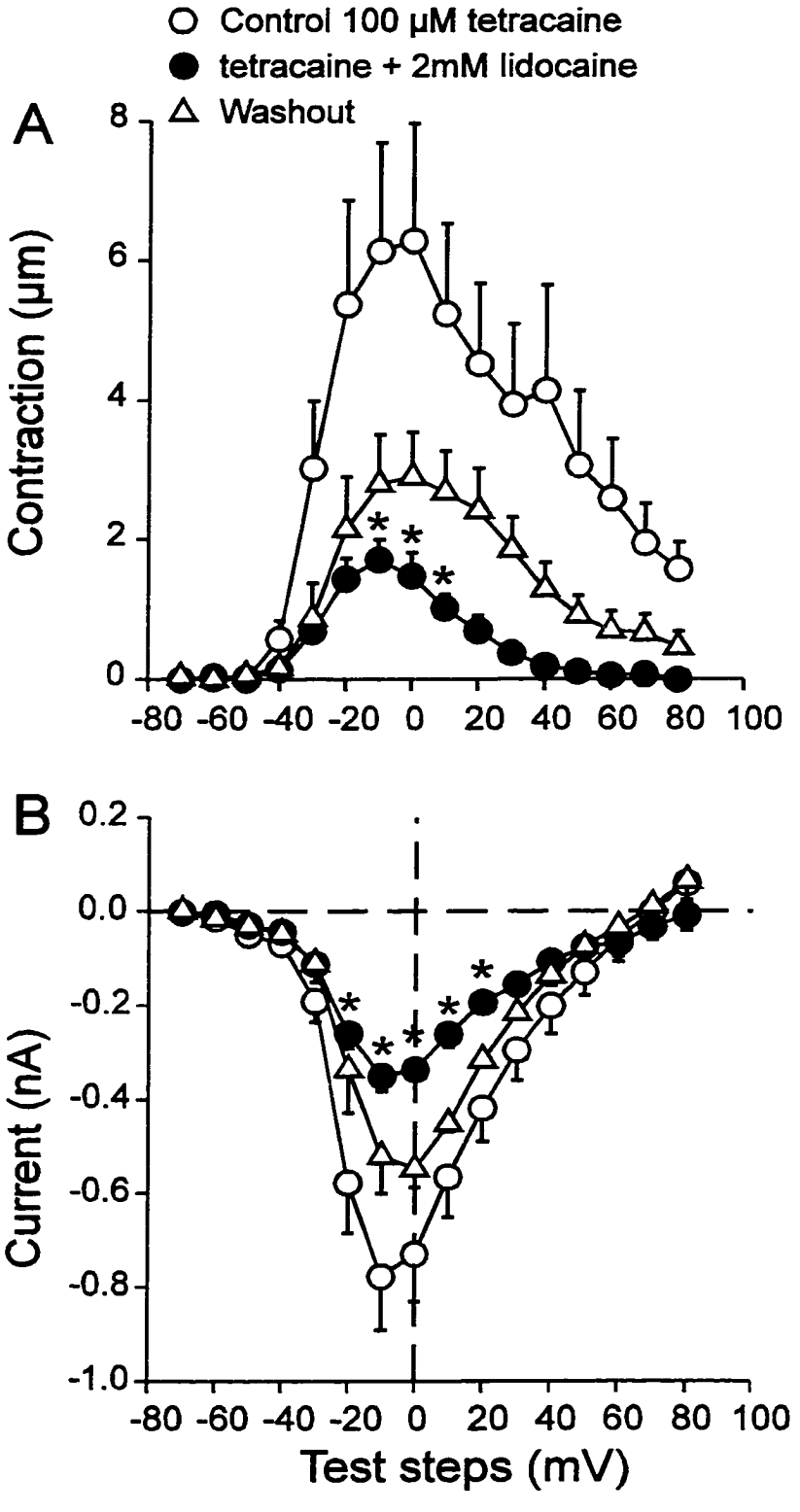

Control 100 µM tetracaine
tetracaine + 2mM lidocaine
Washout
A
Contraction (µm)
8
6
4
2
0
-80 -60 -40 -20 0 20 40 60 80 100
B
Current (nA)
0.2
0.0
-0.2
-0.4
-0.6
-0.8
-1.0
-80 -60 -40 -20 0 20 40 60 80 100
Test steps (mV)

Figure 3-17

Figure 3-18. Inhibition of the VSRM by tetracaine is accompanied by inhibition of staircase phenomena. Panel A illustrates the conditioning pulse train that precedes the test steps of the voltage-clamp protocol. Panel B shows that under control conditions the initial contraction (rest contraction) was large and was followed by a smaller second contraction. Each successive contraction increased in amplitude creating a staircase. Panel C shows that continuous exposure to tetracaine (100 μM) abolished the positive staircase, but did not effect the rest contraction.

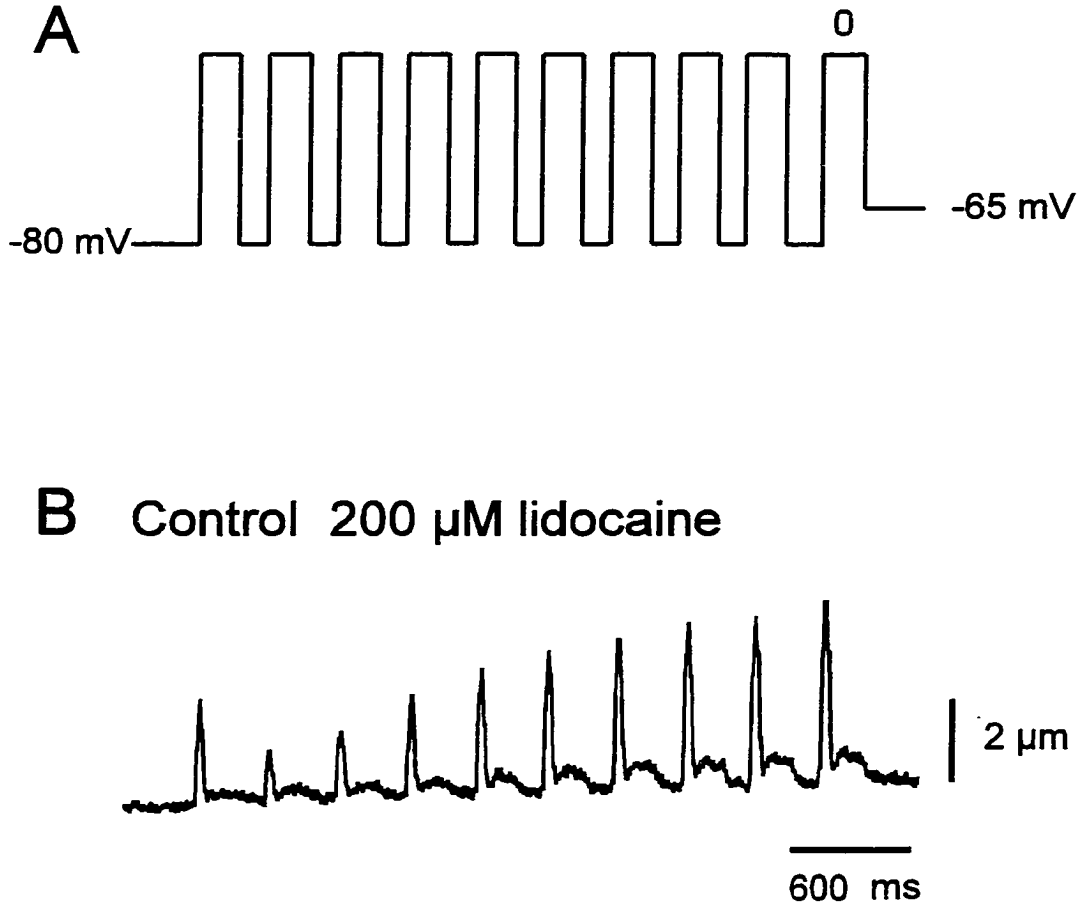

A
0
-80 mV
-65 mV
B Control 200 µM lidocaine
2 µm
600 ms
C 200 µM lidocaine + 100 µM tetracaine

Figure 3-18

elimination of the positive staircase. Further studies are required to determine the significance of these effects. Similar observations were made in 13 of 13 experiments.

Effects of tetracaine on the sustained (non-inactivating) component of VSRM contractions. Through the course of this study it was observed that full relaxation of contraction occurred upon repolarization of the membrane only. At depolarized potentials contractions appeared to have two components, a phasic component followed by a sustained component that lasted for the duration of the depolarization. Both components of contraction are readily observed when contraction traces are expanded to show more of the baseline cell length before and after test steps. An example of this is illustrated by the representative trace in figure 3-19A. The typical phasic contractions representing activation of the VSRM and CICR were elicited by steps to -40 and 0 mV, respectively. However, the expanded time frame also illustrates a sustained contraction following both phasic contractions. The sustained contractions were maintained for the duration of the depolarization with complete relaxation occurring upon repolarization of the myocyte to the -80 mV holding potential.

There are several possible mechanisms which may be responsible for the generation of sustained contractions. These include: CICR either coupled to $I_{Ca\text{-}L}$ or Na-Ca_{EX}, or the VSRM. The first of these possibilities seemed unlikely because sustained contractions were elicited by the step to -40 mV, which activates little $I_{Ca\text{-}L}$. As well, sustained contractions elicited by the step to 0 mV, which is near the peak for $I_{Ca\text{-}L}$, were approximately the same amplitude as those elicited at -40 mV (figure 3-19A). Thus, it appears that this component of contraction is not graded with $I_{Ca\text{-}L}$ as would be expected

Figure 3-19. Depolarization of myocytes elicits sustained contractions which are blocked by 100 μM Cd^{2+}. The voltage clamp protocol is shown at the top of panel A. Panel A. Representative VSRM and CICR contractions initiated by sequential steps to -40 and 0 mV, respectively. Both contractions were composed of a phasic and a sustained component. The step to 0 mV also activated inward I_{Ca-L}. Panel B. Rapid application of Cd^{2+} 3 seconds before and during test steps inhibited I_{Ca-L} and the phasic CICR contraction, but left the phasic VSRM contraction and the sustained contraction which lasted until repolarization to -80 mV.

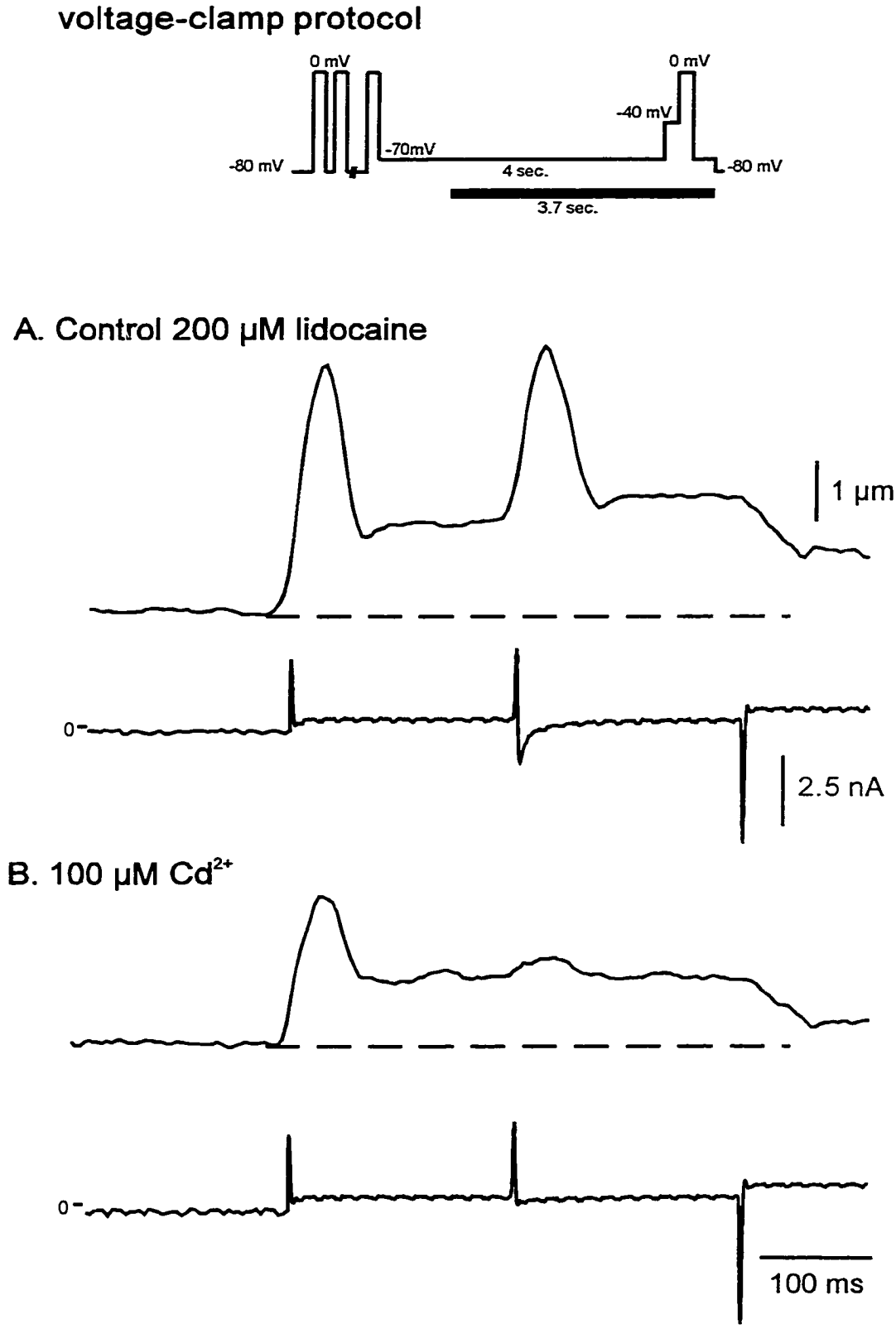

voltage-clamp protocol
0 mV
0 mV
-40 mV
-80 mV
-70mV
4 sec.
-80 mV
3.7 sec.
A. Control 200 µM lidocaine
1 µm
0
2.5 nA
B. 100 µM Cd2+
0
100 ms

Figure 3-19

with CICR coupled to $I_{Ca\text{-}L}$. The transient nature of the trigger for CICR coupled to $I_{Ca\text{-}L}$ also seems incompatible with a sustained contraction.

In order to determine further whether or not $I_{Ca\text{-}L}$ was involved in the generation of sustained contractions Cd^{2+}, a selective Ca^{2+} channel blocker, was used. If sustained contractions were generated by CICR coupled to $I_{Ca\text{-}L}$ then inhibition of $I_{Ca\text{-}L}$ should result in inhibition of these contractions. The voltage clamp protocol is illustrated at the top of figure 3-19. Cd^{2+} (100 μM) was rapidly applied 3 seconds before and during the test steps by way of a rapid solution changer to prevent changes in SR stores due to inhibition $I_{Ca\text{-}L}$ during the condition pulse train. Figure 3-19B shows that rapid application of 100 μM Cd^{2+} abolished $I_{Ca\text{-}L}$ and the phasic contraction associated with this current. However, the sustained contraction associated with the step to 0 mV was virtually unaffected. The phasic and sustained contractions elicited by the step to -40 mV also were unaffected. In fact, figure 3-19B shows that the sustained contraction elicited by the step to -40 mV maintained the same amplitude for the duration of both test steps. These results suggest that the sustained component of contraction is not mediated by CICR coupled to $I_{Ca\text{-}L}$.

Can tetracaine be used to differentiate between VSRM contractions and those elicited by CICR coupled to reverse-mode Na-Ca_{EX}? The VSRM and CICR coupled to reverse mode $NaCa_{EX}$ are two other possible mechanisms responsible for the generation of sustained contractions. Ca^{2+} influx via reverse mode $NaCa_{EX}$ has been shown to initiate ramp-like, sustained contractions somewhat analogous to those described in the present study. Thus, it was possible that the sustained contractions

illustrated in figure 3-19 represent CICR coupled to reverse mode $NaCa_{EX}$. On the other hand, it was equally possible that these contractions were generated by activation of the VSRM upon depolarization of the cell membrane. One way to differentiate between the VSRM and CICR coupled to reverse-mode Na-Ca_{EX} was to selectively inhibit each mechanism and determine the effects on sustained contractions.

The results of the present study demonstrate that rapid application of tetracaine causes preferential inhibition of contractions initiated by VSRM. If sustained contractions are generated by this mechanism then they too should be tetracaine-sensitive. However, it is not known whether rapid application of tetracaine also would inhibit contractions initiated by CICR coupled to reverse mode $NaCa_{EX}$. Previous studies by Overend *et al* (1998) demonstrated $NaCa_{EX}$ current in the presence of tetracaine which suggests that this agent does not inhibit $NaCa_{EX}$. However, their study was conducted in rat myocytes at 22 °C while the present study was carried out at 37 °C with guinea-pig myocytes. Both the temperature and species differences between these two studies make it difficult to extrapolate their results to the present study. Therefore, the effects of tetracaine on CICR contractions coupled to reverse mode $NaCa_{EX}$ were determined in the present study.

In order to examine the effects of tetracaine on $NaCa_{EX}$ contractions conditions which have been previously demonstrated to promote exchanger currents and contractions were used (see methods). As well, a voltage clamp protocol involving a test step to very positive potentials, which favors Ca^{2+} influx via $NaCa_{EX}$, was used. This protocol is illustrated at the top of figure 3-20. Figures 3-20A and B illustrate representative traces

Figure 3-20. CICR coupled to reverse-mode $NaCa_{EX}$ elicits ramp-like contractions upon depolarization and inward tail-currents upon repolarization. The voltage clamp protocol is shown at the top. Panel A. Under control conditions depolarization to +100 from +10 mV initiated a ramp-like contraction. Panel B. Depolarization to +100 mV produced an outward current, while repolarization to -70 mV elicited an inward tail-current representing Na^+ influx and Ca^{2+} efflux via $NaCa_{EX}$.

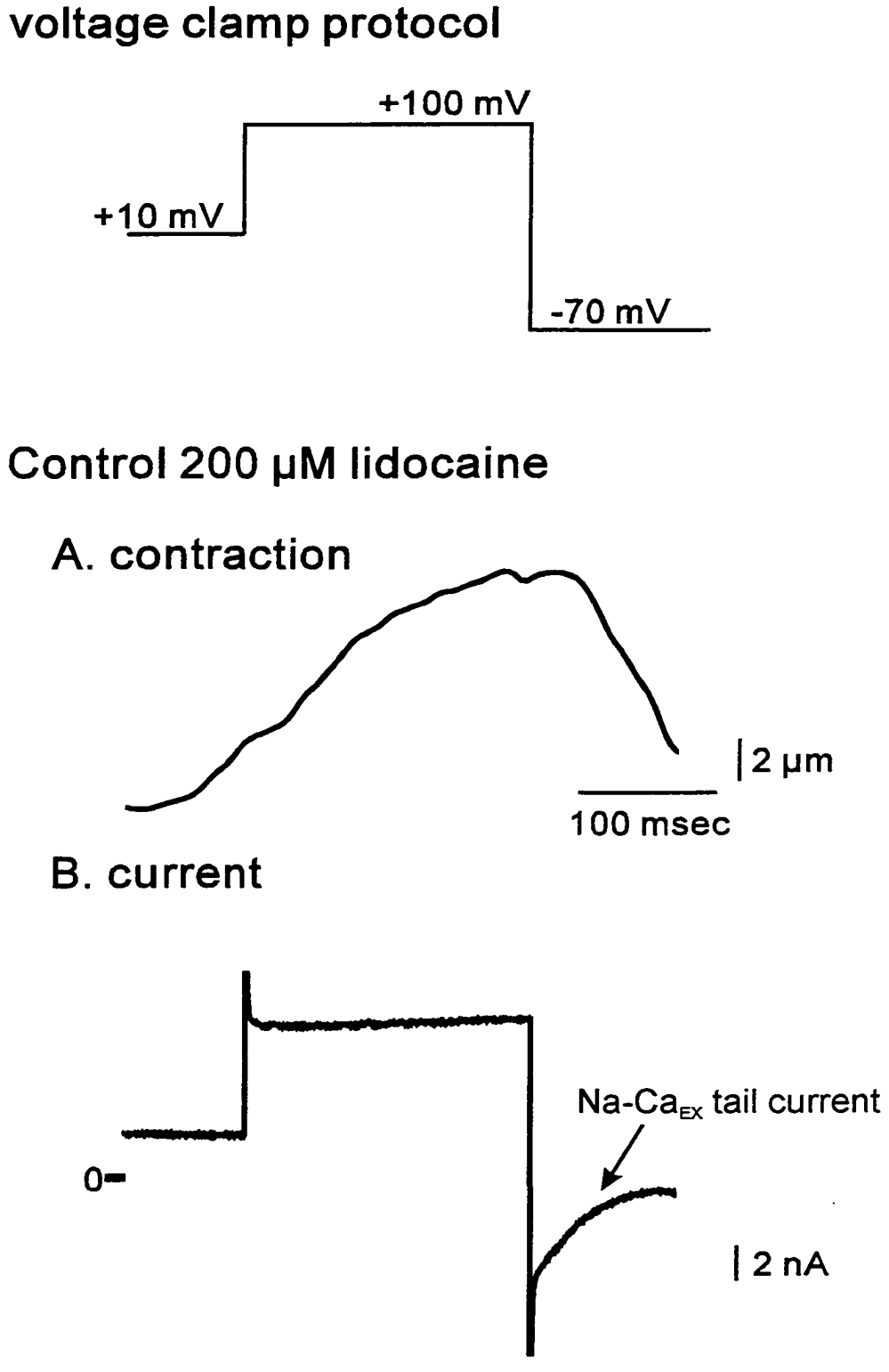

voltage clamp protocol
+100 mV
+10 mV
-70 mV
Control 200 µM lidocaine
A. contraction
2 µm
100 msec
B. current
Na-Ca$_{EX}$ tail current
0
2 nA

Figure 3-20

of contractions and currents generated in this series of experiments. The ramp-like shape of the contraction in figure 3-20A is characteristic of those elicited by CICR coupled to reverse mode Na-Ca_{EX}. Figure 3-20B illustrates that in addition to outward K^+ currents, an outward Na-Ca_{EX} current also was elicited upon depolarization which represents Ca^{2+} influx and Na^+ efflux via this pump. Upon repolarization to -70 mV the driving force for $NaCa_{EX}$ is reversed, promoting relaxation of the myocyte. This is demonstrated by the $NaCa_{EX}$ tail current associated with repolarization which represents Na^+ influx and Ca^{2+} efflux from the cell.

Rapid application of 2 mM Ni^{2+}, a well known $NaCa_{EX}$ inhibitor (Kimura *et al.*, 1987), was used to confirm that the ramp-like contraction and currents represent $NaCa_{EX}$ (figure 3-21). The representative traces illustrated in figure 3-21A show that 2 mM Ni^{2+} virtually abolished the ramp-like contraction elicited under control conditions. The right side of panel A shows the current elicited in response to the voltage step. The outward current associated with depolarization also was depressed in the presence of Ni^{2+}. Thus, inhibition of the ramp-like contraction by this agent represents inhibition of transsarcolemmal Ca^{2+} influx via $NaCa_{EX}$. In the absence of a contraction there is no longer a driving force for inward Na-Ca_{EX} tail current, therefore this current was not elicited in the presence of Ni^{2+}. These results indicate that both the ramp-like contraction and the outward current represent Na-Ca_{EX} as they are both Ni^{2+} sensitive. As well, these results illustrate that rapid application of 2 mM Ni^{2+} provides adequate block of $NaCa_{EX}$.

Figure 3-21. Contractions elicited by CICR coupled to reverse-mode $NaCa_{EX}$ are Ni^{2+}- sensitive but not tetracaine-sensitive. The voltage clamp protocol is shown at the top. Panel A. The large ramp-like contraction elicited under control conditions was inhibited by rapid application (3 seconds before and during test steps) of 2 mM Ni^{2+}. The right side of panel A illustrates inhibition of the inward tail-current elicited upon repolarization in the presence of Ni^{2+}. The outward current associated with depolarization also was depressed in the presence of Ni^{2+}. Panel B. Rapid application of 300 μM tetracaine had no effect on the ramp-like contraction or associated tail-current. In panel C both the ramp-like contraction and tail-current were virtually abolished with a rapid application of both tetracaine and Ni^{2+}.

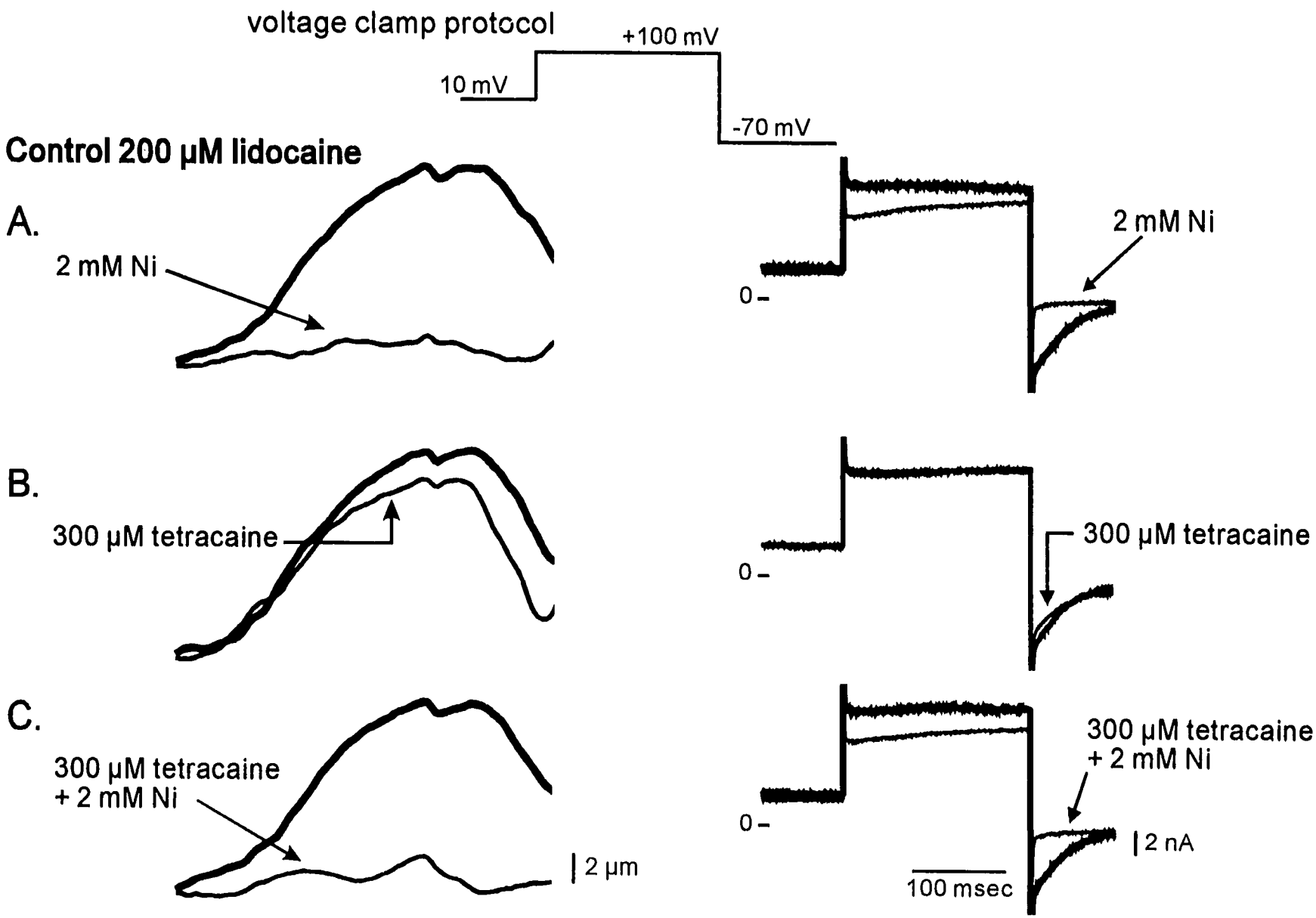

voltage clamp protocol
+100 mV
10 mV
-70 mV
Control 200 µM lidocaine
A.
2 mM Ni
2 mM Ni
0
B.
300 µM tetracaine
300 µM tetracaine
0
C.
300 µM tetracaine
+ 2 mM Ni
300 µM tetracaine
+ 2 mM Ni
0
2 µm
2 nA
100 msec

Figure 3-21

Next the effects of tetracaine on contractions and currents elicited by the step to 100 mV were determined. Figure 3-21B shows that, in contrast to the effects of Ni^{2+}, neither the ramp-like contraction nor the associated outward current were substantially affected by rapid application of 300 μM tetracaine. Tetracaine also did not affect the inward $NaCa_{EX}$ tail current associated with relaxation confirming that this agent is not an inhibitor of this pump. In the presence of both tetracaine and Ni^{2+} (figure 3-21C) the ramp-like contraction and the outward current associated with depolarization were virtually abolished.

Mean data for the effects of tetracaine and Ni^{2+} on $NaCa_{EX}$ contractions and tail currents are shown in figure 3-22. Tetracaine decreased mean contraction amplitude by 32 % whereas Ni^{2+} alone or in combination with tetracaine resulted in 85 and 88 % reduction in contraction amplitude, respectively. Figure 3-22B shows that there was no significant effect of tetracaine on tail currents associated with repolarization whereas Ni^{2+} alone or in combination with tetracaine resulted in a significant (65% and 72%, respectively) inhibition of this current. These results are in agreement with previous studies which demonstrate ~60 % inhibition of Na-Ca_{EX} by 2 mM Ni^{2+} (Kimura *et al.*, 1987).

The present results indicate that 300 μM tetracaine, which provides an effective block of the VSRM component of contraction, does not inhibit the Na-Ca_{EX}. Thus, tetracaine would be a useful pharmacological tool for differentiating between sustained ramp-like contractions elicited by reverse-mode Na-Ca_{EX} and sustained contractions elicited by the VSRM. These results also indicate that 2 mM Ni^{2+} is an effective blocker

Figure 3-22. Rapid application of tetracaine can be used to differentiate between sustained VSRM contractions and contractions elicited by reverse-mode $NaCa_{EX}$. The voltage clamp protocol was the same as that shown at the top of figure 3-20. Panel A. Mean data illustrating the differential effects of tetracaine and Ni^{2+} on contractions elicited by reverse-mode $NaCa_{EX}$. Rapid application of tetracaine caused a small but significant decrease in contraction amplitude (32 %), whereas Ni^{2+} alone or in combination with tetracaine virtually abolished contraction (85 and 88 % inhibition, respectively). Mean data illustrated in panel B shows substantial inhibition of tail-current by rapid application of Ni^{2+} alone or in combination with tetracaine. Rapid application of tetracaine alone had no significant effect on inward tail-current. (n=10 myocytes) (* denotes $p<0.01$; ** denotes $p<0.001$)

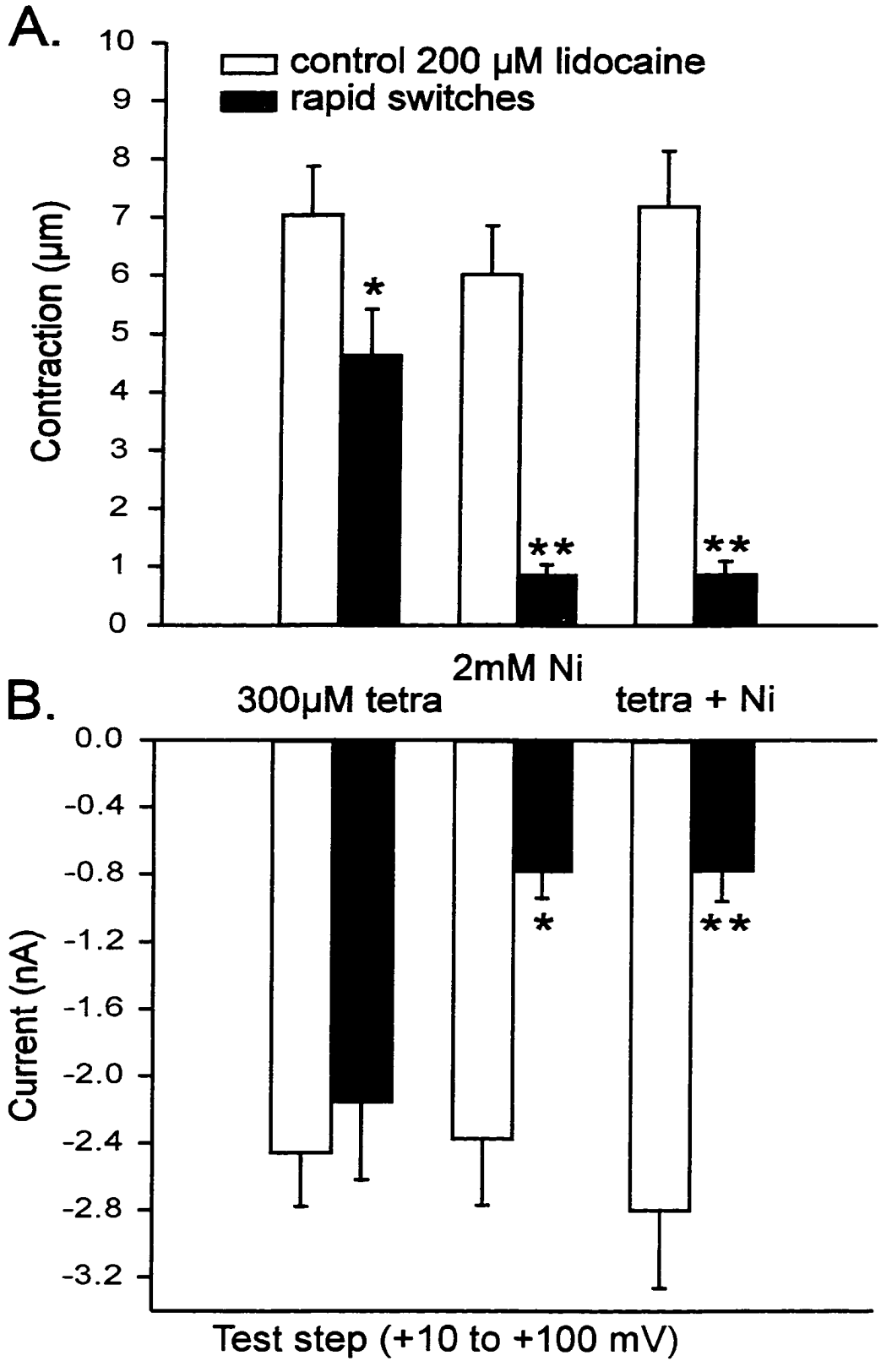

A.
control 200 µM lidocaine
rapid switches
Contraction (µm)
10
9
8
7
6
5
4
3
2
1
0
*
**
**
2mM Ni
B.
300µM tetra
tetra + Ni
0.0
-0.4
-0.8
-1.2
-1.6
-2.0
-2.4
-2.8
-3.2
Current (nA)
*
**
Test step (+10 to +100 mV)

Figure 3-22

of contractions elicited by CICR coupled to reverse mode $NaCa_{EX}$. Therefore, in the next series of experiments both of these agents were used to investigate whether the sustained contractions illustrated in figure 3-19 represent activation of VSRM or reverse mode $NaCa_{EX}$.

The voltage clamp protocol shown at the top of figure 3-23 was used to promote activation of large sustained contractions. The protocol began with a train of conditioning pulses followed by a step to 0 mV (250 msec) from a V_{PC} of -65 mV. The step to 0 mV was then followed by a long (6 seconds) depolarizing step to -20 mV which elicited large sustained contractions that lasted for the duration of the step. A 4 second application of 200 μM tetracaine or 2 mM Ni^{2+} was carried out during the step to -20 mV to determine the effects of these agents on sustained contractions. Thus, the effects of tetracaine and Ni^{2+} on the sustained component of contractions could be observed in isolation from any effects these agents may have on the phasic component of contractions.

Figure 3-23A shows that rapid application of Ni^{2+} had virtually no affected on the amplitude of the sustained contraction. On the other hand, figure 3-23B shows that compared to control, rapid application of tetracaine virtually abolished the sustained component of contraction. This effect of tetracaine was reversible as the sustained contraction began to recover following termination of the tetracaine application. The observation that sustained contractions are insensitive to a selective blocker of $NaCa_{EX}$ suggests that they are not triggered by Ca^{2+} influx via the Na-Ca_{EX}. By contrast, inhibition of this component of contraction by rapid application of tetracaine suggests

Figure 3-23. Sustained contractions are virtually abolished by rapid application of tetracaine but not 2 mM Ni^{2+}. The voltage clamp protocol shown at the top of panel A elicited both a phasic and a sustained contraction. Panel A. A 4 second application of 2 mM Ni^{2+} during the step to -20 mV did not inhibit the sustained contraction. Panel B. A similar 4 second application of 200 μM tetracaine reversibly inhibited the sustained contraction. Similar effects of Ni^{2+} and tetracaine were observed in 20 of 20 cells.

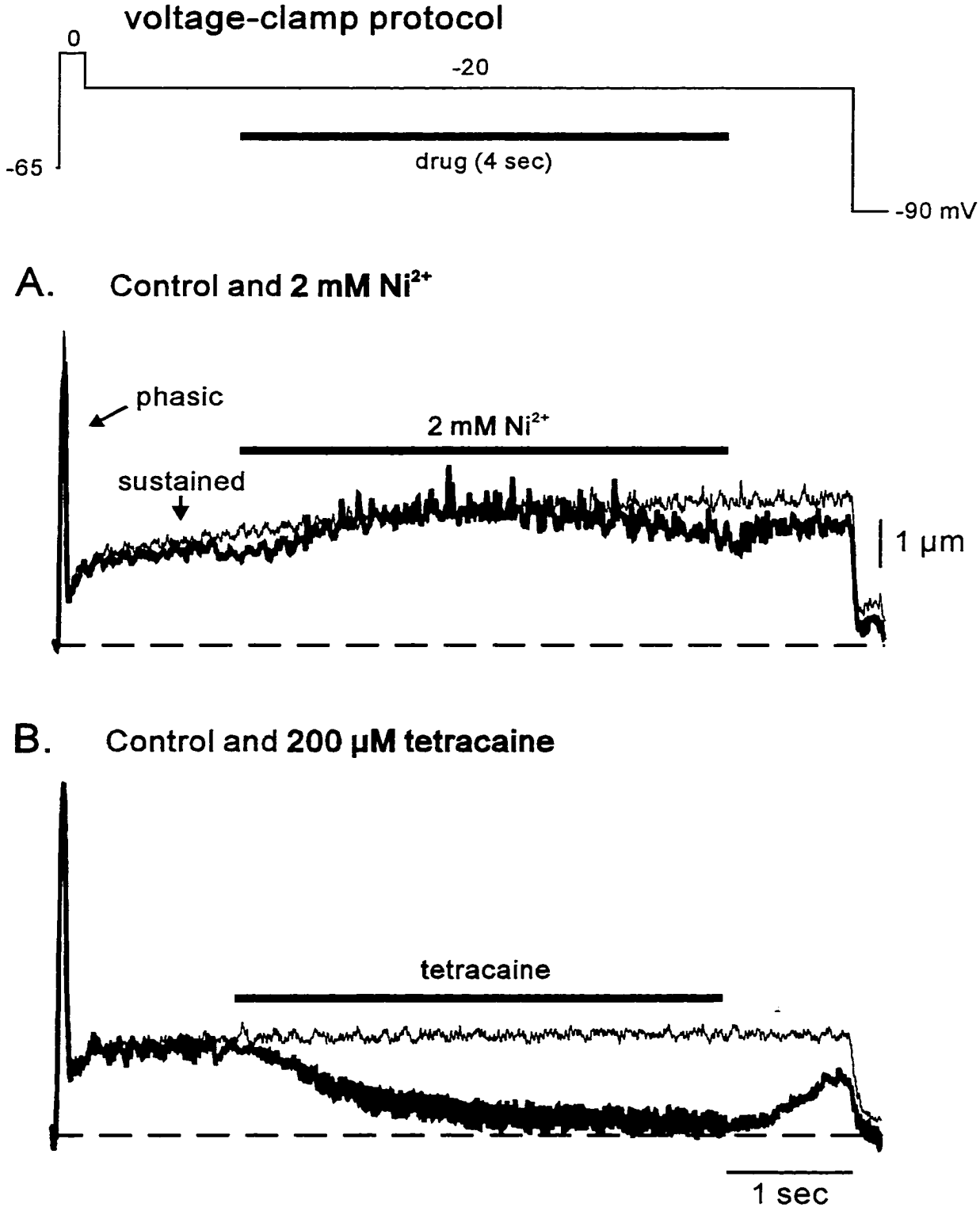

voltage-clamp protocol
0
-20
-65
drug (4 sec)
-90 mV
A. Control and 2 mM Ni2+
phasic
2 mM Ni2+
sustained
1 µm
B. Control and 200 µM tetracaine
tetracaine
1 sec

Figure 3-23

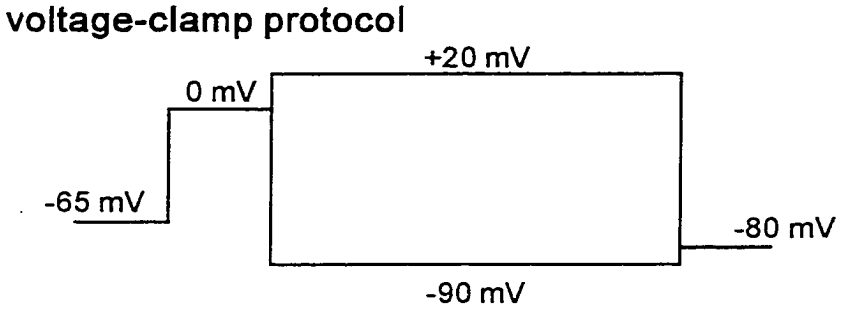
that sustained contractions are generated by the VSRM. Similar results were observed in 20 out of 20 cells tested.

Ca^{2+} transients also demonstrate a tetracaine-sensitive sustained component. The occurrence of sustained contractions suggests that continuous activation of the VSRM results in a maintained SR Ca^{2+} release during depolarization. In support of this experiments carried out using myocytes loaded with the Ca^{2+}-sensitive dye fura-2 demonstrated Ca^{2+} transients with both a phasic and sustained component (Ferrier *et al.*, 1999). The mechanism of inhibition of sustained contractions by tetracaine may represent inhibition of maintained SR Ca^{2+} release by this agent. Thus, the next series of experiments were carried out to investigate the effects of tetracaine on SR Ca^{2+} release.

In order to determine the effects of tetracaine on SR release cells were loaded with the Ca^{2+}-sensitive dye fura-2. The voltage clamp protocol is shown at the top of figure 3-24. This protocol was run twice, the first time the step to 0 mV was followed by a long step to +20 mV to promote large sustained Ca^{2+} transients. The second time the step to 0 mV was followed by repolarization of the myocyte to -90 mV to determine the baseline Ca^{2+} transient at rest. The resulting Ca^{2+} transients were superimposed on one another to demonstrate the amplitude of the sustained Ca^{2+} transient elicited by the step to +20 mV compared to the baseline fluorescence at the rest potential (-90 mV).

Figure 3-24A shows representative traces of Ca^{2+} transients elicited under control conditions by this protocol. The step to 0 mV produced a phasic Ca^{2+} transient while the step to +20 mV produced a sustained transient which resembled the sustained contractions illustrated in figures 3-19 and 3-23. In contrast, repolarizing to -90 mV after

Figure 3-24. Tetracaine inhibits a sustained component of Ca^{2+} transients. The voltage clamp protocol is shown at the top. A test step to 0 mV was followed by a step to +20 mV or repolarization to -90 mV. Panel A. Sequential steps to 0 and +20 mV elicited a phasic and sustained transient, respectively. Repolarization to -90 mV following a step to 0 mV did not elicit a sustained transient. Panel B. The sustained transient associated with the step to +20 mV was inhibited in the presence of tetracaine (200 μM) applied continuously through the experimental chamber (n=9 myocytes).


voltage-clamp protocol
+20 mV
0 mV
-65 mV
-80 mV
-90 mV

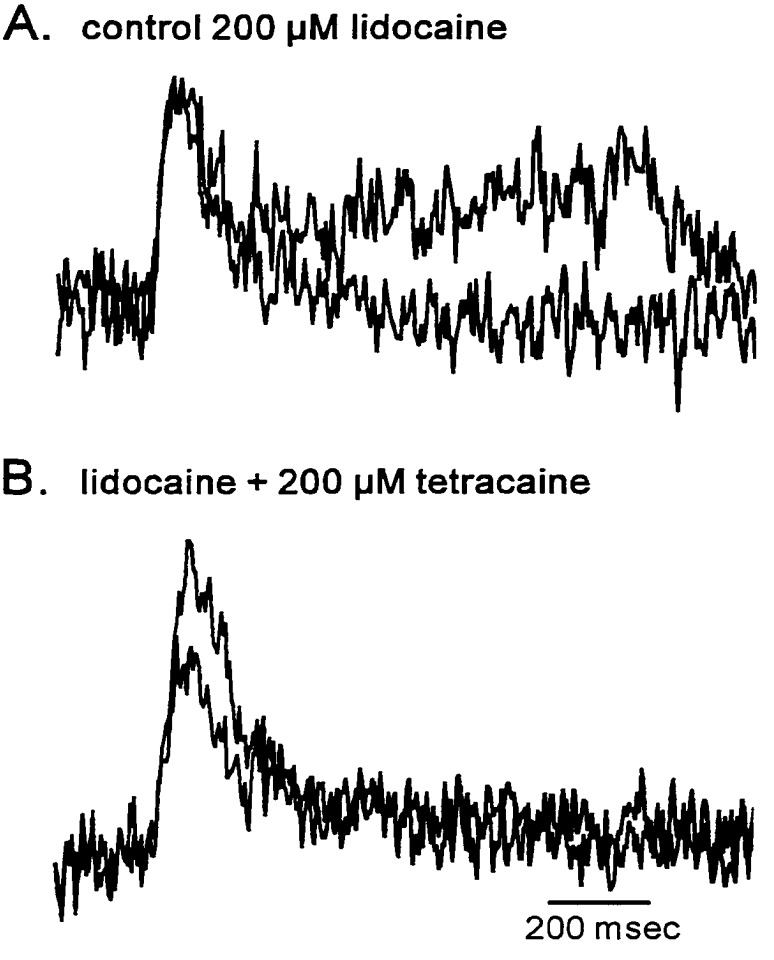

A. control 200 µM lidocaine
B. lidocaine + 200 µM tetracaine
200 msec

Figure 3-24

the step to 0 mV resulted in a decline in $[Ca^{2+}]_i$ to the baseline level at -80 mV. In the presence of 200 μM tetracaine added to the bulk solution the sustained transient associated with the step to +20 mV was abolished (figure 3-24B) (mean length of exposure was 6 minutes). These results suggest that inhibition of the sustained component of contraction by tetracaine is due to a decrease in $[Ca^{2+}]_i$. This decrease in $[Ca^{2+}]_i$ likely represents inhibition of SR Ca^{2+} release by tetracaine.

Is inhibition of the VSRM by tetracaine due to a shift in the properties of inactivation of this component of contraction? In the next series of experiments the protocol illustrated at the top of figure 3-25 was used to determine the effects of tetracaine on the inactivation properties of VSRM contractions in isolation from CICR coupled to $I_{Ca\text{-}L}$. The tetracaine concentration used in these experiments was 100 μM which provides incomplete inhibition of the VSRM so that a shift in the inactivation properties of this mechanism could be observed. This protocol involves a test step to -35 mV which predominantly elicits VSRM contractions as shown by the experiments carried out with the 2 step protocol and earlier studies whereby very little if any $I_{Ca\text{-}L}$ was activated at this potential (Howlett, Zhu & Ferrier, 1998). With each repetition of the voltage clamp protocol the V_{PC} was made progressively more negative from -40 to -70 mV in 5 mV increments. The duration of each V_{PC} was 600 msec, long enough to obtain steady-state inactivation before depolarizing to the test step (-35 mV). A brief repolarization to -70 mV (10 msec) immediately followed the V_{PC}, therefore the activation step was from the same potential each time (-70 mV).

Figure 3-25A illustrates representative traces of contractions elicited under control conditions by each repetition of the voltage clamp protocol. These traces were superimposed on one another to illustrate changes in cell length in response to changes in V_{PC}. The area of the traces labeled *a* illustrates that at a V_{PC} of -40 mV the cell length was shorter than that at -70 mV. In fact, the cell length increased progressively with each -5 mV change in the V_{PC}. Thus, the superimposed contraction traces in figure 3-25A illustrate the presence of a sustained contraction at potentials positive to -70 mV. As well, the phasic contractions (labeled *b*) elicited by the step to -35 mV also varied in response to changes in the V_{PC}, with the largest amplitude contraction being associated with a V_{PC} of -70 mV and the smallest with a V_{PC} of -40 mV. Thus, the larger the contraction during the V_{PC} (*a*) the smaller the amplitude of the phasic contraction (*b*) at the test step.

The superimposed traces also demonstrate that each phasic contraction did not fully relax, but instead maintained the same partial contraction until the end of the test step. Thus, the contractions associated with the test step to -35 mV had both a phasic and a sustained component. In contrast to the sustained contractions elicited by the V_{PC} (labeled *a*) and the phasic contractions elicited by the test step (labeled *b*), the sustained contractions associated with the test step (labeled *c*) did not vary in response to changes in the V_{PC}. Complete relaxation of contractions occurred upon repolarization of the myocyte to the holding potential of -80 mV (labeled *d*).

The decrease in the maximum amplitude of phasic contractions with more depolarized V_{PC} resembles a process known as inactivation. Inactivation is normally used

Figure 3-25. Effects of tetracaine on both the phasic and sustained components of VSRM contractions elicited by a step to -35 mV from various different V_{PC}. The voltage clamp protocol is shown at the top of panel A. The V_{PC} decreased from -40 to -70 mV in 5 mV increments with each repetition of the voltage clamp protocol. Each V_{PC} was followed by a brief repolarization to -70 mV before stepping to -35 mV. Panel A. Representative traces of contractions elicited by each successive V_{PC} and test step superimposed on one another. A phasic component (*b*) initiated by the step to -35 mV was followed by a sustained component (*c*) which lasted for the duration of the step. A sustained contraction (*a*) also was associated with each V_{PC}. The amplitude of the phasic contractions increased as the V_{PC} decreased. In contrast, the amplitude of the sustained contractions (*a*) associated with each V_{PC} decreased as the V_{PC} decreased. Panel B illustrates representative traces of contractions elicited by the same protocol except in the presence of continuously applied 100 μM tetracaine. There was virtually no change in any of the components of these contractions in the presence of tetracaine compared to control.

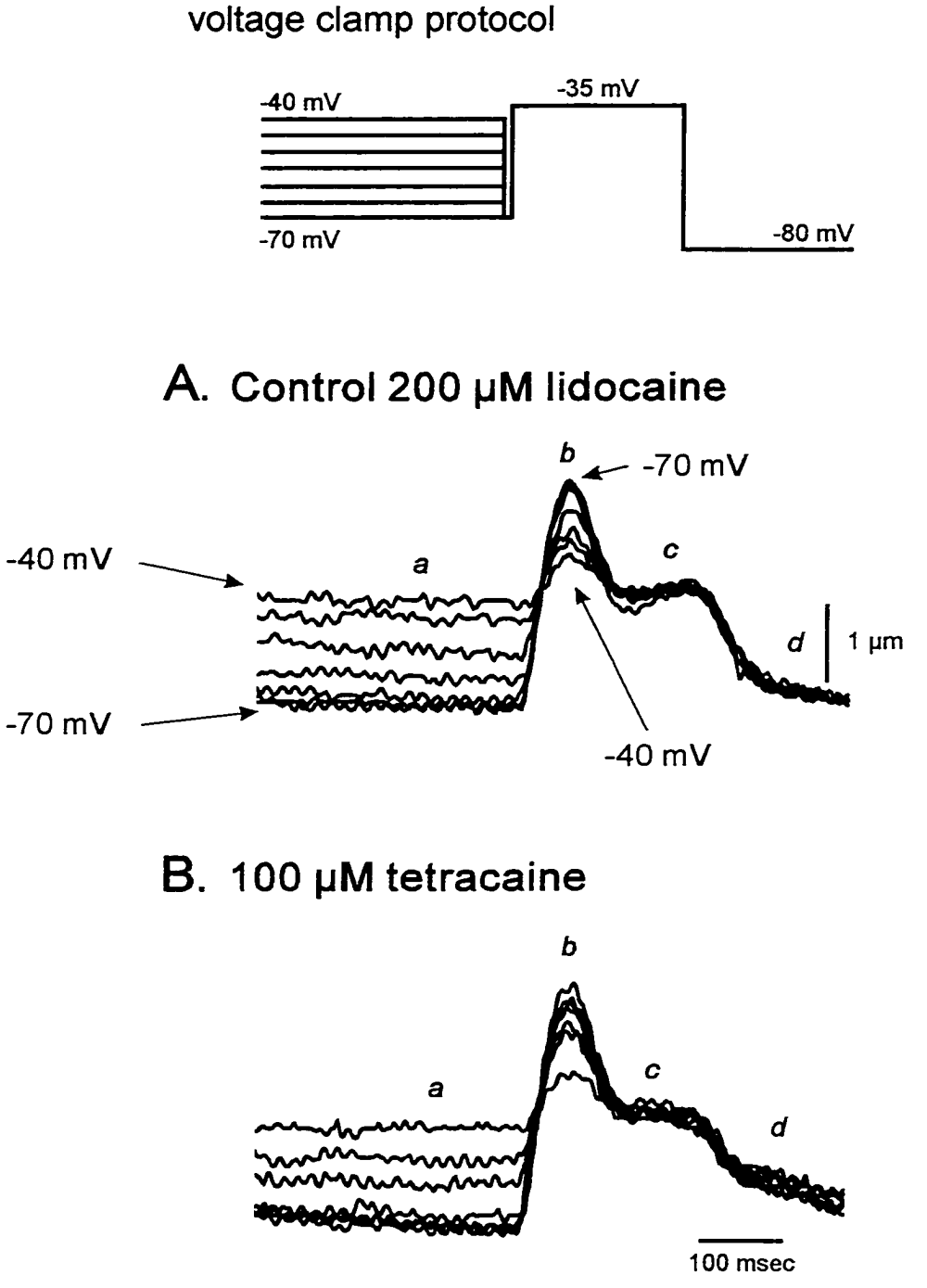

voltage clamp protocol
-40 mV
-35 mV
-70 mV
-80 mV
A. Control 200 µM lidocaine
b
-70 mV
-40 mV
a
c
d
1 µm
-70 mV
-40 mV
B. 100 µM tetracaine
b
a
c
d
100 msec

Figure 3-25

to describe the transition of a voltage-gated ion channel from a conducting to a non-conducting state in response to depolarization. A measure of the change in conductance through a channel in response to changes in V_{PC} produces a sigmoidal curve which is used to describe the properties of inactivation of that channel. By comparison, previous studies have used the term inactivation to describe the decrease in amplitude of the phasic component of VSRM contractions as the V_{PC} was made progressively more positive (Ferrier & Howlett, 1996; Howlett, Zhu & Ferrier, 1998). A measure of the change in amplitude of the phasic component of VSRM contractions in response to changes in V_{PC} produces a sigmoidal curve which is consistent with use of the term inactivation to describe this process.

The observation that VSRM contractions show properties of inactivation raises the possibility that inhibition of this mechanism by tetracaine results from a shift in the voltage dependence of the inactivation process. For example, in the presence of tetracaine the VSRM may inactivate at more negative potentials so that contractions which are normally elicited at -40 mV are inactivated by the V_{PC} of -70 mV. Thus, the absence of contraction at -40 mV in the presence of tetracaine may not represent inhibition by this agent, but rather may represent a shift in activation of contraction to more negative potentials.

In order to determine the effects of tetracaine on steady-state inactivation of phasic VSRM contractions the protocol illustrated at the top of figure 3-25A was repeated in the presence of 100 μM tetracaine. Figure 3-25B illustrates representative traces of contractions elicited in the presence of tetracaine. As observed under control conditions,

a sustained contraction was associated with a V_{PC} of -40 mV. The amplitude of this sustained contraction decreased as the V_{PC} was made progressively more negative. However, in contrast to control, cell length no longer varied with V_{PC} from -55 mV to -70 mV in the presence of tetracaine. The corresponding phasic contractions elicited by the step to -35 mV were similar to those elicited under control conditions. As well, the maximum amplitude of the sustained contractions associated with the test step to -35 mV (labeled *c*) appeared to be slightly depressed in the presence of tetracaine compared to control. As in control, complete relaxation occurred upon repolarization to the holding potential.

To generate inactivation curves, the amplitudes of the phasic contractions (*b*) were measured with reference to the cell length at *a* and plotted against the corresponding V_{PC}. Figure 3-26A illustrates the mean data representing inactivation of the phasic component of VSRM contractions under control and tetracaine treated conditions. A V_{PC} of -70 mV elicited maximum amplitude contractions while progressively more positive V_{PC} elicited smaller amplitude contractions with nearly complete inactivation occurring around -40 mV. In the presence of tetracaine the mean contraction amplitude was slightly decreased over the entire voltage range although this effect was not significant.

To determine whether tetracaine shifted the properties of inactivation the control and tetracaine treated curves were normalized to their respective maximal contraction. The normalized data are illustrated in figure 3-26B. The normalized curves were fit with a Boltzman function of the following form; $y = 1/\{1+exp[(V - V_h)/k]\}$. V is the test potential, V_h is the potential at which VSRM contractions are half maximal, and k is the slope factor. In the present study the Boltzman function describes the availability of

Figure 3-26. Inhibition of VSRM contractions by tetracaine is not due to a shift in the properties of either the phasic or sustained components. The voltage clamp protocol is the same as that illustrated in figure 3-25. The representative traces illustrated in figure 3-25A are shown at the top of figure 3-26. Panel A illustrates the mean data for the effects of continuous application of tetracaine on inactivation of the phasic component of VSRM contractions. Tetracaine caused a slight decrease in contraction amplitude over the entire voltage range. Panel B. Mean data from panel A were normalized to the maximum contraction and fit with a Boltzman function. There was no shift in the inactivation curve in the presence of tetracaine compared to control. Panel C shows the mean CV relationships for activation of sustained contractions. There was no shift in the activation properties of sustained contractions in the presence of tetracaine. (n=11 cells)

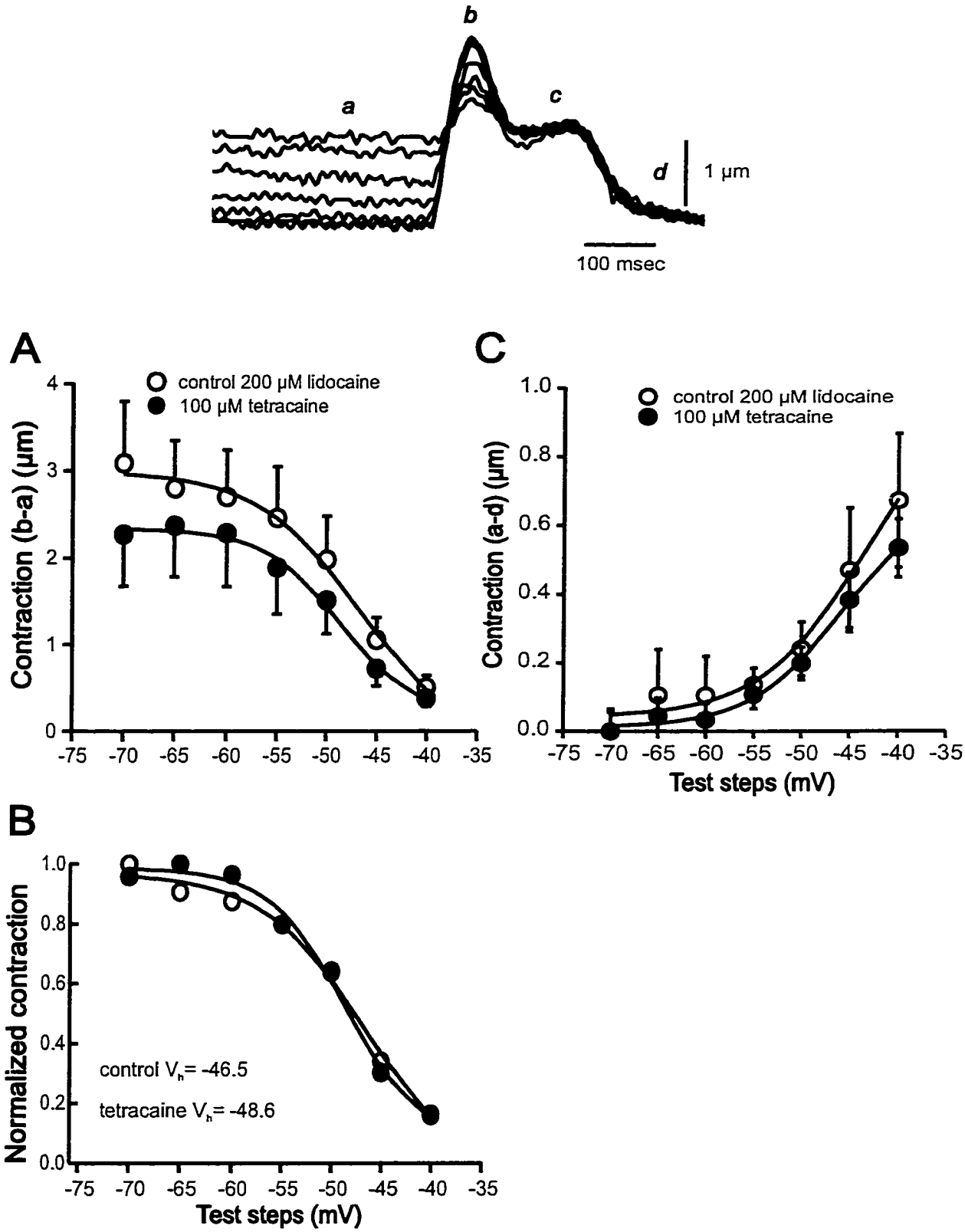

b
a
c
d
1 µm
100 msec
A
control 200 µM lidocaine
100 µM tetracaine
Contraction (b-a) (µm)
C
control 200 µM lidocaine
100 µM tetracaine
Contraction (a-d) (µm)
Test steps (mV)
B
Normalized contraction
control V$_h$= -46.5
tetracaine V$_h$= -48.6
Test steps (mV)

Figure 3-26

phasic VSRM contractions over the range of V_{PC}. The results in figure 3-26B show that the inactivation curve obtained in the presence of tetracaine was virtually identical to that obtained under control conditions. In the presence of tetracaine V_h was -48.6 compared to -46.5 mV in control (n=11 cells from 11 hearts). Thus, the decline in mean contraction amplitude in the presence of 100 μM tetracaine (figure 3-26A) does not reflect a shift in the properties of inactivation of this component of EC-coupling.

Are the effects of tetracaine on VSRM contractions due to a shift in the properties of the sustained, non-inactivating component of these contractions? The properties of steady-state inactivation of VSRM contractions describe only one component of these contractions. The representative traces illustrated in figure 3-25 show that at the more positive V_{PC} the cell maintained some level of contraction (labeled *a*) which lasted for the duration of the V_{PC}. As the V_{PC} was made progressively more negative the amplitude of the maintained contraction decreased. This variation in cell length represents the sustained, non-inactivating component of VSRM contractions which shows properties of activation/deactivation (Ferrier *et al.*, 1999). The present study has illustrated that similar to the phasic inactivating component, the sustained component of VSRM contractions also is tetracaine-sensitive. Thus, it was possible that the effects of tetracaine on VSRM contractions resulted from a shift in the properties of activation.

Previous studies have reported that the variation in cell length at each V_{PC} represents activation of the sustained component of VSRM contractions (Ferrier *et al.*, 1999). Therefore, the effects of tetracaine on the properties of this component of contraction could be determined from the voltage clamp protocol shown at the top of

figure 3-25. The amplitude of the sustained contractions (labeled *a*) were measured with respect to the cell length at -80 mV (labeled *d*) as this was thought to represent complete relaxation. A plot of these contractions versus V_{PC} is shown in figure 3-26C. Under control conditions sustained contractions were activated at potentials positive to -70 mV and the amplitude of these contractions continued to increase throughout the entire voltage range. There was virtually no change in the mean CV relationship in the presence of tetracaine. This curve represents only the initial portion of an activation curve as contraction amplitude did not reach a plateau. However, the control and tetracaine curves are virtually identical which suggests that tetracaine caused little if any shift in the properties of activation of sustained VSRM contractions. Thus, inhibition of VSRM contractions by tetracaine does not likely result from a shift in the activation or inactivation properties of either the phasic or the sustained components of these contractions.

Effects of tetracaine on inactivation of contractions elicited by a test step to 0 mV.

The effects of tetracaine on inactivation of total contraction initiated by test steps to 0 mV also was determined. The protocol used in this series of experiments is illustrated at the top of figure 3-27. This protocol was similar to that shown in figure 3-25 except that the activation step was to 0 mV which allows activation of both the VSRM and CICR coupled to $I_{Ca\text{-}L}$ (Howlett, Zhu & Ferrier, 1998). As well, with each repetition of the voltage clamp protocol the V_{PC} was made more negative in 5 mV increments from -20 to -75 mV as opposed to -40 to -70 mV in figure 3-25. Figure 3-27A illustrates representative examples of contractions elicited by each repetition of this protocol superimposed on one another. The phasic contractions (labeled *b*) represent

Figure 3-27. Effects of tetracaine on the properties of contractions elicited by activation of both CICR coupled to $I_{Ca\text{-}L}$ and VSRM. The voltage clamp protocol is shown at the top. Panel A. Representative traces of phasic and sustained contractions elicited under control conditions. Contractions elicited by the step to 0 mV had both a phasic and a sustained component. Sustained contractions also were elicited by V_{PC} positive to -60 mV. Panel B illustrates a representative example of contractions elicited in the presence of 100 μM tetracaine. The maximum amplitude of the sustained component of contractions decreased while the phasic contractions increased slightly in the presence of tetracaine.

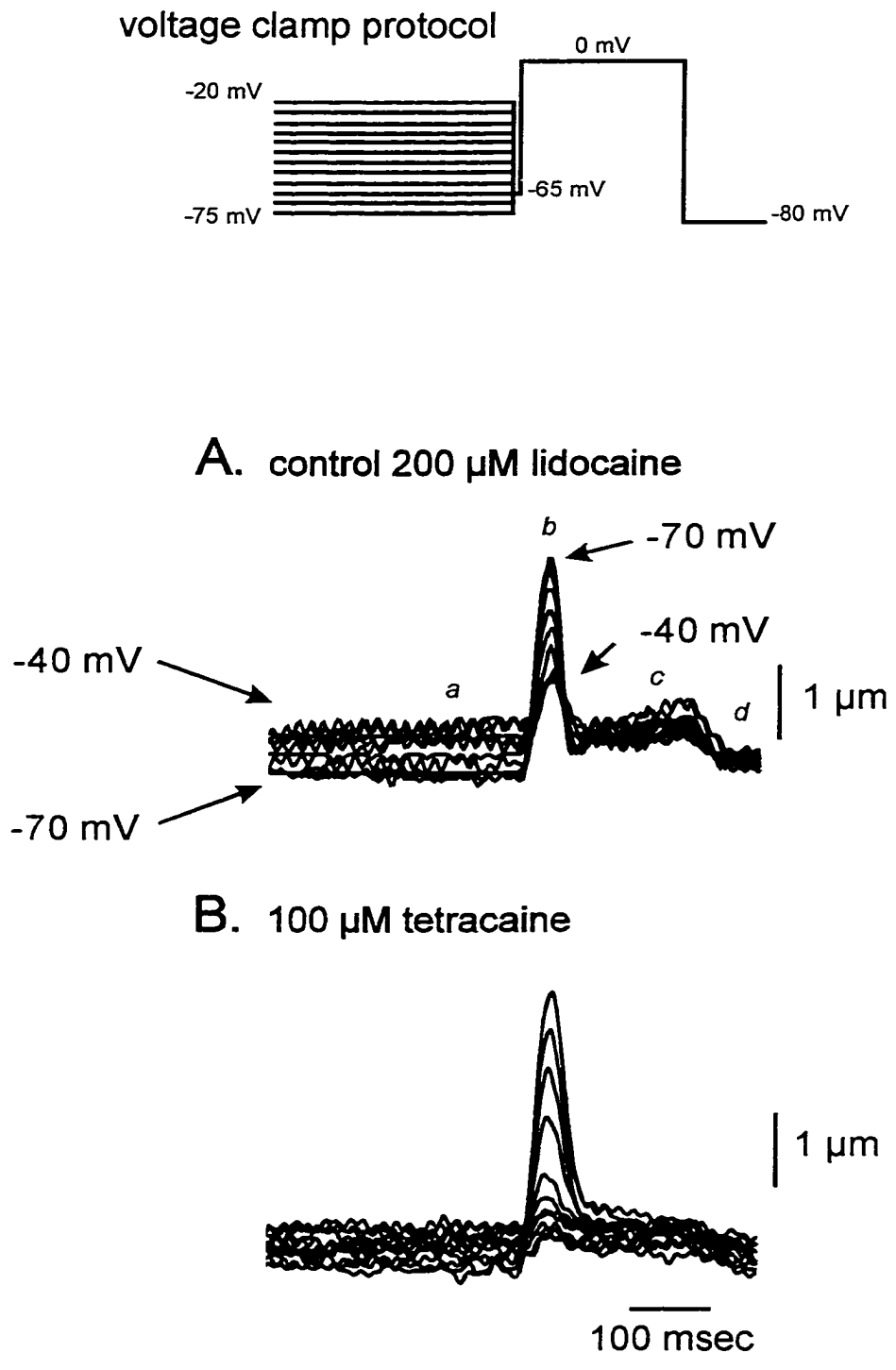

voltage clamp protocol
0 mV
-20 mV
-65 mV
-75 mV
-80 mV
A. control 200 µM lidocaine
b
-70 mV
-40 mV
-40 mV
a
c
d
1 µm
-70 mV
B. 100 µM tetracaine
1 µm
100 msec

Figure 3-27

activation of both the VSRM and CICR coupled to $I_{Ca\text{-}L}$. The amplitudes of these contractions also changed in response to the preceding conditioning potentials in a manner consistent with inactivation. These phasic contractions also did not reach full relaxation during the depolarizing test step but instead maintained a level of partial contraction (labeled *c*) until repolarization of the membrane to -80 mV.

Figure 3-27B illustrates representative traces of contractions elicited in the presence of tetracaine. The maximum amplitude of the sustained contractions elicited by each V_{PC} (labeled *a*) appeared to be slightly depressed compared to control. As well, the sustained contractions elicited by the test step to 0 mV also appeared to be depressed in the presence of tetracaine. By contrast, the phasic contractions elicited by the step to 0 mV were of larger amplitude than those elicited under control conditions. Thus, the sustained component of contractions which represent activation of the VSRM were depressed in the presence of tetracaine while the amplitudes of the phasic component which represents both VSRM and CICR increased. The increase in the amplitude of the phasic component is consistent with the effects of tetracaine on SR Ca^{2+} stores which was previously demonstrated (figure 3-8).

Inactivation of contraction was determined by measuring the phasic contraction with respect to the cell length near the end of V_{PC} (labeled *a*) and plotting this value against the V_{PC}. The mean data are illustrated in figure 3-28A. Because these curves represent activation of two components of cardiac EC-coupling, in theory it is not valid to fit them with a Boltzman function. However, the Boltzman fits help visualize shifts in voltage dependent phenomena, therefore a Boltzman function ($y = 1/\{1+exp[(V-V_h)/k]\}$)

Figure 3-28. Inhibition of cardiac contractions by tetracaine does not result from a shift in the properties of inactivation of VSRM or CICR contractions. The voltage clamp protocol is the same as that illustrated at the top of figure 3-27. Panel A. Mean data showing the voltage dependence of phasic contractions (*b*) elicited by a step to 0 mV from a V_{PC} of -70 mV in the presence and absence of 100 μM tetracaine added continuously through the experimental chamber. Tetracaine caused a slight increase in contraction amplitude over most of the voltage range. Panel B. The control and tetracaine treated curves in panel A were normalized to their respective maximum contraction and fit with a Boltzman function of the following form; $y = 1/\{1+exp[(V-V_h)/k]\}$. Both curves were virtually identical with V_h of -44.9 and -43.3 mV under control and tetracaine treated conditions, respectively. Thus, tetracaine did not shift the properties of inactivation of phasic contractions elicited by activation of both VSRM and CICR. (n=8 myocytes)

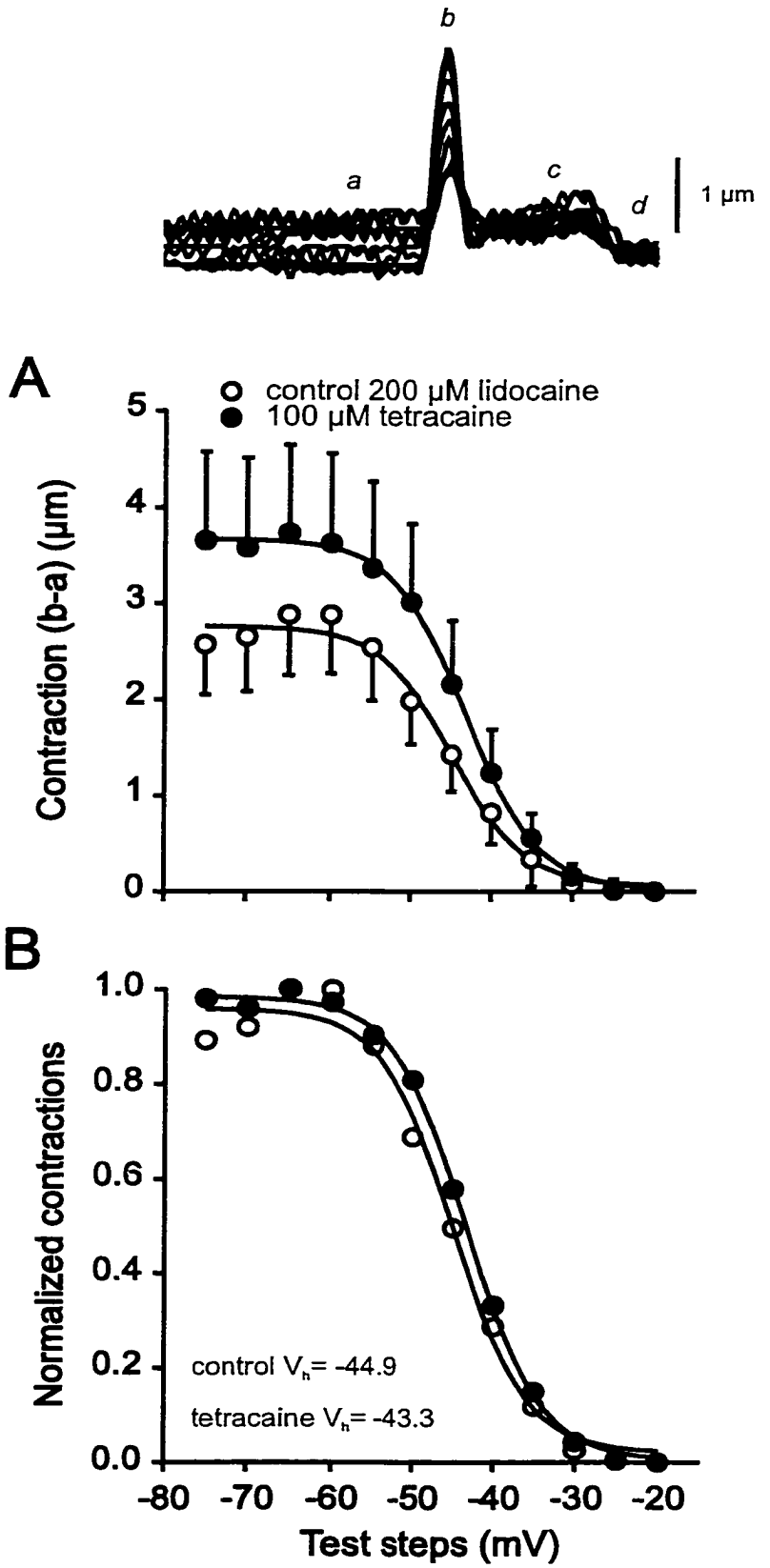

b
a
c
d
1 µm
A
control 200 µM lidocaine
100 µM tetracaine
Contraction (b-a) (µm)
5
4
3
2
1
0
B
Normalized contractions
1.0
0.8
0.6
0.4
0.2
0.0
control V_h= -44.9
tetracaine V_h= -43.3
-80 -70 -60 -50 -40 -30 -20
Test steps (mV)

Figure 3-28

was used to determine whether tetracaine shifted the inactivation properties of overall contraction. In the presence of tetracaine the maximum amplitude of contractions increased over most of the voltage range compared to control. Thus, in contrast to the results illustrated in figure 3-25 which demonstrate a slight decline in the amplitude of VSRM contractions, the present results show an increase in overall contraction amplitude in the presence of tetracaine. Contractions were normalized to the maximum contraction to determine whether tetracaine caused a shift in the properties of inactivation. Figure 3-28B shows the CV relationships for the normalized data. CV curves obtained in the presence of tetracaine were similar to those obtained under control conditions, therefore tetracaine did not shift the properties of inactivation of contractions elicited by a step to 0 mV.

The effects of tetracaine on sustained contractions elicited by the V_{PC} also was determined. The cell length at each V_{PC} was measured with respect to the cell length upon repolarization of the membrane to -80 mV (labeled *d*) as full relaxation appears to occur at this potential. As shown in figure 3-29A, a plot of these changes in cell length against the V_{PC} gives the activation-deactivation curves for these contractions elicited in the presence and absence of tetracaine. Although both the VSRM and CICR are available on the test step to 0 mV, the sustained contractions likely represent activation of the VSRM alone since CICR coupled to $I_{Ca\text{-}L}$ does not exhibit a sustained component. The mean data illustrated in figure 3-29A show that in agreement with the result in figure 3-26, tetracaine caused a slight decrease in the amplitude of sustained VSRM contractions over the entire voltage range.

Figure 3-29. Tetracaine does not shift the properties of activation of sustained contractions. The voltage clamp protocol is the same as that shown at the top of figure 3-27. The representative traces illustrated in figure 3-27A are shown at the top of figure 3-29. Panel A. Mean data showing the effects of tetracaine on the properties of activation of sustained VSRM contractions (labeled *a*) elicited by each V_{PC} compared to control. Tetracaine caused a slight decrease in contraction amplitude over most of the V_{PC} range. Panel B. The mean data from panel A were normalized to the maximum contraction and fit with a Boltzman function of the following form; $y = 1/\{1+exp[(V-V_h)/k]\}$. The V_h for the normalized tetracaine curve was -45.5 mV compared to -46.8 mV in control, therefore tetracaine did not shift the properties of activation of sustained contractions (n=8 cells).

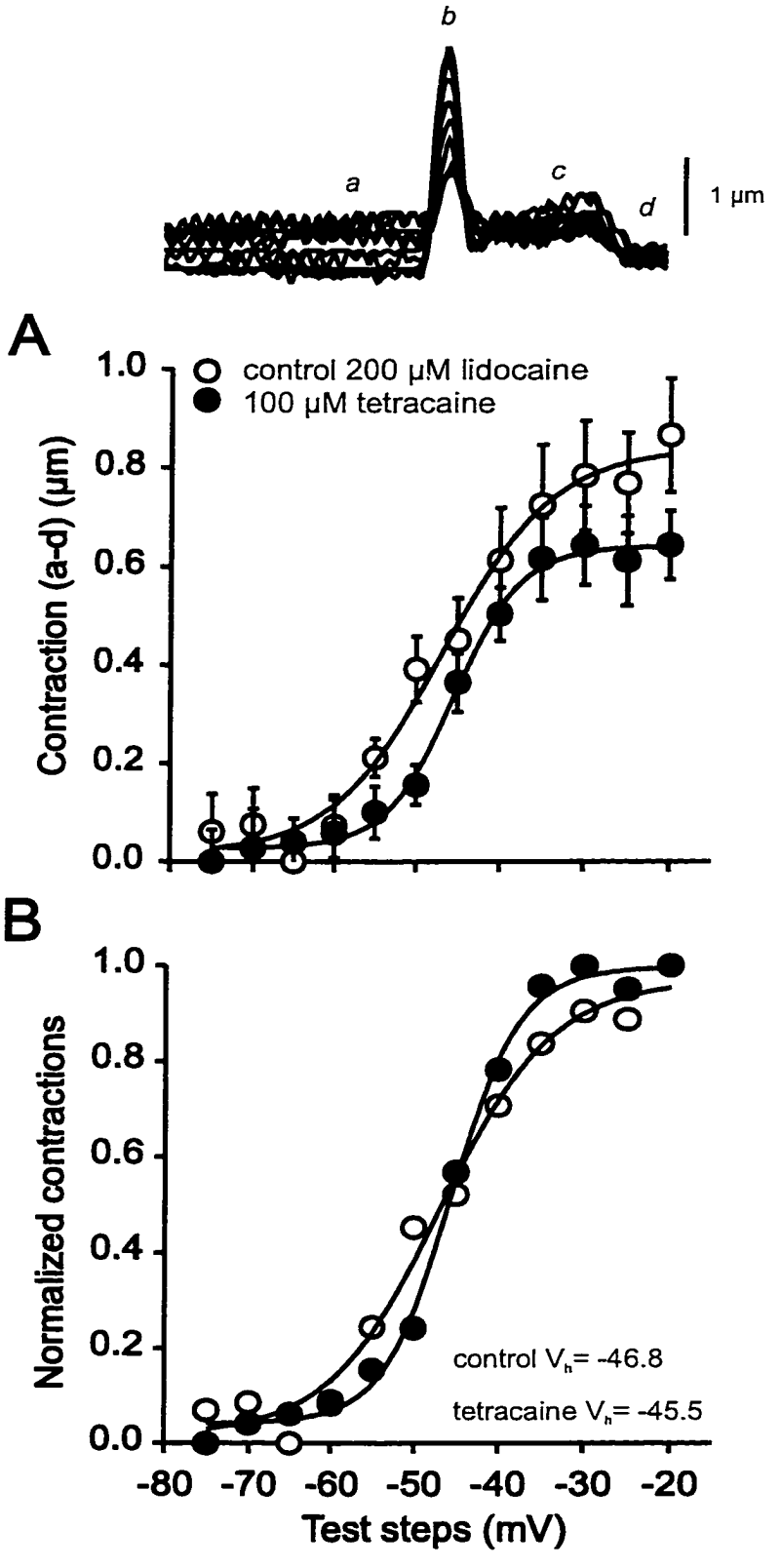

b
a
c
d
1 µm
A
control 200 µM lidocaine
100 µM tetracaine
Contraction (a-d) (µm)
1.0
0.8
0.6
0.4
0.2
0.0
B
Normalized contractions
control V_h= -46.8
tetracaine V_h= -45.5
-80 -70 -60 -50 -40 -30 -20
Test steps (mV)

Figure 3-29

To visualize the effects of tetracaine on the properties of activation/deactivation of contractions these curves were normalized to the maximum contraction as illustrated in figure 3-29B. Since these curves represent only the VSRM it was valid to fit them with a Boltzman function ($y = 1/\{1+exp[(V - V_h)/k]\}$). Under control conditions V_h was -46.8 mV compared to -45.5 mV in the presence of tetracaine. Therefore, similar to the results shown in figure 3-26, those illustrated in figure 3-29B indicate that tetracaine does not shift the properties of activation/deactivation of VSRM contractions.

Together the results in figures 3-25 to 3-29 indicate that the effects of tetracaine on ventricular contractions are not mediated by changes in the properties of either activation-deactivation or inactivation. The results illustrated in figures 3-26 and 3-28 indicate that tetracaine does not shift the properties of inactivation of VSRM contractions in isolation (figure 3-26) nor does it shift the properties of inactivation of overall cardiac contractions (VSRM and CICR) (figure 3-28). The results shown in figure 3-28 also indicate that tetracaine does not affect inactivation of CICR contractions coupled to $I_{Ca\text{-}L}$ (n= 8 cells from 8 hearts). The increase in contraction amplitude in figure 3-28A likely reflects an effect of tetracaine on CICR coupled to $I_{Ca\text{-}L}$ since this effect was not observed when VSRM contractions were elicited in isolation (figure 3-26). The increase in contraction amplitude is consistent with a tetracaine induced increase in SR Ca^{2+} stores as demonstrated earlier (figure 3-8). Overall, the effects of tetracaine on VSRM contractions, as well as effects on SR Ca^{2+} stores, do not cause a shift in the inactivation properties of VSRM contractions or overall cardiac contractions. The voltage-independence of tetracaine effects on cardiac contractions suggests that inhibition of

VSRM contractions by this agent results from a decrease in the ability of the VSRM to release SR Ca^{2+}.

Do 8-Br-cAMP supported VSRM contractions share the same pharmacological characteristics as those elicited in intact myocytes? Most of the experiments reported in this study were carried out with high resistance electrodes which minimize dialysis of the intracellular solution. Many electrophysiological studies are conducted with a voltage clamp technique known as patch clamp which uses electrodes with large diameter tips that allow exchange of the intracellular solution with the patch electrode filling solution. This technique is useful for introducing compounds to the interior of cells to determine the effects of inhibiting or stimulating signaling pathways on the overall functioning of the cell. However, endogenous compounds within the intracellular milieu also may be dialyzed out of cells thereby disrupting normal functioning (Isenberg & Wendt-Gallitelli, 1989). Previous reports from our lab have demonstrated that contractions elicited by the VSRM are rapidly inhibited in experiments carried out with low resistance patch electrodes (Ferrier *et al.*, 1998b). However, VSRM contractions could be restored by addition of 8-Br-cAMP to the patch electrode filling solution. The observation that addition of 8-Br-cAMP restored VSRM contractions suggested that endogenous cAMP might be dialyzed out of cells by patch electrodes.

The inactivation characteristics of VSRM contractions supported by 8-Br-cAMP were found to be similar to those previously described for VSRM contractions in undialysed myocytes (Ferrier *et al.*, 1998b). In the present study the effects of tetracaine on cAMP supported VSRM contractions were investigated to determine whether the

pharmacological characteristics of these contractions also were similar. The voltage clamp protocol is illustrated at the top of figure 3-30. The duration of the V_{PC} was 4 second to allow time for rapid application of tetracaine before the test steps. The switch clamp technique was used because the membrane potential can be continuously monitored with this technique. Representative recordings of contractions and currents elicited by this protocol in the presence of 50 μM 8-Br-cAMP in the electrode filling solution are shown in figure 3-30A. A VSRM contraction was elicited by the step to -40 mV while the step to 0 mV elicited a phasic contraction and $I_{Ca\text{-}L}$ characteristic of CICR coupled to $I_{Ca\text{-}L}$. The mean amplitude of the $I_{Ca\text{-}L}$ elicited by the step to 0 mV (approximately 3 nA) in the present study is larger than that reported in experiments with high resistance electrodes (typically 1 to 1.5 nA). This increase in $I_{Ca\text{-}L}$ amplitude likely represents an increase in cAMP-dependent phosphorylation of L-type Ca^{2+} channels due to addition of 50 μM 8-Br-cAMP to the pipette (McDonald *et al.*, 1994; Ferrier *et al.*, 1998b).

Surprisingly, rapid application of tetracaine had virtually no effect on the VSRM contraction elicited by the step to -40 mV. A representative trace of contractions and currents elicited in the presence of a rapid switch to 300 μM tetracaine is shown in figure 3-30B. The amplitude of the CICR contraction, as well as $I_{Ca\text{-}L}$ appeared to be depressed in the presence of tetracaine in this example. However, mean data shown in figure 3-30C indicate that there was no significant effect of rapid application of tetracaine on either VSRM or CICR contractions, or on mean current amplitudes.

Figure 3-30. Effects of rapid application of 300 μM tetracaine on 8-Br-cAMP supported contractions and currents. The voltage clamp protocol is shown at the top. Panel A. Steps to -40 and 0 mV elicited VSRM and CICR contractions, respectively. As well, the step to 0 mV elicited $I_{Ca\text{-}L}$. Panel B. Representative traces of contractions and currents elicited in the presence of rapid application of 300 μM tetracaine 3 seconds before and during test steps. Panel C illustrates the mean data showing no significant effect of rapid application of tetracaine on contractions or currents supported by 8-Br-cAMP (n=8 myocytes).

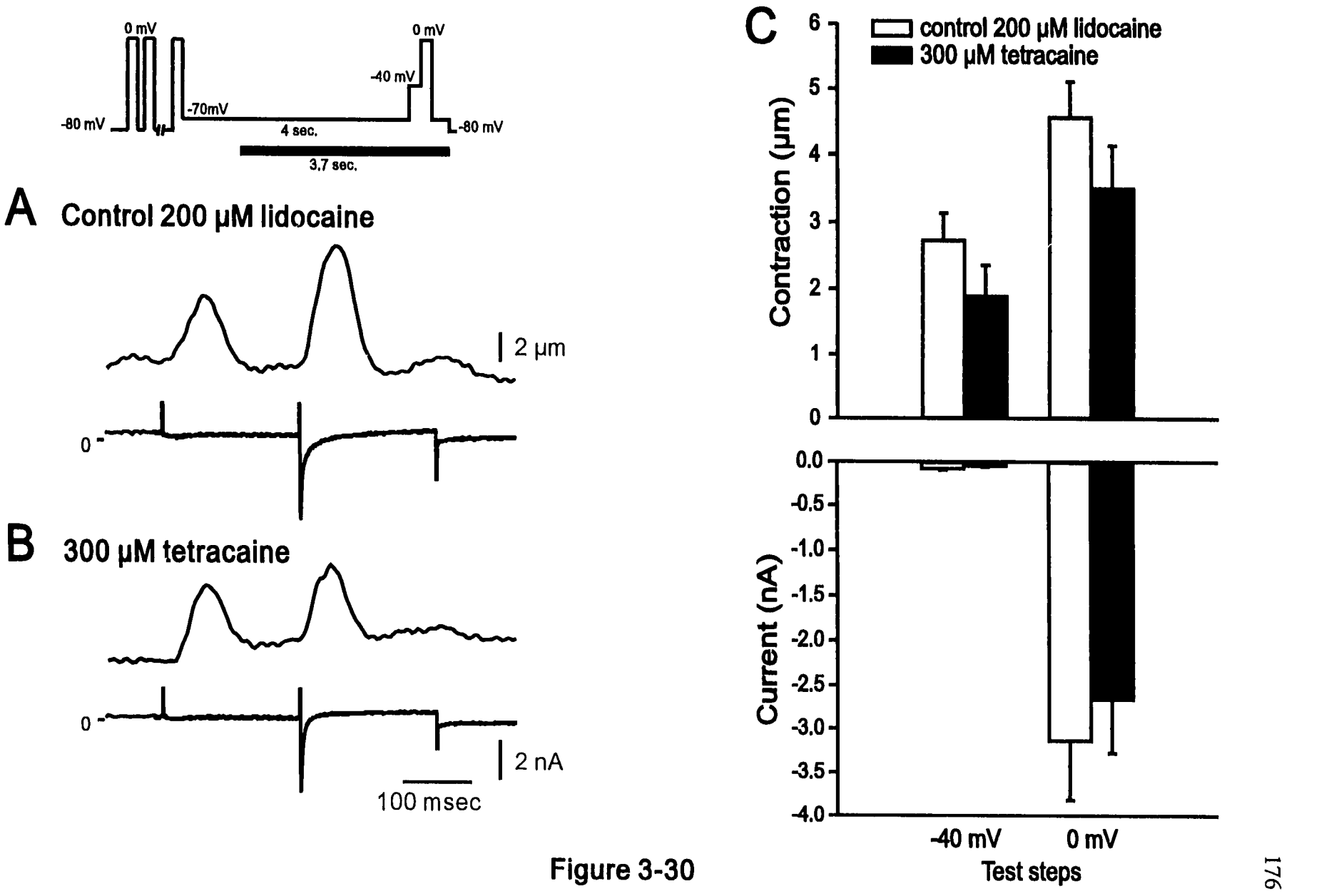

0 mV
0 mV
-40 mV
-70mV
-80 mV
4 sec.
-80 mV
3.7 sec.
A
Control 200 µM lidocaine
2 µm
0
B
300 µM tetracaine
0
2 nA
100 msec
C
control 200 µM lidocaine
300 µM tetracaine
Contraction (µm)
Current (nA)
-40 mV
0 mV
Test steps

Figure 3-30

Tetracaine preferentially inhibited 8-Br-cAMP supported VSRM contractions when applied continuously. The effects of continuously applied tetracaine also were investigated. Representative recordings of contractions and currents elicited under control and tetracaine treated conditions are illustrated in figure 3-31. Exposure of myocytes to 300 μM tetracaine abolished the VSRM contraction within 3 minutes (figure 3-31B). In this example, the $I_{Ca\text{-}L}$-induced contraction was virtually unaffected. However, the mean data illustrated in figure 3-31C show that although CICR contractions remained in the presence of tetracaine the mean amplitude was significantly depressed. Panels B and C also demonstrate significant inhibition of $I_{Ca\text{-}L}$ in the presence of tetracaine. By contrast, the mean data in figure 3-31C illustrate that VSRM contractions were virtually abolished in all cells, as were the residual currents associated with the step to -40 mV.

To further characterize the effects of tetracaine on contractions supported by 8-Br-cAMP, CV and IV relationships were determined in the presence of 8-Br-cAMP added to the pipette solution (figure 3-32). The CV relationship became bell-shaped and reflected the inward current in the presence of continuously applied tetracaine (mean exposure time 3 minutes). Similar to previous results with high resistance electrodes (see figures 3-4 and 3-7), the maximum amplitude of the CV relation remained the same in the presence and absence of tetracaine. The lack of inhibition of the peak CV relation observed in earlier studies was accompanied by a tetracaine-induced increase in SR Ca^{2+} stores, therefore effects of tetracaine on SR stores likely explains the similar results in the

Figure 3-31. Continuous application of tetracaine through the experimental chamber causes preferential inhibition of 8-Br-cAMP supported VSRM contractions. The voltage clamp protocol was the same as that shown in figure 3-30. Panel A. Representative traces of VSRM and CICR contractions elicited by sequential steps to -40 and 0 mV, respectively, as well as $I_{Ca\text{-}L}$ initiated by the step to 0 mV. Panel B. Representative recordings of contractions and currents elicited in the presence of tetracaine added to the solution perfusing the experimental chamber (mean length of exposure 2.5 minutes). Panel C. Mean data illustrating significant inhibition of VSRM contractions in the presence of tetracaine. CICR contraction amplitude also was significantly decreased (* denotes $p<0.02$) in the presence of tetracaine but the magnitude of inhibition was less (41 % compared to 90 % inhibition of VSRM contractions). Tetracaine also caused significant inhibition of the mean current associated with each step (* denotes $p<0.01$) (n=9 myocytes).

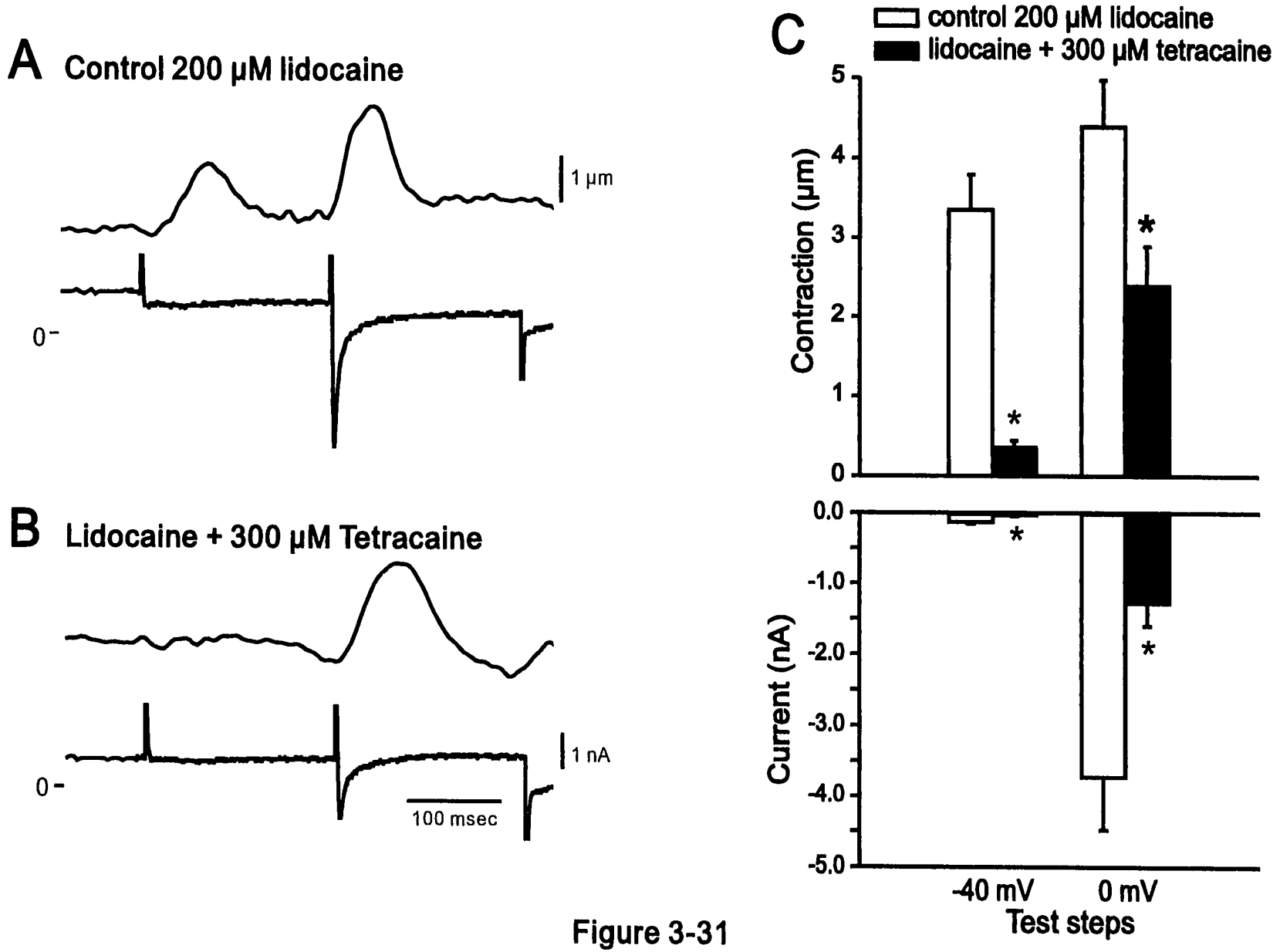

A Control 200 µM lidocaine
1 µm
0
B Lidocaine + 300 µM Tetracaine
1 nA
0
100 msec
C
control 200 µM lidocaine
lidocaine + 300 µM tetracaine
Contraction (µm)
5
4
3
2
1
0
*
*
0.0
-1.0
-2.0
-3.0
-4.0
-5.0
Current (nA)
*
*
-40 mV
0 mV
Test steps

Figure 3-31

Figure 3-32. Effects of tetracaine on CV and IV relationships determined in the presence of 50 μM 8-Br-cAMP in the pipette. The voltage clamp protocol is illustrated at the top. Panel A. Under control conditions the CV relationship was relatively sigmoidal with contractions beginning at -60 mV and reaching a peak near -20 mV. The CV relationship became bell-shaped but reached the same maximum amplitude as control in the presence of continuous exposure to tetracaine (mean length of exposure 3.5 minutes). Panel B. The peak of the IV relationship was significantly decreased in the presence of tetracaine although peak contraction amplitude did not decrease (n=6 myocytes) (* denotes $p<0.05$).

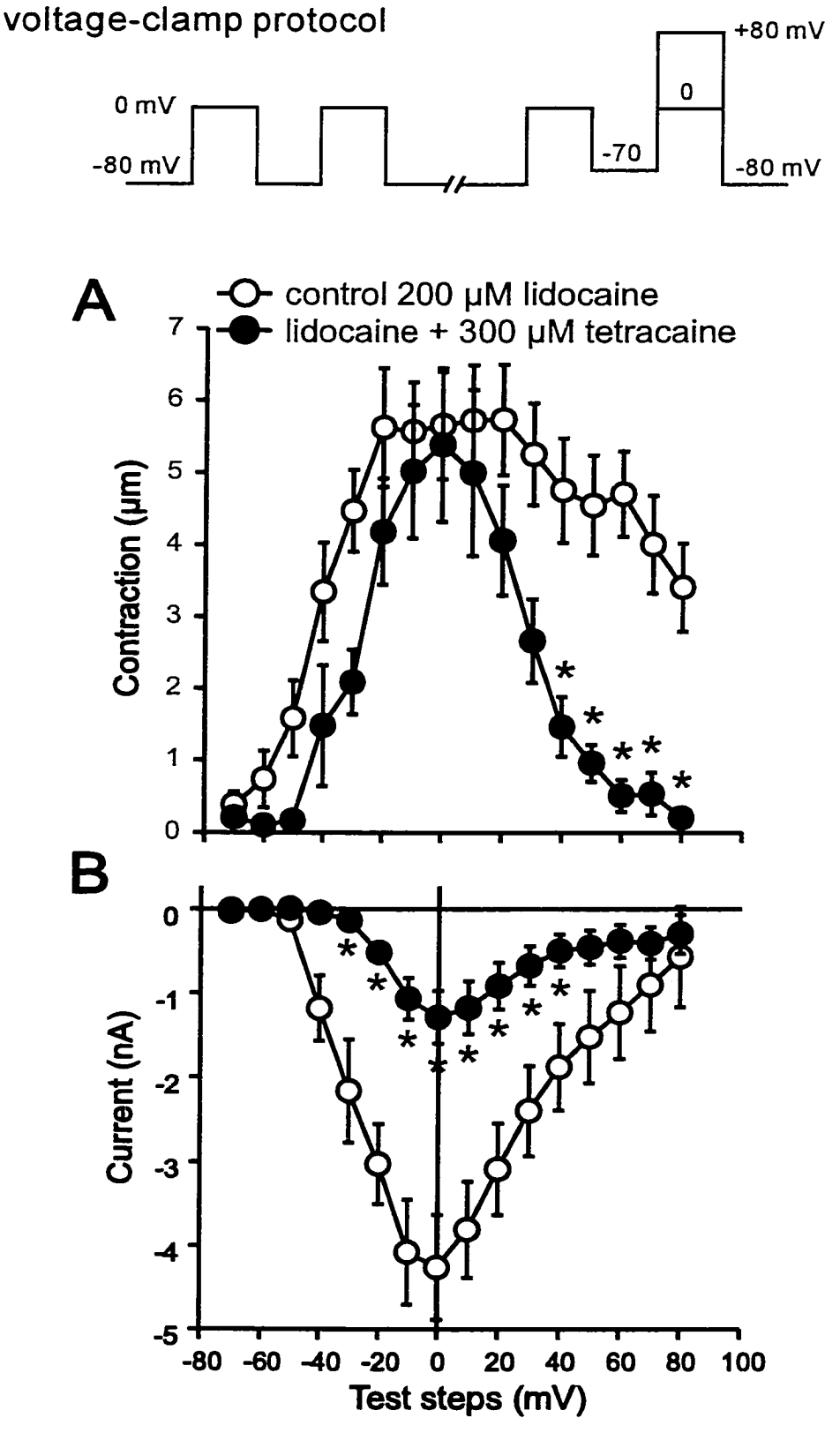

voltage-clamp protocol
+80 mV
0
0 mV
-80 mV
-70
-80 mV
A
control 200 µM lidocaine
lidocaine + 300 µM tetracaine
Contraction (µm)
B
Current (nA)
Test steps (mV)

Figure 3-32

present experiment. Thus, tetracaine appears to inhibit the VSRM component of 8-Br-cAMP supported contractions while simultaneously causing an increase SR Ca^{2+} stores.

Do depression of peak $I_{Ca\text{-}L}$ and $I_{Ca\text{-}L}$-induced contractions represent inhibition by tetracaine? Experiments carried out with low resistance patch electrodes demonstrated a significant decrease in the maximum amplitude of the IV relationship in the presence of 300 μM tetracaine (figure 3-32B). By contrast, experiments carried out with high resistance electrodes illustrated no significant effect of a short term or rapid application of 300 μM tetracaine on mean current. This effect could represent a difference in the effects of tetracaine under these two experimental conditions, or possibly a non-specific effect of dialysis on $I_{Ca\text{-}L}$ (run-down). Therefore, the next series of experiments was carried out to determine the effects of time on contractions and currents stimulated with low resistance patch electrodes.

Figure 3-33 illustrates mean data for CV and IV relationships measured at time zero (control) and after approximately 11 minutes of recording with patch electrodes. Control recordings were taken once the contractions and currents had reached a steady state (approximately 5 minutes). Recordings were made again at 11 minutes, therefore the filled circles in figure 3-33A and B represent 11 minutes after the control recordings were taken. There was a substantial decrease in the maximum amplitude of contractions over time as shown in figure 3-33A. Figure 3-33B also illustrates a substantial decrease in the peak IV relationship over time. These results suggest that the decrease in the peak IV relationship in the presence of tetracaine can be explained by run-down of $I_{Ca\text{-}L}$ over time.

Figure 3-33. Effects of time on the amplitude of 8-Br-cAMP supported contractions and currents. The voltage clamp protocol for panels A and B is the same as that shown in figure 3-32. Control recordings were taken once contractions and currents reached a steady-state, (approximately 5 minutes) and represent time zero. Panel A. Mean data showing the decrease in contraction amplitude over time in experiments carried out with patch electrodes. Filled circles represent a mean recording time of approximately 11 minutes after control. These results show that the maximum amplitude of the CV relationship was substantially decreased over time. Panel B. Similarly, the maximum amplitude of the IV relationship was significantly decreased over time (* denotes $p<0.05$) (n= 4 myocytes). For panels C and D the voltage clamp protocol is the same as that shown at the top of figure 3-30. Panel C. Mean data illustrating a significant decrease in the amplitude of both VSRM and CICR contractions after 5 minutes and again after 10 minutes of recording. Panel D. $I_{Ca\text{-}L}$ associated with the step to 0 mV was also significantly decreased after 5 minutes and again after 10 minutes of recording (* denotes $p<0.01$; ** denotes $p<0.001$) (n= 10 myocytes).

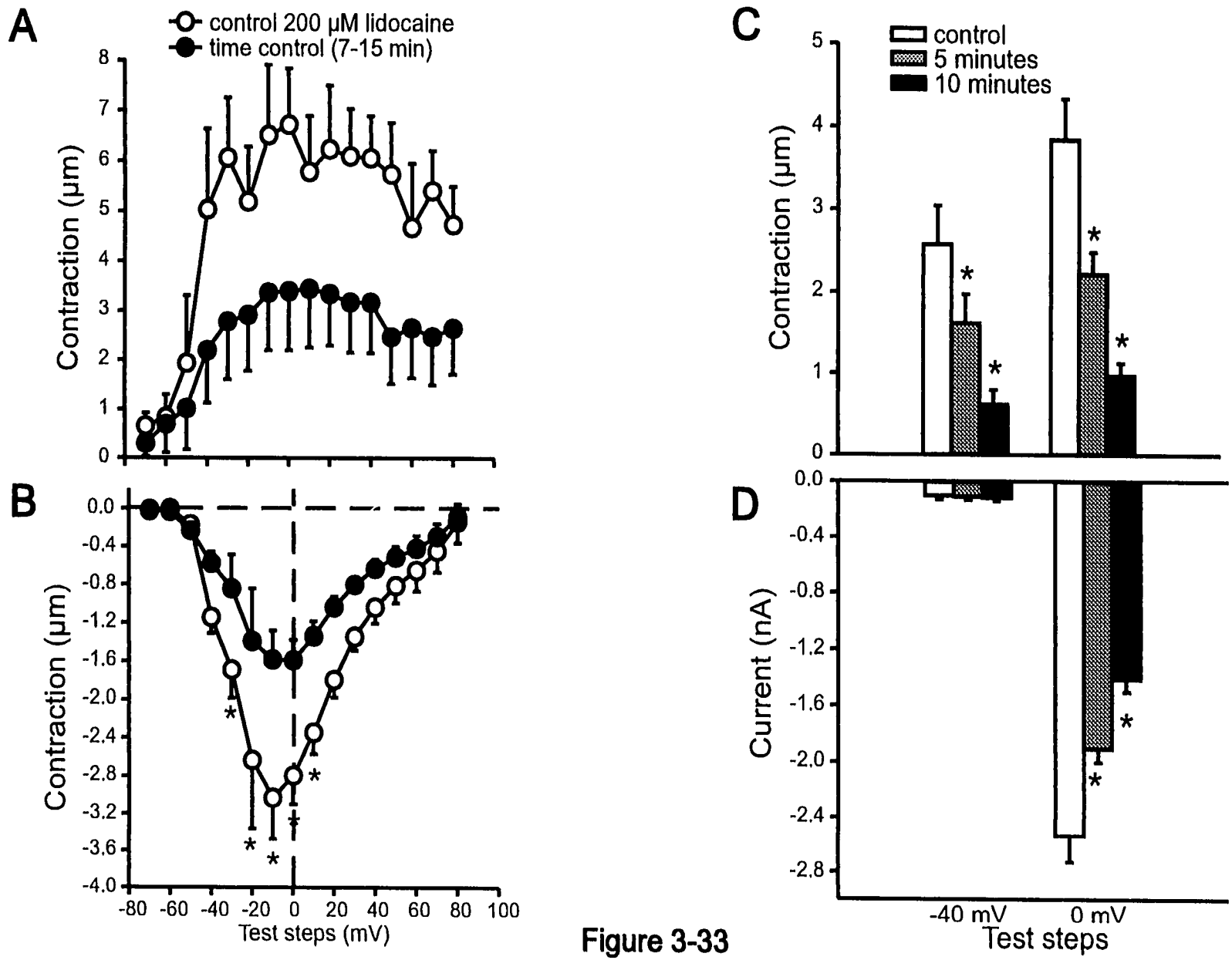

A
control 200 µM lidocaine
time control (7-15 min)
Contraction (µm)
B
Contraction (µm)
Test steps (mV)
C
control
5 minutes
10 minutes
Contraction (µm)
D
Current (nA)
-40 mV
0 mV
Test steps

Figure 3-33

Figure 3-33C illustrates the mean data showing the effects of time on contractions and currents elicited by sequential steps to -40 and 0 mV. There was a similar, significant decrease in both VSRM and CICR contractions, as well as $I_{Ca\text{-}L}$ over time. Thus, the significant decrease in the contraction and current associated with the step to 0 mV in the presence of tetracaine may reflect a non-specific effect of run-down rather than an effect of tetracaine.

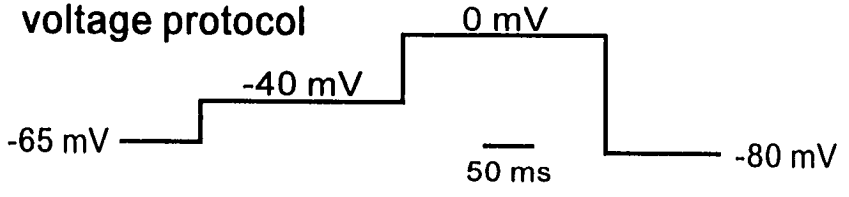
Like tetracaine, ryanodine also interacts with the SR Ca^{2+} release channels in striated muscle. However, while tetracaine has been shown to inhibit opening of isolated RyRs, low concentrations of ryanodine (30 nM) cause RyRs to open to a subconducting state resulting in depletion of SR Ca^{2+} stores (Rousseau, Smith & Meissner, 1987; Fleischer *et al.*, 1985; Lattanzio *et al.*, 1987; Pessah *et al.*, 1985; Shattock & Bers, 1987). The ability of ryanodine to deplete SR stores along with the high selectivity and affinity with which it binds to the SR Ca^{2+} release channels has led to the wide spread use of this agent as a pharmacological tool to study EC-coupling (Balke & Wier, 1991; Alderson & Feher, 1987; McGrew *et al.*, 1989; Meissner, 1986).

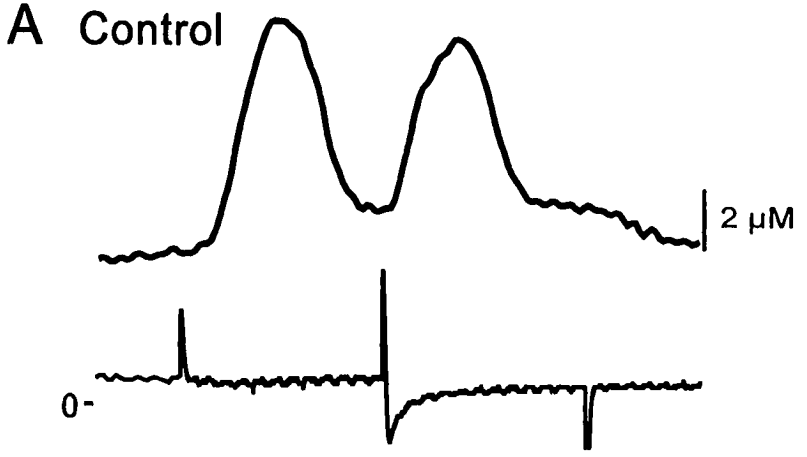
Ryanodine was used as a tool in early experiments investigating the cardiac VSRM in order to determine whether contractions elicited by this mechanism were dependent on SR Ca^{2+} release (Ferrier & Howlett, 1995). In those studies VSRM and CICR contractions were elicited in the presence and absence of 30 nM ryanodine to deplete SR Ca^{2+} stores. The results of this study showed that VSRM contractions were

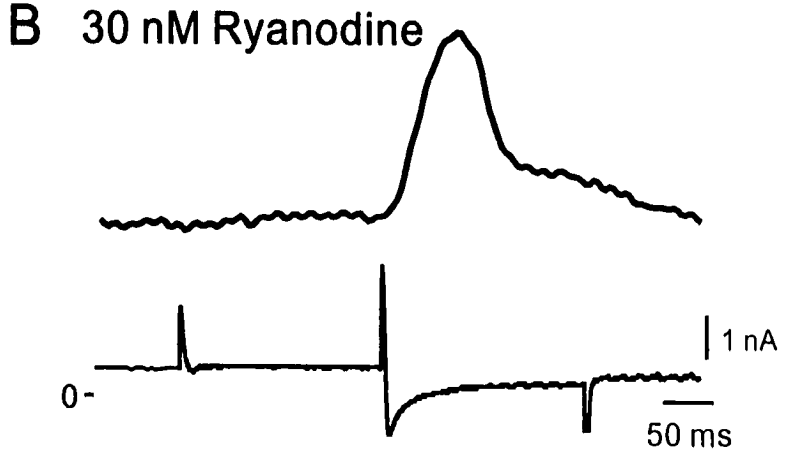
inhibited in the presence of ryanodine suggesting this component of contraction was dependent on releasable stores of Ca^{2+}. However, CICR contractions which also are dependent on SR Ca^{2+} release, were not inhibited in the presence of 30 nM ryanodine. This result was quite surprising and made it difficult to explain inhibition of VSRM contractions on the basis of depletion of SR Ca^{2+} stores. It is possible that 30 nM ryanodine had an effect on the VSRM component of contractions which was unrelated to depletion of SR Ca^{2+} stores. This led to the first objective of the present study which was to determine whether inhibition of the VSRM component of cardiac contractions by low concentration ryanodine was accompanied by a depletion of SR Ca^{2+} stores.

Is inhibition of VSRM contractions by ryanodine (30 nM) accompanied by depletion of SR Ca^{2+} stores in ventricular myocytes at 37 °C? SR Ca^{2+} stores can be evaluated with rapid application of 10 mM caffeine. Rapid application of caffeine results in a large contracture, the amplitude of which can be used as an index of SR Ca^{2+} content (Rousseau & Meissner, 1989; O'Neill, Donoso & Eisner, 1990; Bers, 1991). In the present study caffeine was used to evaluate changes in SR Ca^{2+} stores in the presence and absence of inhibition of the VSRM by 30 nM ryanodine. The 2 step protocol was used to illustrate the differential effects of ryanodine on the VSRM and CICR contractions. Figure 3-34, panels A and B, show representative recordings of contractions and currents elicited under control conditions and in the presence of 30 nM ryanodine, respectively. The VSRM component of contraction was strongly inhibited in the presence of 30 nM ryanodine while the CICR contraction coupled to $I_{Ca\text{-}L}$ was virtually unaffected in this example. Mean data for the effects of low concentration ryanodine on VSRM and CICR

Figure 3-34. Differential effects of 30 nM ryanodine on two components of cardiac EC-coupling. Contractions and currents were initiated by the voltage clamp protocol shown at the top of panel A. In panel A the step to –40 mV elicited a VSRM contraction in the absence of inward current. The step to 0 mV elicited $I_{Ca\text{-}L}$, as well as an $I_{Ca\text{-}L}$-induced contraction. Panel B demonstrates that in the presence of 30 nM ryanodine (applied continuously through the experimental chamber) the VSRM contraction was abolished, while $I_{Ca\text{-}L}$ and the $I_{Ca\text{-}L}$-induced contraction were unaffected. Panel C illustrates the mean data for the effects of ryanodine on contractions and currents elicited by this protocol (mean length of exposure, 20 minutes; the voltage clamp protocol illustrated at the top-left, which began with 10 conditioning pulses to 0 mV at 2 Hz, was run continuously throughout this time period to constantly stimulate myocytes). The VSRM contractions were virtually abolished in the presence of ryanodine (** denotes $p< 0.0001$) while $I_{Ca\text{-}L}$-induced contractions were decrease by less than 50 % (* denotes $p<0.01$). There was no significant effect on inward currents. Myocytes were continuously stimulated (n=11 myocytes).


voltage protocol
0 mV
-40 mV
-65 mV
50 ms
-80 mV


A Control
2 µM
0


B 30 nM Ryanodine
1 nA
0
50 ms

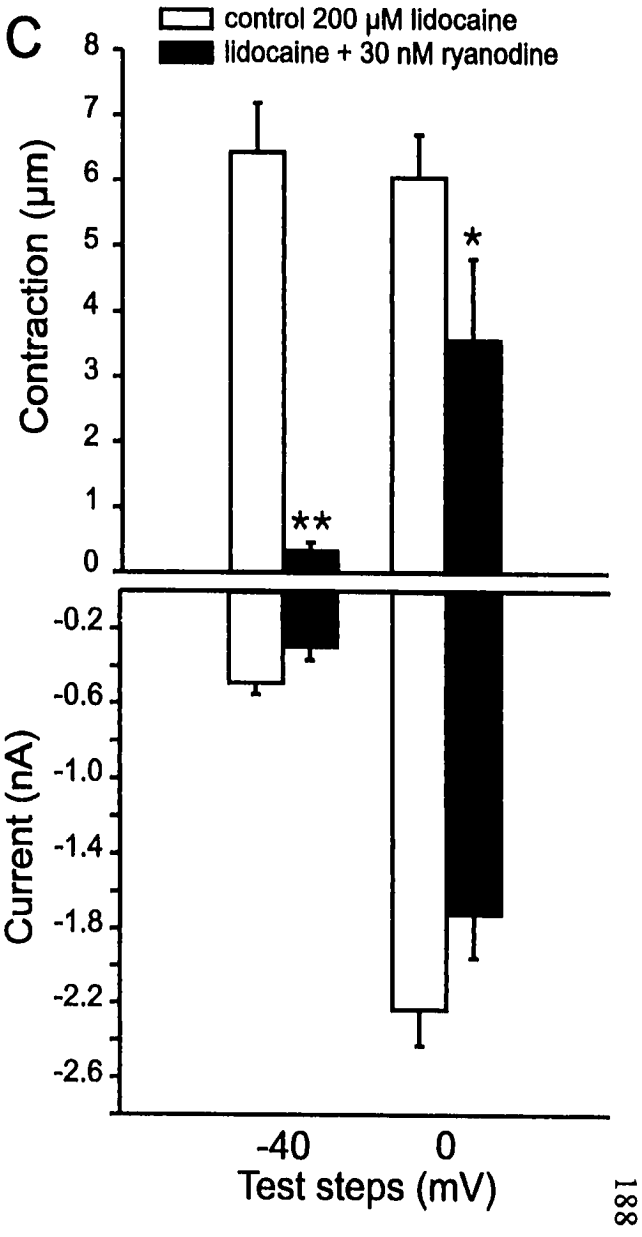

C
control 200 µM lidocaine
lidocaine + 30 nM ryanodine
Contraction (µm)
8
7
6
5
4
3
2
1
0
**
*
-0.2
-0.6
-1.0
-1.4
-1.8
-2.2
-2.6
Current (nA)
-40
0
Test steps (mV)

Figure 3-34

contractions are presented in figure 3-34C. Contractions elicited by the VSRM were strongly and significantly inhibited in the presence of 30 nM ryanodine (95 % inhibition) while those elicited by CICR were only moderately depressed (41 % inhibition). Ryanodine had no significant effect on inward current. These results show that 30 nM ryanodine preferentially inhibits contractions elicited by the VSRM in cardiac myocytes.

Figure 3-35 shows representative caffeine contractures recorded under control conditions and after inhibition of VSRM contractions with 30 nM ryanodine. In the voltage clamp protocol for these experiments (top of figure 3-35) the conditioning pulse train was followed by a 12 second V_{PC} of -70 mV, during which 10 mM caffeine was applied for a period of 4 seconds. Figure 3-35A shows that under control conditions a large caffeine contracture was elicited. The control reached a peak then declined to a steady-state before returning to baseline at the end of the caffeine application. In the presence of 30 nM ryanodine (mean length of exposure, 20 minutes), rapid application of 10 mM caffeine still was able to elicit a large caffeine contracture (figure 3-35B). These results show that low concentration ryanodine does not deplete SR Ca^{2+} stores in guinea-pig ventricular myocytes at 37 °C. Thus, depletion cannot explain inhibition of the VSRM component of contraction by this concentration of ryanodine.

Do higher concentrations of ryanodine deplete SR stores of Ca^{2+} under the present experimental conditions? Effects of ryanodine on SR stores are known to depend on the experimental conditions (e.g. temperature, ryanodine concentration, species) (Jenden & Fairhurst, 1969; Sutko & Kenyon, 1983; Sutko, Ito & Kenyon, 1985). Therefore, it was possible that at 37 °C a higher concentration of ryanodine is required to deplete SR stores.

Figure 3-35. Ryanodine concentrations which inhibit the VSRM component of contraction do not deplete SR Ca^{2+} stores. The voltage clamp protocol is shown at the top. The V_{PC} was held at –70 mV for 12 seconds before returning to the holding potential of –80 mV. During the V_{PC} 10 mM caffeine was applied for a period of 4 seconds. The resulting contracture was used as an index of SR Ca^{2+} stores. Panel A illustrates a caffeine contracture elicited under control conditions. Panel B illustrates a caffeine contracture in the presence of 30 nM ryanodine indicating that SR Ca^{2+} stores are not depleted (similar results were observed in 11 out of 11 myocytes tested).

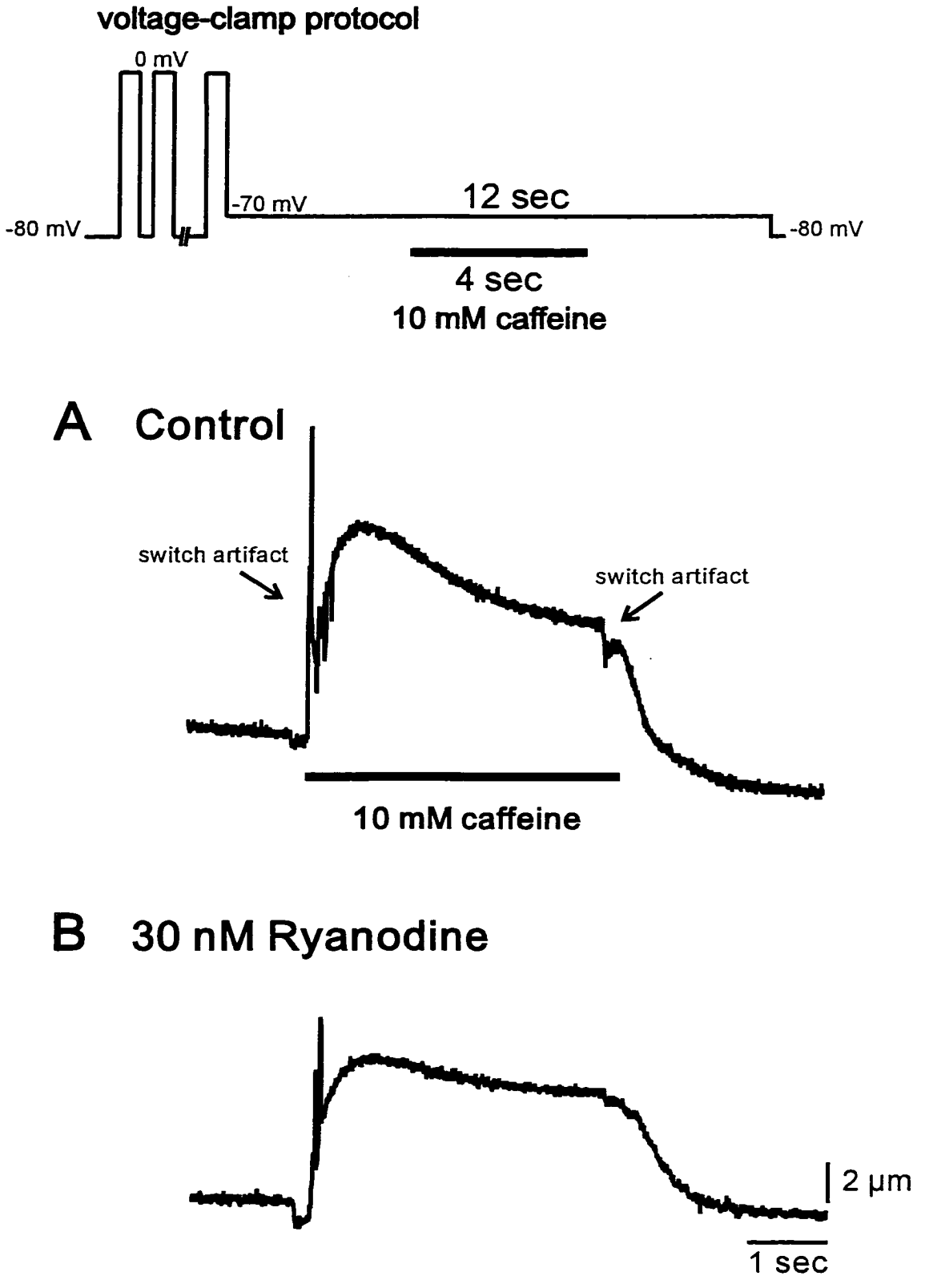

voltage-clamp protocol
0 mV
-80 mV
-70 mV
12 sec
-80 mV
4 sec
10 mM caffeine
A Control
switch artifact
switch artifact
10 mM caffeine
B 30 nM Ryanodine
2 µm
1 sec

Figure 3-35

Figure 3-36 shows representative traces of caffeine contractures recorded from the same cell under control conditions and in the presence of increasing concentrations of ryanodine (0.03 to 10 μM). Each concentration of ryanodine required approximately 15 minutes to reach a maximal effect, therefore caffeine applications were carried out after 15 minutes exposure to each concentration. The voltage clamp protocol was the same as that shown in figure 3-35. Under control conditions a large caffeine contracture was elicited. In the presence of 30 nM ryanodine the maximum amplitude of caffeine contracture decreased only slightly. However, with 1 μM ryanodine the peak component of the caffeine contracture was strongly inhibited while the steady-state component was virtually unaffected. At 10 μM ryanodine, the kinetics of the caffeine contracture were slowed and the amplitude decreased dramatically which suggests that the SR Ca^{2+} stores were largely depleted. These results indicate that ryanodine concentrations above 30 nM are required to deplete SR Ca^{2+} stores in myocytes stimulated at 37 °C with high resistance electrodes.

Mean data illustrating the effects of increasing concentrations of ryanodine on contractions and caffeine contractures are illustrated in figure 3-37A. Under control conditions VSRM and CICR contractions were of similar amplitude whereas the caffeine contracture was approximately two-fold larger in amplitude. These results may suggest that each phasic contraction results from the release of approximately one-half of the Ca^{2+} available in the SR. The maximum amplitude of caffeine contractures decreased progressively in response to increasing concentrations of ryanodine. As well, CICR contractions also were inhibited in a graded fashion with progressively higher

Figure 3-36. Representative traces illustrating a progressive depletion of SR Ca^{2+} stores in the presence of increasing concentrations of ryanodine. The voltage clamp protocol is the same as that shown in figure 3-35. The top trace shows a large caffeine contracture elicited under control conditions. The second trace shows a large caffeine contracture elicited in the presence of 30 nM ryanodine which indicates that SR Ca^{2+} stores were not depleted. The last two recordings demonstrate a gradual decline in amplitude and slowed kinetics of caffeine contractures elicited in the presence of 1 and 10 μM ryanodine, respectively. All traces were obtained from the same cell. Mean length of exposure to each concentration of ryanodine was 15 minutes.

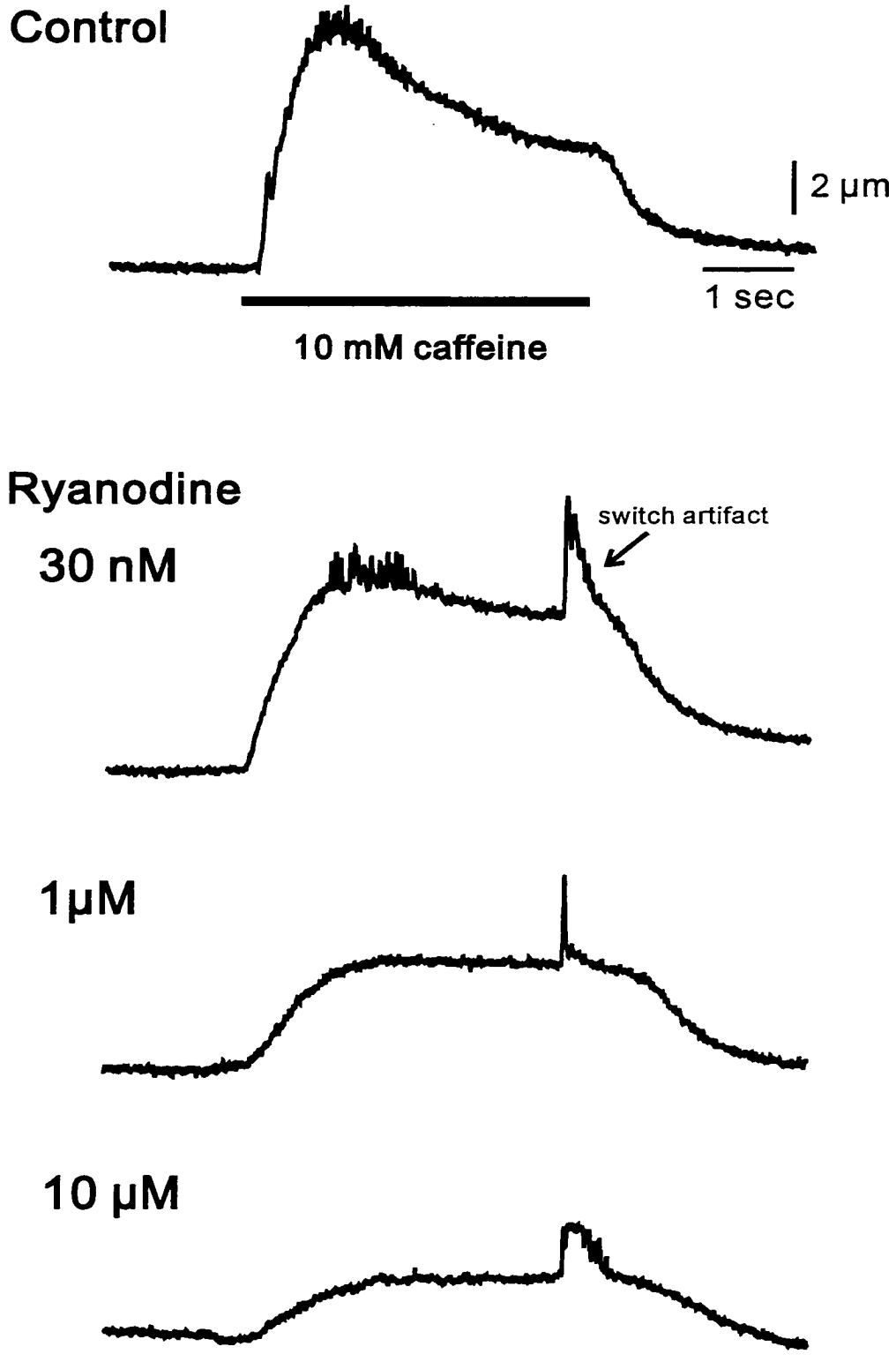

Control
2 µm
1 sec
10 mM caffeine
Ryanodine
30 nM
switch artifact
1µM
10 µM

Figure 3-36

Figure 3-37. Mean data demonstrating the concentration dependence of the effects of ryanodine on caffeine contractures and on the two components of cardiac EC-coupling. Panel A mean data show that VSRM contractions were virtually abolished at all concentrations of ryanodine (** denotes $p< 0.001$). Increasing concentrations of ryanodine caused a gradual decline in the amplitude of the caffeine contractures, as well as $I_{Ca\text{-}L}$-induced contractions (* denotes $p<0.01$). Panel B shows that there was no significant effect of ryanodine on inward currents at any concentration (30 nM ryanodine n= 11; 1 μM ryanodine n=7; 10 μM ryanodine n=10 myocytes).

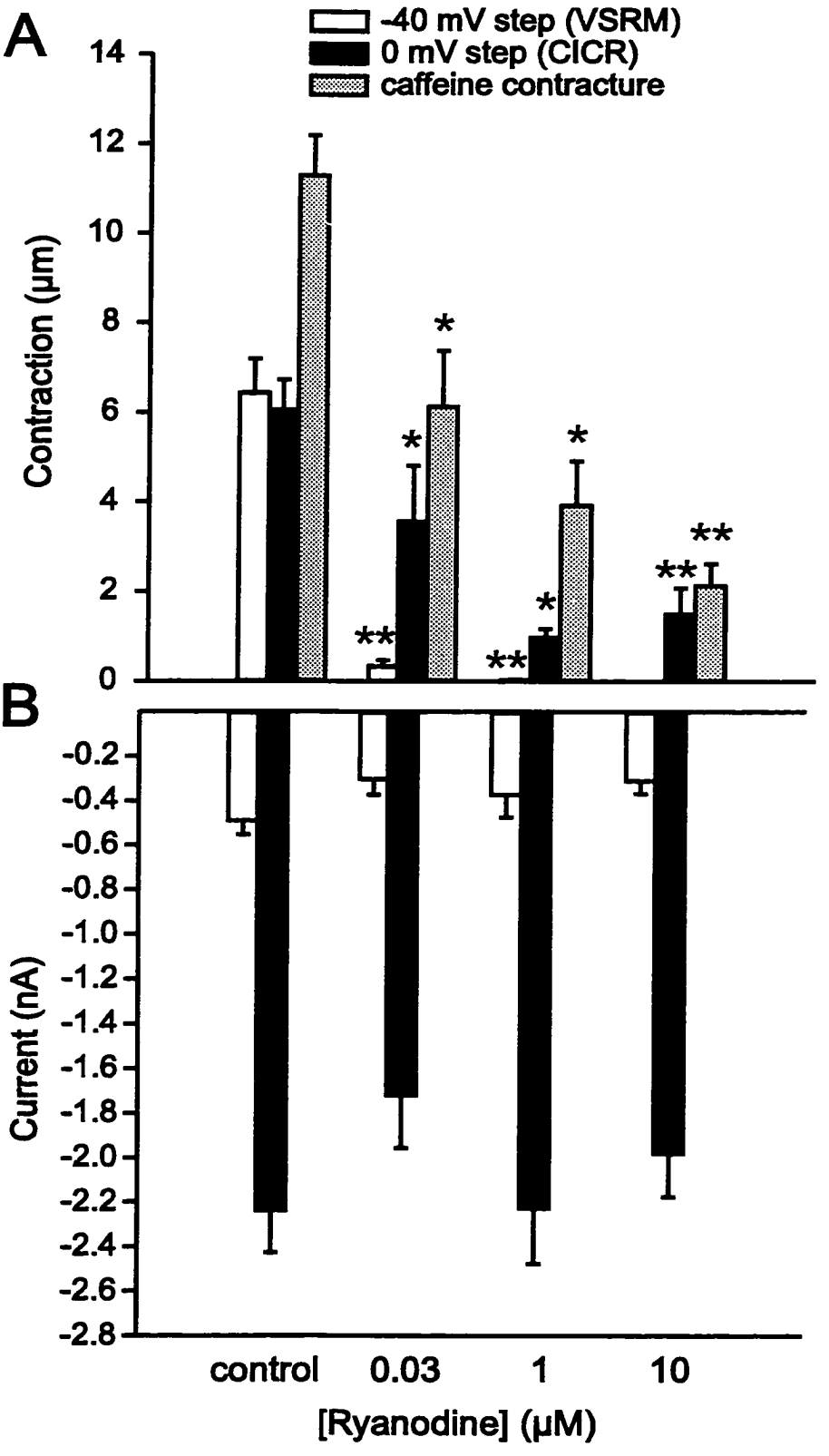

A
-40 mV step (VSRM)
0 mV step (CICR)
caffeine contracture
Contraction (μm)
14
12
10
8
6
4
2
0
B
Current (nA)
-0.2
-0.4
-0.6
-0.8
-1.0
-1.2
-1.4
-1.6
-1.8
-2.0
-2.2
-2.4
-2.6
-2.8
control
0.03
1
10
[Ryanodine] (μM)

Figure 3-37

concentrations of ryanodine. By contrast, even the lowest concentration of ryanodine tested virtually abolished the VSRM component of contractions. VSRM contractions remained inhibited over the entire range of concentrations tested. Thus, the decrease in the amplitude of contractions elicited by CICR coupled to $I_{Ca\text{-}L}$ was proportional to the decrease in caffeine contracture amplitude while VSRM contractions were virtually abolished by the lowest concentration of ryanodine. Figure 3-37B shows that there was virtually no effect of ryanodine on inward current at any of the concentrations tested. Thus, inhibition of CICR contractions cannot be attributed to a decrease in $I_{Ca\text{-}L}$ and likely results from depletion of SR Ca^{2+} stores.

CICR contractions measured in the presence of 10 μM ryanodine were slightly larger in amplitude than those measured in 1 μM ryanodine. This slight increase in CICR contractions may be caused by the method used to generate the mean concentration-response data. Ryanodine concentrations were increased cumulatively while recording from the same cell. However, not all cells survived until 10 μM ryanodine, therefore, some of the cells included in the mean data for this concentration of ryanodine were exposed just to control and 10 μM ryanodine. The shorter total experimental time may have resulted in slightly larger contractions.

The mean amplitudes (figure 3-38) of the VSRM and CICR contractions and caffeine contractures determined in the presence of increasing concentrations of ryanodine were each normalized to their maximum control amplitude to generate concentration-response curves. The three concentration-response curves were plotted together in order to compare the differential effects of ryanodine on the two components

of contraction and the caffeine contractures. Figure 3-38 illustrates that the curves representing CICR contractions and caffeine contractures follow a similar pattern of gradual decline in amplitude with increasing concentrations of ryanodine. By contrast, the VSRM contractions were virtually abolished by 30 nM ryanodine, therefore the concentration-response curve for this mechanism of EC-coupling was shifted to the left. These results demonstrate that the percent change in CICR contractions paralleled that of the caffeine contractures which suggests that a decrease in SR Ca^{2+} stores is responsible for inhibition of CICR coupled to $I_{Ca\text{-}L}$. By contrast, the cumulative effects of increasing concentrations of ryanodine on VSRM contractions followed a different concentration-response curve. Inhibition of these contractions occurred at the lowest concentration and was unrelated to depletion of intracellular Ca^{2+} stores.

Figure 3-38. Concentration-response curve illustrates the differential effect of ryanodine on VSRM contractions compared to CICR contractions and caffeine contractures. The mean contraction and contracture amplitudes from figure 3-37 were normalized to the maximum contraction amplitudes. The percent change in $I_{Ca\text{-}L}$-induced contractions parallels depletion of SR Ca^{2+} as assessed by caffeine contractures whereas inhibition of VSRM contractions occurs at lower concentrations of ryanodine and is independent of SR depletion.

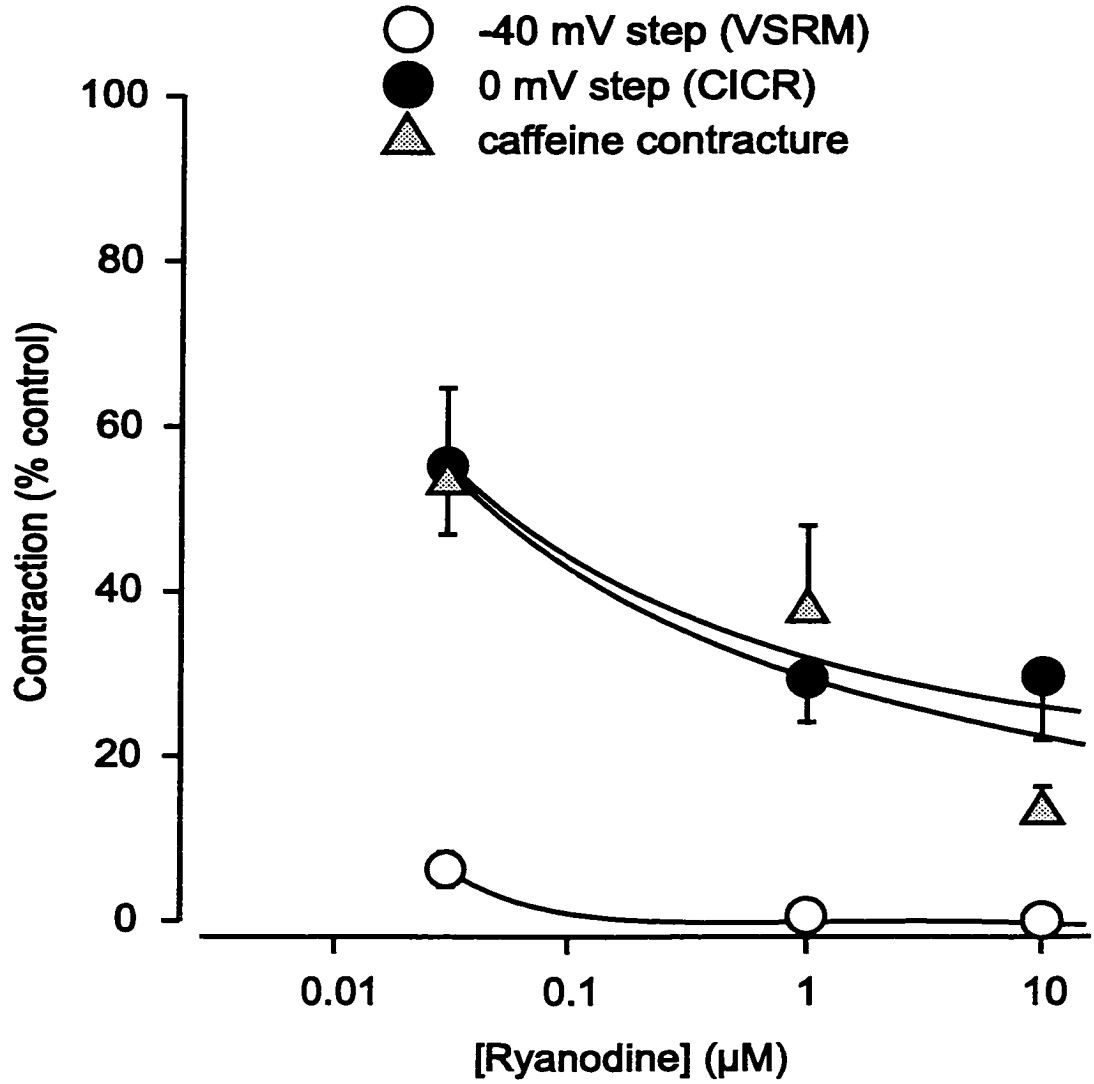

-40 mV step (VSRM)
0 mV step (CICR)
caffeine contracture
100
80
60
40
20
0
Contraction (% control)
0.01
0.1
1
10
[Ryanodine] (μM)

Figure 3-38

DISCUSSION

OVERVIEW:

The overall objective of this study was to determine the effects of tetracaine and ryanodine on two components of cardiac EC-coupling. Two mechanisms which have been proposed for cardiac EC-coupling are the VSRM and CICR. The VSRM involves a process whereby stimulation of the SL directly induces SR release of Ca^{2+}. By contrast, a mechanism of CICR requires transsarcolemmal influx of extracellular Ca^{2+} to trigger release of SR Ca^{2+} stores. Thus, both of these mechanisms ultimately induce release of SR Ca^{2+} stores. Overall, the results of the present study indicate that tetracaine and ryanodine, both of which interact with SR RyRs, can be used as tools to selectively inhibit the VSRM component of contraction. This finding was unexpected because one would postulate that agents that interact with the SR release channels would affect both mechanisms of EC-coupling since both mechanisms mediate SR release. As well, tetracaine and ryanodine have been reported to have opposite effects on SR Ca^{2+} stores, as studies have shown that the former increases SR Ca^{2+}, while the latter is known to decrease SR Ca^{2+} stores. Thus, one would not expect these two agents to have similar actions on VSRM contractions.

It was initially hypothesized that tetracaine would have a negative inotropic effect in cardiac muscle similar to that previously reported in skeletal muscle. However, what was surprising about the present results was that in cardiac muscle tetracaine preferentially inhibited a VSRM, whereas in skeletal muscle it has the opposite effect of preferentially inhibiting a mechanism of CICR. The results of the present study illustrate

that selective inhibition of the VSRM by tetracaine is very reproducible over a reasonable concentration range ($\geq$ 300 μM). This effect of tetracaine was found to be independent of the LA actions of this agent. As well, inhibition of the VSRM component of contraction persisted although SR Ca^{2+} stores increased progressively in the presence of tetracaine. The observation that moderate concentrations of tetracaine ($\leq$ 300 μM) do not inhibit CICR contractions coupled to either $I_{Ca\text{-}L}$ or reverse mode $NaCa_{EX}$ suggests that this agent is a useful pharmacological tool for investigation of the contribution of the VSRM to overall cardiac contraction.

The observation that ryanodine also causes preferential inhibition of the VSRM component of EC-coupling was quite unexpected as well. Also not predicted was that inhibition of the VSRM by ryanodine was independent of depletion of SR stores of Ca^{2+}. Over the years ryanodine at low concentrations has been used as a tool for inhibiting contraction by depleting SR Ca^{2+} stores (Balke & Wier, 1991; Alderson & Feher, 1987; McGrew *et al.*, 1989; Meissner, 1986; Bers, 1991). By contrast, the results of the present study illustrate that ryanodine has an effect on the VSRM component of contraction which is analogous to the actions of tetracaine. Thus, ryanodine has been shown to have an effect on cardiac contraction that has not been previously reported and which is unrelated to the well known effects of this agent. The high selectivity and affinity of this agent for SR RyRs (Pessah *et al.*, 1986; Alderson & Feher, 1987; Fleischer *et al.*, 1985) along with the presently demonstrated effects on the VSRM component of contraction make ryanodine an attractive tool for investigation of cardiac EC-coupling. Overall, the results of this study indicate that both tetracaine and ryanodine are useful

pharmacological tools for examining the contribution of the VSRM to cardiac contraction.

TETRACAINE:

Negative inotropic effect of tetracaine:

The first goal of this study was to determine whether tetracaine affects the strength of contraction in cardiac ventricular myocytes at 37 °C when both CICR and the VSRM are available. This study demonstrates that tetracaine inhibits myocyte contraction under these conditions, however, the actions of this agent are complex as different effects were seen with different concentrations and methods of application. The present experiments showed that a very high concentration of tetracaine abolished contraction in isolated guinea pig ventricular myocytes. More modest concentrations of tetracaine (100-300 μM) changed the shape of the CV curve from sigmoidal to bell-shaped, but did not reduce the maximum amplitude of contractions. This effect was accompanied by an increase in SR Ca^{2+} stores. When tetracaine was applied just before and during test steps, SR Ca^{2+} stores were not significantly elevated and the maximum amplitude of contraction was decreased.

Initial experiments showed that tetracaine exerts a negative inotropic effect in isolated guinea pig ventricular myocytes. Part of this effect may be related to the local anesthetic effect of tetracaine, since sodium channel blockade can exert a negative inotropic effect (Wilson *et al*, 1993). This effect of local anesthetics is generally attributed to a decrease in SR Ca^{2+} load which occurs through reduction of intracellular Na^{+} concentration, which in return results in greater efflux of Ca^{2+} through $Na\text{-}Ca_{EX}$

(Wilson *et al*, 1993). However, our observations demonstrate that tetracaine must have an additional mechanism for its negative inotropic effect, since tetracaine still inhibited contraction when I_{Na} had been blocked previously with lidocaine or lidocaine plus TTX. Thus, this negative inotropic effect is independent of local anesthetic actions of tetracaine. This additional effect was found to be caused by blockade of a component of cardiac EC-coupling related to SR Ca^{2+} release.

Tetracaine preferentially inhibits the VSRM component of cardiac EC-coupling:

Once the negative inotropic effects of tetracaine in cardiac muscle were determined the next objective was to investigate whether this agent would differentiate between EC-coupling mediated either by CICR or by the VSRM. This question was proposed because studies in skeletal muscle have previously demonstrated that tetracaine selectively inhibits CICR, without affecting DICR in that tissue (Pizarró *et al.*, 1992). In contrast, this study showed that tetracaine preferentially inhibited contraction initiated by the VSRM in cardiac myocytes, at concentrations up to 300 μM. It was found that tetracaine only blocked CICR contractions at high concentrations (1 mM) which also inhibited $I_{Ca\text{-}L}$, or during longer exposure of myocytes to an intermediate concentration (300 μM). In some experiments block of CICR contractions triggered by $I_{Ca\text{-}L}$ was accompanied by inhibition of $I_{Ca\text{-}L}$. Thus, inhibition of this mechanism of EC-coupling appeared to be largely attributable to inhibition of the trigger for CICR. Studies by others also have shown that tetracaine can inhibit $I_{Ca\text{-}L}$ in guinea-pig ventricular myocytes (Carmeliet *et al.*, 1986). In the present study, however complete inhibition of contraction also was observed without complete blockade of $I_{Ca\text{-}L}$ (figure 3-15). Therefore, these

results suggest there are additional effects of tetracaine on this component of contraction, which occur downstream to influx of trigger Ca^{2+}. One possibility is that under certain conditions tetracaine interferes with the ability of $I_{Ca\text{-}L}$ to trigger release of Ca^{2+} from the SR through an interaction with RyRs.

Tetracaine caused a rightward shift of approximately +10 mV in the negative limb of the IV relationship for I_{Ca}. Addition of the specific Na^+ channel blocking toxin TTX indicated that this effect of tetracaine represents inhibition of residual I_{Na}. The ability of TTX to inhibit this initial inward current without inhibiting contraction eliminates the possibility that the VSRM contraction represents CICR coupled to reverse $Na\text{-}Ca_{EX}$ (Leblanc & Hume, 1990). This also demonstrates that lidocaine provides adequate block of Na^+ channels activated from a V_{PC} of -65 mV since the residual, TTX sensitive I_{Na} is not enough to activate contraction. These results also suggest that Ca^{2+} influx through Na^+ channels cannot explain activation of VSRM contractions. Reduced selectivity for Na^+ over Ca^{2+} or "slip-mode conductance" has been described recently for Na^+ channels in single, isoproterenol stimulated, rat ventricular myocytes (Santana *et al*, 1998). This mechanism of contraction has been hypothesized to explain the persistence of contraction outside the range of $I_{Ca\text{-}L}$. However, contraction activated by "slip-mode conductance" is blocked by 10 μM TTX whereas the VSRM component of contraction is insensitive to block of Na^+ channels by TTX at a five times greater concentration (50 μM). Also, the VSRM component of contraction in the present study, was observed without β-adrenergic stimulation. Thus, effects of tetracaine on the VSRM component of contraction are not likely mediated by inhibition of Na^+ or Ca^{2+} influx through TTX sensitive channels.

Even at a very high concentration (1 mM) which blocked $I_{Ca\text{-}L}$, tetracaine inhibited the VSRM almost immediately, whereas $I_{Ca\text{-}L}$ and CICR contractions were inhibited only several minutes later. Thus, tetracaine still blocked the VSRM preferentially at this high concentration. Tetracaine effects were reversible at all concentrations tested. Interestingly, the effects of tetracaine on the VSRM persisted longer than the effects on $I_{Ca\text{-}L}$ during washout of the drug. Thus, onset of inhibition of the VSRM was faster, but reversal of inhibition was slower than the corresponding effects on $I_{Ca\text{-}L}$. This suggests that the effects of tetracaine on the VSRM and on $I_{Ca\text{-}L}$ are most likely mediated by separate receptors with different affinities for tetracaine, rather than a single receptor distributed in different compartments.

Differential effects of tetracaine on EC-coupling mechanisms in striated muscle:

The observation that tetracaine preferentially blocks the VSRM in cardiac myocytes, but CICR in skeletal muscle provides evidence that these two components of EC-coupling must differ between muscle types. As well, these results suggest that the tetracaine receptors and/or protein components of EC-coupling must differ between muscle types. It is not clear whether the different actions of tetracaine in cardiac and skeletal muscles are related to different isoforms of the Ca^{2+} release channel in cardiac (RyR2) and skeletal (RyR1) muscle, or to different voltage sensors for the voltage coupled mechanisms in these tissues. Binding sites for tetracaine have been suggested to exist on SR Ca^{2+} release channels from both cardiac and skeletal muscles (Volpe *et al*, 1983). It is possible that the two mechanisms of EC-coupling in each tissue are mediated by different isoforms of Ca^{2+} release channel within each tissue. For example, cardiac

tissue may have two isoforms of RyR2, one that mediates CICR and another that mediates a VSRM. The RyR isoform that mediates a VSRM in cardiac muscle may closely resemble the isoform that mediates CICR in skeletal muscle. Thus, tetracaine binding receptors may be present on one isoform of the release channel, but not on the other, and the selectivity may be related to which isoform is coupled either to the voltage sensor or to CICR, in each tissue. Thus, it is possible that the different actions of tetracaine in cardiac and skeletal muscles are related to different isoforms of the Ca^{2+} release channel in each type of muscle. It also is possible that the actions of tetracaine in each striated muscle are related to different voltage sensors for the voltage coupled mechanisms in these tissues. That is, tetracaine may bind to the voltage sensor for the cardiac VSRM but not the voltage sensor for the skeletal DICR.

Can tetracaine be used as a tool to differentiate between the VSRM and CICR?

The third objective of this study was to determine whether tetracaine can be used as a pharmacological tool to identify and differentiate between components of cardiac EC-coupling attributable to CICR or the VSRM. Experiments with high resistance electrodes and rapid solution switches showed that VSRM and CICR contractions elicited by sequential test steps to -40 and 0 mV could be inhibited differentially by tetracaine and Cd^{2+}. Addition of tetracaine through the rapid solution changer, at concentrations up to 300 μM, preferentially blocked VSRM contractions with little or no effect on contractions triggered by $I_{Ca\text{-}L}$. In contrast, 100 μM Cd^{2+} abolished $I_{Ca\text{-}L}$ and CICR contractions, with little or no effect on VSRM contractions. When VSRM contractions were inhibited with short-term, continuous application of tetracaine in experiments

carried out with either high resistance or patch electrodes, CV relations determined from a V_{PC} of -65 mV became bell-shaped and mirrored the IV relation for $I_{Ca\text{-}L}$. Contractions remaining in the presence of tetracaine were significantly inhibited by ryanodine or by Ca^{2+} channel blockade, and must therefore represent CICR coupled to $I_{Ca\text{-}L}$. Thus, our results indicate that tetracaine can be used as a pharmacological tool to identify and separate the VSRM and CICR mechanisms of EC-coupling in heart muscle.

The results of the present study also indicate that tetracaine is a useful pharmacological tool for separating contractions generated by reverse mode $NaCa_{EX}$ from the sustained contractions generated by the VSRM. The phasic component of VSRM contractions show properties of inactivation, therefore these contractions can be readily distinguished from contractions initiated by CICR coupled to reverse mode $NaCa_{EX}$ which do not show properties of inactivation. As well, the kinetics of reverse mode $NaCa_{EX}$ contractions tend to be slower and ramp-like in contrast to phasic VSRM contractions. However, sustained VSRM contractions do not inactivate and have a shape that could be considered somewhat analogous to that of $NaCa_{EX}$ contractions. Thus, preferential inhibition of this component of VSRM contractions by tetracaine makes this agent a useful tool for identifying sustained contractions. As well, the strong inhibition of the sustained contractions by tetracaine and the lack of inhibition by 2 mM Ni^{2+} support the finding that these contractions are elicited by the VSRM and not by $NaCa_{EX}$ working in reverse mode.

Experiments carried out with high resistance electrodes illustrate that continuous application of tetracaine causes an increase in SR Ca^{2+} stores while rapidly applied

tetracaine provides selective inhibition of the VSRM without changing SR Ca^{2+} stores. Thus, to achieve a selective effect of tetracaine on the VSRM component of contractions, rapid application of tetracaine is an ideal technique to use. However, experiments carried out with patch electrodes filled with 50 μM 8-Br-cAMP demonstrated that tetracaine must be applied continuously under these experimental conditions as rapid application 3 seconds before the test steps did not inhibit contractions. Therefore, although tetracaine can be used as a tool to identify the VSRM component of cAMP supported contractions the effects of this agent on SR Ca^{2+} stores cannot be separated from effects on contractions. As well, contractions and currents tended to run-down within 10-20 minutes of recording in experiments carried out with low resistance patch electrodes which further complicates the use of tetracaine as a tool under these experimental conditions.

Comparison of the present results with those previously reported by others:

Previous studies have shown that the VSRM contraction is sensitive to conditions that presumably decrease SR Ca^{2+} load such as: exposure to ryanodine, thapsigargin or conditioning protocols that prevent influx of Ca^{2+} through L-type Ca^{2+} channels (Howlett, Zhu & Ferrier, 1998). Thus, one could suggest that inhibition of the VSRM by tetracaine might result from reduction of SR Ca^{2+} load by tetracaine rather than a selective inhibition of this component of contraction. However, the amplitude of caffeine contractures in the presence of tetracaine was several fold larger than those elicited in the absence of tetracaine. This indicates that tetracaine caused a substantial increase in the SR Ca^{2+} load. Overend *et al* (1997; 1998) as well as Györke *et al* (1997), also reported

an increase in SR Ca^{2+} in the presence of tetracaine. Overend *et al* suggested that tetracaine may increase SR Ca^{2+} stores secondarily to inhibition of release. They reported that tetracaine initially decreased the magnitude of contraction by decreasing SR release. However, because release was reduced, SR stores accumulated and eventually largely reversed the negative inotropic effect. A similar effect may account for the maintenance of maximum peak amplitude of contraction in CV relations observed with short-term (2 minutes) continuous application of tetracaine in experiments carried out with both high resistance (figures 3-4 and 3-7) and patch electrodes (figure 3-32) in the present study. Thus, although tetracaine inhibited release of SR Ca^{2+} by the VSRM, the initial reduction in Ca^{2+} release might lead to build up of SR Ca^{2+} stores and thereby a compensatory increase in the amplitude of CICR contractions coupled to $I_{Ca\text{-}L}$. However, a longer duration exposure (12 minutes) of myocytes to the same concentration of tetracaine resulted in a significant decrease in the maximum amplitude of contraction as well as a decrease in $I_{Ca\text{-}L}$. Thus, initially tetracaine caused an increase in SR Ca^{2+} load so that the maximum amplitude of contractions remained the same. However, with longer exposure, the trigger for CICR was reduced, which resulted in a net reduction in the amplitude of contraction. Overend *et al* did not observe a decrease in $I_{Ca\text{-}L}$ with protocols in which tetracaine had a negative inotropic effect.

The initial decrease in CICR contraction amplitude reported by Overend *et al* (1998) was not observed in the present study. However, one would not necessarily expect to see a transient decrease with the type of protocol used in this study. The voltage clamp protocols used to determine CV curves include trains of conditioning pulses before the

activation steps. Several repetitions of the protocol would have been made before the increments in test step voltage would reach potentials which elicit substantial CICR contractions. Thus, a transient inhibition could occur and reverse during the conditioning trains and not register in the CV curves. In addition, the studies of Overend *et al* were conducted at 22 °C, and the effects of tetracaine may not be identical to those at 37 °C. We have reported previously that the VSRM is inhibited at room temperature (Ferrier, 1996). Thus, only CICR may have been operative in the experiments of Overend *et al.*

The effects of tetracaine in the present study may be complicated by interaction between two release mechanisms, the VSRM and CICR, which makes comparison to the results of Overend *et al* more difficult. In the present study inhibition of the VSRM may contribute to accumulation of SR Ca^{2+}. However, VSRM contractions did not return to control amplitude in response to increased SR Ca^{2+}, but remained inhibited. This result contrasts that of Overend *et al* who reported a transient inhibition of CICR contractions in the presence of tetracaine (Overend *et al.*, 1998). As well, inhibition of VSRM contractions by tetracaine was not always associated with changes in SR Ca^{2+}. Rapid application of tetracaine just before and during activation steps did not affect SR stores (figure 3-13) although VSRM contractions were still inhibited. Rapid application also did not affect $I_{Ca\text{-}L}$ contractions which depend on release of SR Ca^{2+} as well. Therefore, the effects of tetracaine on SR Ca^{2+} stores are not related to inhibition of VSRM contractions. The discrepancies between the results of the present study and those of Overend *et al* may reflect differences in drug concentrations, duration of exposure and experimental conditions.

Modulation of CICR contractions by changes in SR Ca^{2+} stores also was demonstrated in the series of experiments investigating the effects of tetracaine on inactivation of VSRM contractions. The maximum amplitudes of both the phasic and sustained components of VSRM contractions were moderately depressed in the presence of 100 μM tetracaine as expected. By contrast, the amplitude of contractions initiated by both CICR and the VSRM increased in the presence of tetracaine. These studies suggest that CICR contractions respond to changes in SR Ca^{2+} stores such that an increase in SR Ca^{2+} causes an increase in CICR contraction amplitude (Han, Schiefer & Isenberg, 1994; López-López & Wier, 1994; Eisner *et al.*, 1998). Thus, the ability of tetracaine to increase SR Ca^{2+} stores while simultaneously inhibiting VSRM contractions has provided further insight into the characteristics of this mechanism. Although both mechanisms of cardiac contraction are thought to induce release of SR stores, only CICR is readily modulated by changes in the amount of Ca^{2+} available to be released from this storage site.

Possible mechanisms of action of tetracaine:

The mechanism by which tetracaine causes preferential inhibition of the VSRM component of cardiac contraction is not known, but several possibilities are suggested by the observations of this study. The decrease in the level of sustained $[Ca^{2+}]_i$ transients observed in the presence of tetracaine likely represents inhibition of SR Ca^{2+} release by this agent. It is interesting to note that the Ca^{2+} transient records demonstrated both a phasic and a sustained component of SR Ca^{2+} release similar to cell shortening records. The similarities in these two records suggests that a rapid increase in $[Ca^{2+}]_i$ is

responsible for generation of phasic contractions while a maintained increase in $[Ca^{2+}]_i$ is responsible for generation of sustained contractions.

The effects of tetracaine on 8-Br-cAMP supported contractions presently illustrated may also give some insight into the mechanism of action of this agent. Previous studies in skeletal muscle have reported that tetracaine must cross the membrane of these cells in order to reach the site of action for inhibition of contractions (Pizarro *et al.*, 1989; Ríos, Pizarro & Stefani, 1992). It is possible that tetracaine also must cross the membrane of cardiac myocytes in order to reach the site of action for inhibition of the VSRM. Thus, in experiments carried out with patch pipettes rapidly applied tetracaine may be dialyzed out the cell before reaching this site. On the other hand, continuous exposure of myocytes to tetracaine might ensure that an adequate concentration of tetracaine crossed the membrane and reached the site of action for inhibition of contractions. Thus, continuous exposure may overcome the problem of dialysis of the intracellular solution with the use of patch electrodes.

It is possible that the inhibitory effects of tetracaine occur through inhibition of phosphorylation pathways which have been shown to be involved in stimulation of VSRM contractions. Both the cAMP-PKA and CamK pathways are thought to be involved in phosphorylation of the VSRM (Ferrier *et al.*, 1998; Zhu & Ferrier, 1999). Full activation of this mechanism seems to require functioning of both phosphorylation pathways since H-89, a selective inhibitor of PKA (Chijiwa *et al.*, 1990), and KN-62, a selective inhibitor of CamK (Tokumitsu *et al.*, 1990) each caused approximately 50 % inhibition of VSRM contractions in undialyzed myocytes (Zhu & Ferrier, 1999).

Activation of the VSRM was virtually abolished by simultaneous addition of both inhibitors. Thus, the mechanism of action of tetracaine on VSRM contractions may involve inhibition of phosphorylation through an interaction with calmodulin. In support of this hypothesis, local anesthetics have been reported to inhibit calmodulin activity through an interaction with the Ca^{2+}-calmodulin complex (Tanaka & Hidaka, 1981; Volpi *et al.*, 1981; Muto, Kudo & Nozawa, 1983). However, inhibition of the CamK pathway by tetracaine would not be expected to abolish contraction as phosphorylation via the cAMP-PKA pathway would still occur in the presence of 50 μM 8-Br-cAMP in the patch electrode solution. The same could be said for the effects of tetracaine in undialyzed myocytes whereby endogenous levels of cAMP would likely be sufficient to activate the VSRM in the absence of activation via CamK. One cannot rule out the possibility that some of the effects of tetracaine on cardiac contractions may be attributable to inhibition of CamK, although the observations of the present study suggest that this affect alone does not explain preferential inhibition of the VSRM by this agent.

Other LAs do not share the same pharmacological profile as tetracaine:

It is not clear why lidocaine does not share the inhibitory effect of tetracaine on the VSRM. In skeletal muscle, lidocaine also does not block SR Ca^{2+} release, and may even promote SR Ca^{2+} release at very high concentrations (Bianchi, 1968). It has been hypothesized that differences in the effects of these local anesthetics on SR Ca^{2+} release in skeletal muscle are related to the existence of two binding sites within the local anesthetic receptor on the SR release channel, one stimulatory and the other inhibitory. Short molecules like lidocaine might bind only to the stimulatory site and cause release of

Ca^{2+} from the SR. On the other hand, tetracaine, which has a longer carbon chain, may bind to both sites with the inhibitory effect dominating (Shoshan-Barmatz & Zchut, 1993). These considerations led us to investigate whether a 20 fold excess of lidocaine might interfere with tetracaine by competitively displacing tetracaine from its binding site, or by exerting a stimulatory effect. However, lidocaine increased inhibition of contraction rather than reversing the effects of tetracaine. This effect of lidocaine may represent inhibition of $I_{Ca\text{-}L}$, which we observed with the high concentration of lidocaine used in these experiments, and which also has been reported previously in skeletal muscle (Ríos & Pizarró, 1991). On the other hand, it also is possible that the affinity of RyRs for tetracaine is so much higher than that for lidocaine that a 20 fold excess concentration of lidocaine was not enough to overcome the effects of tetracaine. In this situation an even higher concentration of lidocaine might reverse the effects of tetracaine, however the effects of lidocaine on $I_{Ca\text{-}L}$ would complicate the results of such an experiment making it difficult to interpret.

Can tetracaine be used as a tool to evaluate the contribution of the VSRM to overall cardiac contraction?:

The final objective of the tetracaine study was to determine whether tetracaine could be used to evaluate the contribution of the VSRM to cardiac EC-coupling. $I_{Ca\text{-}L}$ contractions can still be elicited when the VSRM component of contraction is inhibited with tetracaine. As well, contractions initiated by CICR coupled to reverse mode $NaCa_{EX}$ are insensitive to tetracaine. Overall, when I_{Na} has been blocked in advance, the effects of tetracaine on the configuration, magnitude, and threshold of the contraction-voltage

relationship most likely reflect removal of the contribution of the VSRM to EC-coupling. This is supported by the observation that similar effects on the CV relationship are observed when the VSRM is eliminated through voltage-dependent inactivation (Ferrier & Howlett, 1995; Howlett, Zhu & Ferrier, 1998). These results suggest that tetracaine is a valuable tool for investigation of the contribution of VSRM to cardiac EC-coupling.

Characteristics of the VSRM which have been determined by tetracaine inhibition:

When the VSRM is selectively inhibited by tetracaine, the amplitude of the remaining contraction varies with membrane potential in concert with $I_{Ca\text{-}L}$. Thus, small changes in membrane potential or $I_{Ca\text{-}L}$ during the initial phases of the cardiac action potential would affect the magnitude of contraction when the VSRM is unavailable. It is interesting to note that $I_{Ca\text{-}L}$ is maximal near 0 mV whereas the initial phase of the action potential plateau of the guinea pig ventricular myocyte is approximately +40 mV, at which both $I_{Ca\text{-}L}$ and CICR contractions are clearly submaximal. However, when the VSRM is available for activation, the CV relationship is approximately sigmoidal and the magnitudes of contraction are no longer proportional to the magnitude of inward $I_{Ca\text{-}L}$. Thus, when the VSRM is available, the initial phases of the action potential would provide a maximal trigger for contraction. The trigger for contraction also would be less sensitive to beat-to-beat variations in action potential configuration or magnitude of $I_{Ca\text{-}L}$.

The tetracaine sensitive component of contraction also contributed significantly to the magnitude of contraction. The maximum contraction initiated when contraction was triggered in the absence of the VSRM was less than that triggered when both the VSRM and CICR were available, even for test steps which elicited the maximum $I_{Ca\text{-}L}$. This

suggests that $I_{Ca\text{-}L}$ may elicit less than maximal release of SR Ca^{2+}, and that the VSRM contributes importantly to release of SR Ca^{2+}. The magnitude of this contribution was very large at positive potentials corresponding to the overshoot and plateau voltages of the guinea pig action potential (+50 to +20 mV). The VSRM also is an important determinant of the threshold for activation of cell shortening. Inhibition of the VSRM with 300 μM tetracaine resulted in a shift in the threshold for contraction of approximately +20 mV. All of these characteristics of the VSRM were observed by selective inhibition of this mechanism by tetracaine. Thus, tetracaine has been shown to be a valuable tool for investigation of the contribution of the VSRM to overall cardiac myocyte functioning.

The VSRM also may play an important role in force-interval relations as indicated by the effects of tetracaine on staircase phenomena. The staircase phenomenon, first observed by Bowditch in 1871 (Langer, 1968), is generally attributed to an interplay between recovery of SR Ca^{2+} release following a previous activation, changes in SR loading related to the rate of repetitive activation of $I_{Ca\text{-}L}$, and to changes in extrusion and/or uptake of Ca^{2+} during diastole (Bers, 1991). One could speculate that the ability of tetracaine to strongly inhibit the staircase suggests that the VSRM plays a significant role in beat-to-beat regulation of cardiac contraction. Recent studies have reported that VSRM contractions increase markedly with the number of conditioning pulses while the amplitudes of CICR contractions and caffeine contractures increased only slightly (Zhu & Ferrier, 1999). As well, VSRM contraction amplitude was increased by an increase in the conditioning pulse frequency from 0.1 to 5 Hz. It was concluded from these observations

that force-interval relations and staircases strongly depend on changes in the fractional release of SR Ca^{2+} by the VSRM but less so than on changes in SR stores (Zhu & Ferrier, 1999). At present it is not clear why the VSRM appears to play a greater role than CICR in determining staircase phenomena. It will be necessary to explore physiological regulation of the VSRM further before this question can be answered, and the results of the present study indicate that tetracaine will be very useful in this respect.

RYANODINE:

The present study illustrates a previously unreported negative inotropic effect of ryanodine which is unrelated to depletion of SR Ca^{2+} stores and which is mediated by selective inhibition of the VSRM component of cardiac EC-coupling. Ryanodine is known to bind with high selectivity and affinity to SR Ca^{2+} release channels (Pessah *et al.*, 1986; Alderson & Feher, 1987; Fleischer *et al.*, 1985), therefore inhibition of VSRM contractions by this agent likely reflects an interaction of ryanodine with these channels. Previous reports have indicated that low concentration ryanodine binds to SR release channels and locks them in an open-subconducting state without affecting SR Ca^{2+} uptake (Rousseau, Smith & Meissner, 1987; Bers, 1991). Thus, in the presence of ryanodine, SR Ca^{2+} can leak out into the cytosol over a period of several hundred milliseconds. The discrepancy between the results of the present study and those illustrating depletion of SR stores in the presence of 30 nM ryanodine likely represent differences in experimental conditions. In support of this hypothesis, the effects of ryanodine have been reported to vary with the experimental conditions and temperature (Meissner, 1986; Shattock & Bers, 1987). As well, many previous studies were carried out under conditions which inhibit

activation of the VSRM, whereas the results of the present study are complicated by the interaction of two mechanisms of cardiac EC-coupling.

Although the present study demonstrates that VSRM contractions are virtually abolished in the presence of 30 nM ryanodine, the mean amplitude of CICR contractions also was significantly depressed. It is possible that the slight depression in the maximum amplitude of $I_{Ca\text{-}L}$ observed in the presence of ryanodine contributed to the decrease in CICR contraction amplitude. However, the magnitude of the decrease in contraction was greater than that of $I_{Ca\text{-}L}$, therefore the slight depression of $I_{Ca\text{-}L}$ alone cannot explain inhibition of this component of contraction. Like the VSRM, the $I_{Ca\text{-}L}$ -induced component of contraction also involves release of SR Ca^{2+} stores. The present study shows that the magnitude of CICR contractions decreased in parallel with that of caffeine contractures in the presence of increasing concentrations of ryanodine. These results suggest that the decrease in the amplitude of CICR contractions coupled to $I_{Ca\text{-}L}$ results from a gradual depletion of SR stores in the presence of ryanodine.

One could hypothesize that the selective effects of ryanodine on VSRM contractions reflect a greater sensitivity of this mechanism to changes in SR Ca^{2+} concentration compared to $I_{Ca\text{-}L}$-induced contractions. Thus, any small decrease in SR Ca^{2+} stores may result in inhibition of VSRM contractions whereas contractions elicited by CICR coupled to $I_{Ca\text{-}L}$ may require large decreases in SR Ca^{2+} load before any substantial decrease in contraction amplitude is observed. However, previous studies have shown that when SR stores were reduced by conditions promoting loss of Ca^{2+} through the $NaCa_{EX}$, VSRM and CICR contractions decreased in parallel (Richard &

Ferrier, 1999). These results suggest that both mechanisms of EC-coupling are equally sensitive to changes in SR Ca^{2+} stores. Therefore, it is unlikely that the preferential inhibition of VSRM contractions observed in the present study are due to a greater sensitivity of this mechanism to changes in SR Ca^{2+}.

Several hypotheses can be proposed to explain the differential effects of ryanodine on contractions elicited by the VSRM compared to those elicited by CICR. Two of the hypotheses proposed earlier to explain the effects of tetracaine could also be used to explain the effects of ryanodine since both agents are known to interact with SR RyRs. For example, SR Ca^{2+} release channels involved in mediating voltage dependent Ca^{2+} release may have a different conformation which increases the affinity of these channels for ryanodine. On the other hand, the previous proposal that there may be two isoforms of RyR, one mediating SR release by the VSRM and the other by CICR, could also be used to explain the selective effects of ryanodine. This hypothesis suggests that RyRs involved in voltage dependent release have a higher affinity for ryanodine than those mediating CICR contractions.

The selective effects of ryanodine on the VSRM component of EC-coupling also may result from interaction of this agent with two different binding sites on one isoform of RyR. Cardiac RyRs have been reported to have both a low and a high affinity binding site for ryanodine (Pessah *et al.*, 1986, 1987; Inui *et al.*, 1987, 1988; McGrew *et al.*, 1989). It is possible that binding of ryanodine to the high affinity site interferes with voltage dependent opening of SR RyRs which would ultimately inhibit contractions initiated by the VSRM. Conversely, binding of ryanodine to a low affinity site may be

responsible for locking the channel in an open subconducting state which leads to depletion of SR stores and inhibition of CICR contractions. This interaction would explain the selective inhibition of contractions generated by activation of the VSRM by nanomolar concentrations of ryanodine.

The final objective of this portion of the thesis was to determine whether ryanodine could be used as a pharmacological tool to investigate the contribution of the VSRM to cardiac contraction. The results presented here indicate that nanomolar concentrations of ryanodine can be used to preferentially inhibit the VSRM component of EC-coupling in the absence of depletion of SR stores. The selectivity of this agent for SR RyRs along with its high affinity for inhibition of the VSRM are ideal characteristics of a useful pharmacological tool.

Comparison of ryanodine and tetracaine as pharmacological tools:

The high selectivity and affinity of ryanodine make this agent very attractive as a pharmacological tool for investigation of the contribution of the VSRM to overall cardiac contraction. However, the use of ryanodine is not without complications as this agent has a slow mechanism of action and, therefore requires several minutes to reach a maximal effect. By comparison, rapid application studies have shown that complete inhibition of the VSRM by tetracaine occurs within seconds. This characteristic of tetracaine makes this agent a major asset for investigation of the VSRM. However, like ryanodine, tetracaine also has its complications. For example, the present study has shown that tetracaine increases SR Ca^{2+} stores at moderate concentrations which inhibit the VSRM. As well, selective inhibition of VSRM contractions by tetracaine has been shown to be

time and concentration dependent with longer exposures to moderate to high concentrations resulting in inhibition of $I_{Ca\text{-}L}$ and CICR contractions. These side effects of tetracaine can be avoided with rapid application, and therefore this agent can be used as a pharmacological tool for evaluating the cardiac VSRM as long as one is aware of its limitations and complications.

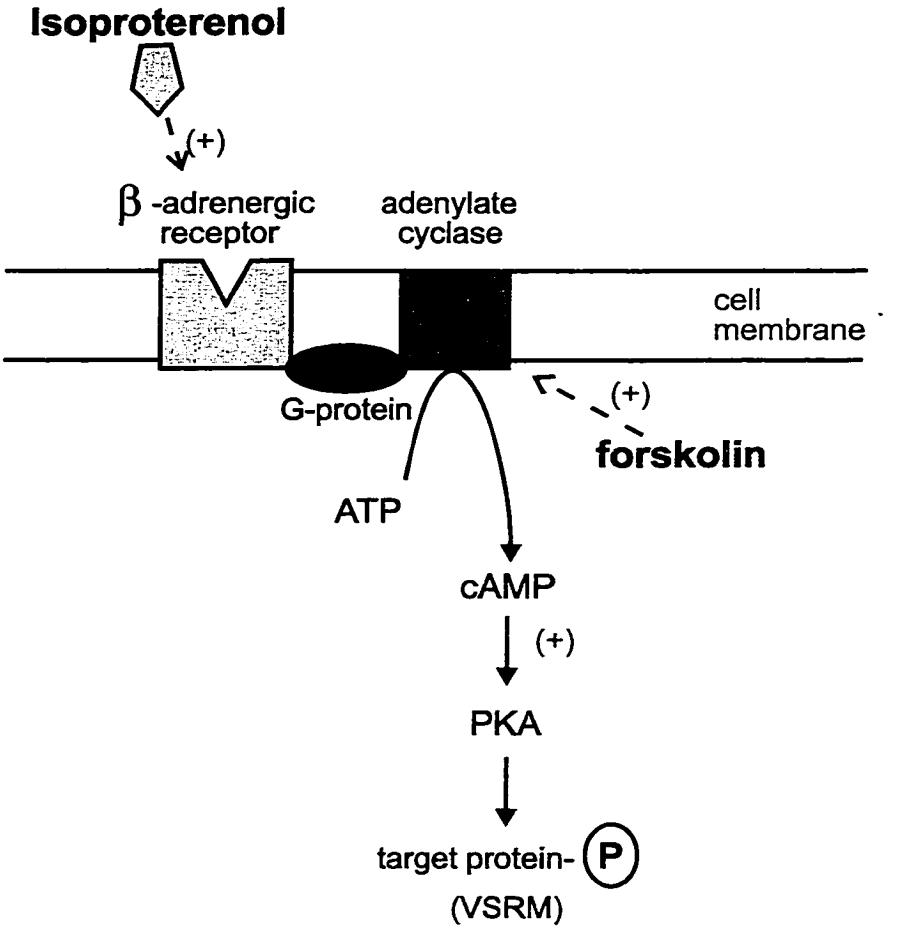
CONCLUSIONS:

The present study demonstrates that tetracaine has a negative inotropic effect in guinea pig ventricular myocytes and that this effect involves inhibition of the VSRM. Over a wide range of concentrations, tetracaine is a selective blocker of the VSRM and can be used to identify and separate the VSRM from the component of contraction initiated by CICR. Furthermore, tetracaine provides a useful pharmacological tool which can be used to evaluate the contribution of the VSRM and CICR to cardiac contraction and force-interval relations. Similarly, ryanodine has been shown to selectively inhibit the VSRM component of EC-coupling by a mechanism which is independent of changes in SR stores. As well, an important finding from the ryanodine study is that inhibition of contraction by low concentrations of ryanodine may identify a role for the SR in contraction but cannot be used as an index of SR depletion.

The relative contributions of the VSRM and CICR to initiation of contraction are not yet known. The voltage dependence of the VSRM indicates that it is available over the range of potentials associated with a cardiac action potential, in contrast to CICR, which is only available at potentials which initiate Ca^{2+} current. Conditions which dampen the staircase phenomenon, such as conditioning pulses to -40 mV, inhibit

contractions elicited by the VSRM but not those elicited by CICR. These findings suggest that the VSRM plays an important role in initiating contractions and in beat-to-beat modulation of contraction under normal physiological conditions. It has not yet been determined what contribution the VSRM makes to cardiac EC-coupling compared to CICR. Extrapolating the findings in skeletal muscle to cardiac muscle would lead to a prediction that the VSRM will be determined to play the predominant role in cardiac EC-coupling just as DICR does in skeletal muscle. However, more research is needed to answer this question. This research will be greatly aided by the use of pharmacological tools such as tetracaine and ryanodine which selectively inhibit the VSRM component of contraction.

Appendix



Activation of the VSRM is believed to involve phosphorylation. One way for this phosphorylation to occur is via activation of the cAMP-PKA pathway. Studies have shown that stimulation of this pathway at various sites can support VSRM contractions. For example, activation of β-receptors with isoproterenol stimulates VSRM contractions and transients as does direct stimulation of adenylate cyclase by forskolin (Chartier *et al.*, 1999; Moore *et al.*, 1999).

REFERENCES

Adachi Akahane, S., Lu, L., Li, Z. Frank, J.S., Philipson, K.D. & Morad, M. (1997). Calcium signaling in transgenic mice overexpressing cardiac Na^{+} -Ca^{2+} exchanger. *Journal of General Physiology* **109**, 717-729.

Adelman, J.P. (1995). Proteins that interact with the pore-forming subunits of voltage-gated ion channels. *Current Opinion in Neurobiology* **5**, 286-295.

Agnew, W. S. (1988). Excitation-contraction coupling. Proteins that bridge the gap. *Nature* **334**, 299-300.

Alderson, B.H. & Feher, J.J. (1987) The interaction of calcium and ryanodine with cardiac sarcoplasmic reticulum. *Biochimica et Biophysica Acta.* **900**, 221-229.

Almers, W. & Best, P. M. (1976). Effects of tetracaine on displacement currents and contraction of frog skeletal muscle. *Journal of Physiology (London)* **262**, 583-611.

Almers, W. & Best, P. M. (1976b). Effects of tetracaine on contraction and 'gating currents' in frog skeletal muscle. *Biophysical Journal* **16**, 152a.

Arreola, J., Dirksen, R.T., Shieh, R.C., Williford, D.J. & Shea, S.S. (1991). Ca^{2+} current and Ca^{2+} transients under action potential clamp in guinea pig ventricular myocytes. *American Journal of Physiology* **261**, C939-7.

Ashley, R.H. & Williams, A.J. (1990). Divalent cation activation and inhibition of single calcium release channels from sheep cardiac sarcoplasmic reticulum. *Journal of General Physiology* **95**, 981-1005.

Balke, C.W. & Wier, W.G. (1991). Ryanodine does not affect calcium current in guinea pig ventricular myocytes in which Ca2+ is buffered. *Circulation Research* **68**, 897-902.

Barcenas-Ruiz, L., Beuckelmann, D.J. & Wier, W.G. (1987). Sodium-calcium exchange in heart: membrane currents and changes in $[Ca^{2+}]_i$. *Science* **238**, 1720-1722.

Barcenas-Ruiz, L.& Wier, W.G. (1987). Voltage dependence of intracellular $[Ca^{2+}]_i$ transients in guinea pig ventricular myocytes. *Circulation Research* **61**, 148-154.

Bean, B. (1985). Two kinds of calcium channels in canine atrial cells. *Journal of General Physiology* **86**, 1-30.

Beeler, G.W. & Reuter, H. (1970). The relation between membrane potential, membrane currents, and activation of contraction in ventricular myocardial fibres. *Journal of Physiology* **207**, 211-229.

Berlin, J.R., Cannell, M.B. & Lederer, W.J. (1987). Regulation of twitch tension in sheep cardiac Purkinje fibers during calcium overload. *American Journal of Physiology* **253**, H1540-1547.

Berne, R.M. & Levy, M.N. (1997). Cardiovascular physiology. Seventh edition. (Mosby-Year Book, Inc. St. Louis, Missouri).

Berridge, M. J. (1997). Elementary and global aspects of calcium signaling. *Journal of Physiology (London)* **499**, 291-306.

Bers, D. M. (1991). Excitation-contraction coupling and cardiac contractile force. Kluwer Academic Publishers, Dordrecht.

Bers, D.M. & Bridge, J. (1989). Relaxation of rabbit ventricular muscle by Na-Ca exchange and sarcoplasmic reticulum Ca-pump: Ryanodine and voltage sensitivity. *Circulation Research* **65**,334-342.

Bers, D.M. & Bridge, J. & Spitzer, K.W. (1989). Intracellular Ca transients during rapid cooling contractures in guinea-pig ventricular myocytes. *Journal of Physiology* **417**, 537-53.

Bers, D.M., Lederer, W.J. & Berlin, J.R. (1990). Intracellular Ca transients in rat cardiac myocytes: role of Na-Ca exchange in excitation-contraction coupling. *American Journal of Physiology* **258**, C944-C954.

Bers, D.M. & Stiffel, V.M. (1993). Ratio of ryanodine to dihydropyridine receptors in cardiac and skeletal muscle and implications for E-C coupling. *American Journal of Physiology* **264**, C1587-C1593.

Beuckelmann, D.J. & Wier, W.G. (1988). Mechanism of release of calcium from s.r. of guinea-pig cardiac cells. *Journal of Physiology* **405**, 233-255.

Beuckelmann, D.J. & Wier, W.G. (1989). Sodium-calcium exchange in guinea-pig cardiac cells: exchange current and changes in intracellular Ca^{2+}. *Journal of Physiology* **414**, 499-520.

Bianchi, C.P. (1968). Pharmacological actions on excitation-contraction coupling in striated muscle. Federation Proceedings **27**, 126-31.

Blatter, L. A. & Niggli, E. (1997). Rapid solution changes in cardiac myocyte T-tubules. *Biophysical Journal* **72**, A45.

Block, B. A., Imagawa, T., Campbell, K. P. & Franzini-Armstrong, C. (1988). Structural evidence for direct interaction between the molecular components of the transverse

tubule/sarcoplasmic reticulum junction in skeletal muscle. *Journal of Cell Biology* **107**, 2587-2600.

Bouchard, R.A., Clark, R.B. & Giles, W.R. (1993). Role of sodium-calcium exchange in activation of contraction in rat ventricle. *Journal of Physiology* **472**, 391-413.

Brady, A.J. (1964). Excitation and excitation-contraction coupling in cardiac muscle. *Annual Review of Physiology* **26**, 341-356

Brill, D.M., Fozzard, H.A., Makielski, J.C. & Wasserstrom, J.A. (1987) Effect of prolonged depolarizations on twitch tension and intracellular sodium activity in sheep cardiac Purkinje fibres. *Journal of Physiology* **384**, 355-75.

Brum, G., Fitts, R., Pizarró, G. & Ríos, E. (1987). Voltage sensors of the frog skeletal muscle membrane require calcium to function in excitation-contraction coupling. *Journal of Physiology (London)* **398**, 475-505.

Brum, G., Stefani, E. & Ríos, E. (1986). Simultaneous measurements of Ca^{2+} currents and intracellular Ca^{2+} concentrations in single skeletal muscle fibers of the frog. *Canadian Journal of Physiology and Pharmacology* **65**, 681-685.

Butterworth, J.F. & Strichartz, G.R. (1990). Molecular mechanisms of local anesthesia: a review. *Anesthesiology* **72**, 711-734.

Callewaert, G. (1992). Excitation-contraction coupling in mammalian cardiac cells. *Cardiovascular Research* **26**, 923-932.

Callewaert, G., Cleemann, L. & Morad, M. (1988). Epinephrine enhances Ca^{2+} current-regulated Ca^{2+} release and Ca^{2+} reuptake in rat ventricular myocytes. *Proceedings of the National Academy of Sciences USA* **85**, 2009-2013.

Campbell, K.P., Knodson, C.M., Imagawa, T., Leung, A.T. Sutko, J.L., Kahl, S.D., Raab, C.R. & Madson, L. (1987). Identification and characterization of the high affinity ryanodine receptor of the junctional sarcoplasmic reticulum Ca^{2+} release channel. *Journal of Biological Chemistry* **262**, 6460-63.

Cannell, M.B., Berlin, J.R. & Lederer, W.J. (1987). Effect of membrane potential changes on the calcium transient in single rat cardiac muscle cells. *Science* **238**, 1419-1423.

Cannell, M.B., Cheng, H. & Lederer, W.J. (1994). Spatial non-uniformities in $[Ca^{2+}]_i$ during excitation-contraction coupling in cardiac myocytes. *Biophysical Journal* **67**, 1942-1956.

Cannell, M.B., Cheng, H. & Lederer, W.J. (1995). The control of calcium release in the heart. *Science* **268**, 1045-1050.

Cannell, M.B., Grantham, C.J., Main, M.J. & Evans, A.M. (1996). The roles of the sodium and calcium current in triggering calcium release from the sarcoplasmic reticulum. *Annals of the New York Academy of Sciences* **779**, 443-50.

Carafoli, E. (1975).Mitochondria, Ca^{2+} transport and the regulation of heart contraction and metabolism. *Journal of Molecular & Cellular Cardiology* **7**, 83-89.

Carafoli, E. & Lehninger, A.L. (1971). A survey of the interaction of calcium ions with mitochondria from different tissues and species. *Biochemical Journal* **122**,618-690.

Carmeliet, E., Morad, M., Van der Heyden, G. & Vereecke, J. (1986). Electrophysiological effects of tetracaine in single guinea-pig ventricular myocytes. *Journal of Physiology (London)* **376**, 143-161.

Chamberlain, B. K., Volpe, P. & Fleischer, S. (1984). Inhibition of calcium-induced calcium release from purified cardiac sarcoplasmic reticulum vesicles. *Journal of Biological Chemistry* **259**, 7547-7553.

Chapman, R.A. (1983). Control of cardiac contractility at the cellular level. *American Journal of Physiology* **245**, H535-H552.

Chapman, R.A. & Miller, D.J. (1974). The effects of caffeine on the contraction of the frog heart. *Journal of Physiology* **242**, 589-613.

Chartier, D., Moore, H.M., Howlett, S.E., Ferrier, G. & Leblanc, N. (1999). Activation of the voltage-sensitive release mechanism by beta-adrenergic stimulation in dialyzed guinea-pig ventricular myocytes. *Biophysical Journal* **76**, A458.

Cheng, H., Lederer, W.J. & Cannell, M.B. (1993). Calcium sparks: elementary events underlying excitation-contraction coupling in heart muscle. *Science* **262**, 740-744.

Chijiwa, T., Mishima, A., Hagiwara, M., Sano, M., Hayashi, K., Inoue, T., Naito, K., Toshioka, T. & Hidaka, H. (1990). Inhibition of forskolin-induced neurite outgrowth and protein phosphorylation by a newly synthesized selective inhibitor of cyclic AMP-dependent protein kinase, N-[2-(p-bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide (H-89), of PC12D pheochromocytoma cells. *Journal of Biological Chemistry* **265**, 5267-5272.

Cleemann, L. & Morad, M. (1991). Role of Ca^{2+} channel in cardiac excitation-contraction coupling in the rat: evidence from Ca^{2+} transients and contraction. *Journal of Physiology* **432**, 283-312.

Cohen, C.J., Bean, B.P., Colatsky, T.J. & Tsien, R.W. (1981). Tetrodotoxin block of sodium channels in rabbit Purkinje fibers. *Journal of General Physiology* **78**, 383-411.

Cohen, N.M. & Lederer, W.J. (1988). Changes in the calcium current of rat heart ventricular myocytes during development. *Journal of Physiology* **406**, 115-146.

duBell, W.H. & Houser, S.R. (1989). Voltage and beat dependence of Ca^{2+} transient in feline ventricular myocytes. *American Journal of Physiology* **257**, H746-59.

duBell, W.H., Lederer, W.J. & Rogers, T.B. (1996). Dynamic modulation of excitation-contraction coupling by protein phosphatases in rat ventricular myocytes. *Journal of Physiology* **493**, 793-800.

Ebashi, S. (1960). Calcium binding and relaxation in the actomyosin system. *Journal of Biochemistry* **48**, 150-151.

Ebashi, S. (1961a). Calcium binding activity of vesicular relaxing factor. *Journal of Biochemistry* **50**, 236-44.

Ebashi, S. (1961b). The role of "relaxing factor" in contraction-relaxation cycle of muscle. *Progress in Theoretical Physiology* **17**, 35-40.

Ebashi, S. (1974). Regulatory mechanism of muscle contraction with special reference to the Ca-troponin-tropomyosin system. In *Essays in Biochemistry*, Vol. 10, ed. P.N. Campbell, F. Dickens, pp. 1-36. London: Academic.

Ebashi, S. (1976). Excitation-contraction coupling. *Annual Review of Physiology* **38**, 293-313.

Ebashi, S. & Endo, M. (1968). Ca ion and muscle contraction. *Progress in Biophysics and Molecular Biology* **18**, 123-83.

Ebashi, S. & Lipmann, F. (1962). Adenosine triphosphate-linked concentration of calcium ions in a particulate fraction of rabbit muscle. *Journal of Cell Biology* **14**, 389-400.

Eisner, D.A., Trafford, A.W., Diaz, M.E., Overend, C.L. & O'Neill, S.C. (1998). The control of Ca release from the cardiac sarcoplasmic reticulum: regulation versus autoregulation. *Cardiovascular Research* **38**, 589-604.

Endo, M. (1964). Entry of a dye into the sarcotublar system of muscle. *Nature* **202**, 1115-16.

Endo, M. (1975). Mechanism of action of caffeine on the sarcolplasmic reticulum of skeletal muscle. *Proceding of the Japanese Academy* **51**, 467-472.

Endo, M. (1977). Calcium release from the sarcoplasmic reticulum. *Physiological Reviews* 57, 71-108.

Endo, M., Tanaka, M. & Ogawa, Y. (1970). Calcium-induced release of calcium from the sarcoplasmic reticulum of skinned skeletal muscle fibres. *Nature* **228**, 34-6.

Fabiato, A. (1985a). Rapid ionic modifications during the aequorin-detected calcium transient in a skinned canine cardiac Purkinje cell. *Journal of General Physiology* **85**,189-246.

Fabiato, A. (1985b). Simulated calcium current can both cause calcium loading in and trigger calcium release from the sarcoplasmic reticulum of a skinned canine cardiac Purkinje cell. *Journal of General Physiology* **85**, 291-320.

Fabiato, A. (1985c). Time and calcium dependence of activation and inactivation of calcium-induced release of calcium from the sarcoplasmic reticulum of a skinned canine cardiac Purkinje cell. *Journal of General Physiology* **85**, 247-290.

Fabiato, A. & Fabiato, F. (1975). Contractions induced by a calcium-triggered release of calcium from the sarcoplasmic reticulum of single skinned cardiac cells. *Journal of Physiology* **249**, 469-495.

Fabiato, A. & Fabiato, F. (1977). Calcium release from the sarcoplasmic reticulum. *Circulation Research* **40**, 119-129.

Fabiato, A. & Fabiato, F. (1978). Effects of pH on the myofilaments and the sarcoplasmic reticulum of skinned cells from cardiac and skeletal muscles. *Journal of Physiology* **276**, 233-255.

Fawcett, D.W. & McNutt, N.S. (1969). The ultrastructure of the cat myocardium. I. Ventricular papillary muscle. *Journal of Cell Biology* **42**, 1-45.

Feher, J.J. & Lipford, G.B. (1985). Mechanism of action of ryanodine on cardiac sarcoplasmic reticulum. Biochimica et Biophysica Acta. **813**, 77-86

Feinstein, M.B. (1963). Inhibition of caffeine rigor and radiocalcium movements by local anesthetics in frog sartorius muslce. *Journal of General Physiology* **47**, 151-172.

Ferrier, G.R. (1996). Low temperature inhibits cardiac contractions initiated by the voltage-sensitive release mechanism. *Journal of Molecular and Cellular Cardiology* **28**, A180.

Ferrier, G.R. & Howlett, S.E. (1995). Contractions in guinea-pig ventricular myocytes triggered by a calcium-release mechanism separate from Na^+ and L-currents. *Journal of Physiology (London)* **484**, 107-122.

Ferrier, G.R. & Howlett, S.E. (1996). Contractions initiated by a cardiac voltage sensitive release mechanism differ from those of Na^{+}-Ca^{++} exchange. *Journal of Molecular and Cellular Cardiology* **28**, (T77)A180.

Ferrier, G.R., Redondo, I.M., Isac, M. & Howlett, S.E. (1998). The cardiac voltage sensitive release mechanism is located in a separate compartment from L-type Ca^{2+} current. *Biophysical Journal* **74**, A54.

Ferrier, G.R., Redondo, I.M., Mason, C.A., Mapplebeck, C.L., and Howlett, S.E. (1999). Membrane potential directly regulates contraction and relaxation in cardiac muscle. *Journal of Physiology*, in press.

Ferrier, G.R., Redondo, I.M., Mason, C.A., Mapplebeck, C. & Howlett, S.E. (1999b). Inactivating and non-inactivating components of the cardiac voltage-sensitive release mechanism. *Biophysical Journal* **76**, A457.

Ferrier, G. R., Zhu, J-Q., Redondo, I. M. & Howlett, S. E. (1998b). Role of cAMP-dependent protein kinase A in activation of a voltage-sensitive release mechanism for cardiac contraction. *Journal of Physiology (London)* **513**, 185-201.

Finkel, A.S. and Redman, S.J. (1984). Theory and operation of a single microelectrode voltage clamp. *Journal of Neuroscience Methods* **11**, 101-127.

Fleischer, S., Ogunbunmi, E.M., Dixon, M.C., & Fleer, E.A.M. (1985). Localization of Ca2+ release channels with ryanodine in junctional terminal cisternae of sarcoplasmic reticulum of fast skeletal muscle. *Procedings of the National Academy of Sciences USA* **82**, 7256-7259.

Franzini-Armstrong, C. (1970). Studies of the triad. I. Structure of the junction in frog twitch fibers. *Journal of Cell Biology* **47**, 488-499.

Frank, G.B. (1980). The current view of the source of trigger calcium in excitation-contraction coupling in vertebrate skeletal muscle. *Biochemical Pharmacology* **29**, 2399-2406.

Ford, L.E. & Podolsky, R.J. (1970). Regenerative calcium release within muscle cells. *Science* **167**, 58-9.

Fozzard, H.A. (1991). Excitation-contraction coupling in the heart. *Advances in Experimental Medicine and Biology* **308**, 135-142.

Fozzard, H.A. & Hellam, D.C. (1968). Relationship between membrane voltage and tension in voltage-clamped cardiac Purkinje fibres. *Nature* **218**, 588-589.

Fuchs, F. (1974). Striated muscle. *Annual Review of Physiology* **36**,461-502.

Giannini, G., Clementi, E., Ceci, R., Marziali, G. & Sorrentino, V. (1992). Expression of a ryanodine receptor-Ca2+ channel that is regulated by TGF-beta. *Science* **257**, 91-94.

Giannini, G., Conti, A., Mammarella, S., Scrobogna, M. & Sorrentino, V. (1995). The ryanodine receptor/calcium channel genes are widely and differentially expressed in murine brain and peripheral tissues. *Journal of Cell Biology* **128**, 893-904.

Györke, S.& Fill, M. (1993). Ryanodine receptor adapation: control mechanism of Ca^{2+}-induced Ca^{2+} release in heart. *Science* **260**, 807-809.

Györke, S., Lukyanenko, V. & Györke, I. (1997). Dual effects of tetracaine on spontaneous calcium release in rat ventricular myocytes. *Journal of Physiology* **500**, 297-309.

Györke, S. & Palade, P. (1993). Role of local Ca^{2+} domains in activation of Ca^{2+}-induced Ca^{2+} release in crayfish muscle. *American Journal of Physiology* **264**, C1505-C1512.

Hagiwara, N., Irisawa, H. & Kameyama, M. (1988). Contribution of two types of calcium currents to the pacemaker potentials of rabbit sino-atrial node cells. *Journal of Physiology* **395**, 233-253.

Hall, Z.W. (1992). An introduction to molecular neurobiology. Sinauer Assoc. Inc. Publishers. Sunderland, Massachusetts.

Han, S., Schiefer, A. & Isenberg, G. (1994). Ca^{2+} load of guinea-pig ventricular myocytes determines efficacy of brief Ca^{2+} currents as trigger for Ca^{2+} release. *Journal of Physiology* **480**, 411-421.

Hancox, J.C. & Levi, A.J. (1995). Calcium transients which accompany the activation of sodium current in rat ventricular myocytes at 37 °C: a trigger role for reverse Na-Ca exchange activated by membrane potential? *Pfluegers Archives* **430**, 887-893.

Hartzell, H.C. (1988). Regulation of cardiac ion channels by catecholamines, acetylcholine and second messenger systems. *Progress in Biophysics and Molecular Biology* **52**, 165-247.

Hille, B. (1977). Local anesthetics: hydrophilic and hydrophobic pathways for the drug-receptor reaction. *Journal of General Physiology* **69**, 497-515.

Hille, B. (1992). Ionic channels of excitable membranes. (Sinauer Assoc. Inc., Sunderland MA, ed. 2) pp. 403-414.

Hirano, Y., Fozzard, H.A. & January, C.T. (1989). Characteristics of L- and T-type Ca^{2+} currents in canine cardiac Purkinje cells. *American Journal of Physiology* **256**, H1478-H1492.

Hobai, I. A., Howarth, F. C., Pabbathi, V. K., Dalton, G. R., Hancox, J. C., Zhu, J-Q., Howlett, S. E., Ferrier, G. R.& Levi, A. J. (1997). "Voltage-activated Ca release" in rabbit, rat and guinea-pig cardiac myocytes, and modulation by internal cAMP. *Pflügers Archives* **435**, 164-173.

Honore, E., Adamantidis, M.M., Dupuis, B.A., Challice, C.E., & Guilbault, P. (1987a). Calcium channels and excitation-contraction coupling in cardiac cells. I. Two components of contraction in guinea pig papillary muscle. *Canadian Journal of Physiology and Pharmacology* **65**, 1821-1831.

Honore, E., Adamantidis, M.M., Dupuis, B.A., Challice, C.E., & Guilbault, P. (1987b). Calcium channels and excitation-contraction coupling in cardiac cells. II. A pharmacological study of the biphasic contraction in guinea pig papillary muscle. *Canadian Journal of Physiology and Pharmacology* **65**, 1832-1839.

Howlett, S.E., Barry, A. & Ferrier, G.R. (1999). Selective inhibition of the cardiac voltage sensitive release mechanism by amiloride. *Biophysical Journal* **76**, A457.

Howlett, S. E., Zhu, J. Q. & Ferrier, G. R. (1998). Contribution of a voltage-sensitive calcium release mechanism to cardiac contraction in cardiac ventricular myocytes. *American Journal of Physiology* **274**, H155-H170.

Howlett, S. E. & Ferrier, G. R. (1997). The voltage-sensitive release mechanism: a new trigger for cardiac contraction. *Canadian Journal of Physiology and Pharmacology* **75**, 1044-1057.

Howlett, S.E. & Mapplebeck, C. (1996). Contractions initiated by the cardiac voltage-sensitive release mechanism are selectively depressed in myocytes from young cardiomyopathic hamsters. *British Journal of Pharmacology* **119**, 99P.

Howlett, S.E., Ferrier, G.R. & Mapplebeck, C. (1997). Depression of contraction activated by the cardiac voltage-sensitive release mechanism in myocytes from myopathic hamsters. *Biophysical Journal* **72**, A161.

Hunter, D.R., Haworth, R.A. & Berkoff, H.A. (1982). Cellular calcium turnover in the perfused rat heart: modulation by caffeine and procaine. *Circulation Research* **51**, 363-370.

Huxley, H.E. (1969). The mechanism of muscular contraction. *Science* **164**, 1356-1366.

Huxley, A.F. & Simmons, R.M. (1971). Proposed mechanism of force generation in striated muscle. *Nature* **233**, 533-538.

Hyrshko, L.V., Stiffel, V.M. & Bers, D.M. (1990). BAY K 8644 may affect cardiac SR via direct communication between sarcolemmal and SR Ca channels. *Biophysical Journal* **57**, 167a.

Inui, M. Saito, A. & Fleischer, S. (1987). Isolation of the ryanodine receptor from cardiac sarcoplasmic reticulum and identity with the feet structures. *Journal of Biological Chemistry* **262**, 15637-42.

Inui, M., Wang, S., Saito, A. and Fleischer, S. (1988). Characterization of junctional and longitudinal sarcoplasmic reticulum from heart muscle. *Journal of Biological Chemistry* **263**, 10843-10850.

Isenberg, G., Beresewicz, A., Maschier, D. & Valenzuela, F. (1985). The two components in the shortening of unloaded ventricular myocytes: their voltage dependence. *Basic Research in Cardiology* **80** (suppl. 1), 117-122.

Isenberg, G. & Wendt-Gallitelli, M.F. (1989). Cellular mechanisms of excitation contraction coupling. *In* Isolated adult cardiomyocytes. Vol. II. eds. H.M. Piper & G. Isenberg. CRC Press Inc., Boca Raton, Fla. pp. 213-248.

Jacquemond, V., Csernoch, L., Klein, M. G. & Schneider, M. F. (1991). Voltage-gated and calcium-gated calcium release during depolarization of skeletal muscle fibers. *Biophysical Journal* **60**, 867-873.

Jenden, D.J. & Fairhurst, A.S. (1969). The pharmacology of ryanodine. *Pharmacological Reviews* **21**, 1-25.

Johnson, P.N. & Inesi, G. (1969). The effect of methylxanthines and local anesthetics on fragmented sarcoplasmic reticulum. *Journal of Pharmacological and Experimental Therapeutics* **169**, 308-14.

Katzung, B.G. (1995). Basic & Clinical Pharmacology. 6th edition. Appleton & Lange, Norwalk, Connecticut, USA.

Kimura, J., Miyamae, S. & Noma, A. (1987). Identification of sodium-calcium exchange current in single ventricular cells of guinea-pig. *Journal of Physiology* **384**, 199-222.

Lai, F.A., Anderson, K., Rousseau, E., Liu, Q.Y. & Meissner, G. (1988a). Evidence for a Ca^{2+} channel within the ryanodine receptor complex from cardiac sarcoplasmic reticulum. *Biochemical and Biophysical Research Communications* **151**, 441-9

Lai, F.A. & Meissner, G. (1989). The muscle ryanodine receptor and its intrinsic Ca^{2+} channel activity. *Journal of Bioenergetics and Biomembranes* **21**, 227-46.

Lamb, G. D. & Stephenson, D. G. (1992). Control of calcium release from the sarcoplasmic reticulum. Excitation-contraction coupling in skeletal, cardiac, and smooth muscle. ed. G.B. Frank *et al.*, Plenum Press, New York.

Langer, G.A. (1968). Ion fluxes in cardiac excitation and contraction and their relation to myocardial contractility. *Physiological Reviews* **48**, 708-757.

Langer, G.A. (1978). The structure and function of the myocardial cell surface. *American Journal of Physiology* **235**, H461-68.

Lattanzio, F.A., Schlatter, R.G., Nicar, M., Campbell, K.P. & Sutko, J.L. (1987). The effects of ryanodine on passive calcium fluxes across sarcoplasmic reticulum membranes. *The Journal of Biological Chemistry* **262**, 2711-2718.

Laver, D.R. & Lamb, G.D. (1998). Inactivation of Ca^{2+} release channels (ryanodine receptors RyR1 and RyR2) with rapid steps in [Ca^{2+}]and voltage. *Biophysical Journal* **74**, 2352-2364.

Leblanc, N. & Hume, J. (1990). Sodium current-induced release of calcium from cardiac sarcoplasmic reticulum. *Science* **248**, 372-378.

Lederer, W.J., Niggli, E. & Hadley, R.W. (1990). Sodium-calcium exchange in excitable cells: fuzzy space. *Science* **248**, 283.

Lehninger, A.L. (1974). Ca^{2+} transport by mitochondria and its possible role in the cardiac excitation-contraction-relaxation cycle. *Circulation Research* **34/35**, Suppl. III, 83-89.

Lehninger, A.L., Carafoli, E. & Rossi, C.S. (1967). Energy linked ion movements in mitochondrial systems. *Advanced Enzymology* **29**, 259-320.

Levesque, P. C., Leblanc, N. & Hume, J. R. (1994). Release of calcium from guinea-pig cardiac sarcoplasmic reticulum induced by sodium-calcium exchange. *Cardiovascular Research* **28**, 370-378.

Levi, A.J., Brooksby, P. & Hancox, J.C. (1993). A role for depolarization induced calcium entry on the Na-Ca exchange in triggering intracellular calcium release and contraction in rat ventricular myocytes. *Cardiovascular Research* **27**, 1677-1690.

Levi, A. J., Hancox, J. C., Howarth, F. C., Croker, J. & Vinnicombe, J. (1996). A method for making rapid changes of superfusate whilst maintaining temperature at 37°C. *Pflügers Archives* **432**, 930-937.

Levi, A.J., Mitcheson, J.S. & Hancox, J.C. (1996b). The effect of internal sodium and caesium on phasic contraction of patch-clamped rabbit ventricular myocytes. *Journal of Physiology* **492**, 1-19.

Levi, A. J., Spitzer, K. W., Kohomoto, O. & Bridge, J. H. B. (1994). Depolarization-induced Ca entry via Na-Ca exchange triggers SR release in guinea-pig cardiac myocytes. *American Journal of Physiology (London)* **266**, H1422-1433.

Lewartowski, B., Hansford, R. G., Langer, G. A., & Lakatta, E. G. (1990). Contraction and sarcoplasmic reticulum Ca^{2+} content in single myocytes of guinea pig heart: effect of ryanodine. *American Journal of Physiology* **259**, H1222-H1229.

Li, L., Satoh, H., Ginsburg, K.S. & Bers, D.M. (1997). The effect of Ca^{2+}-calmodulin-dependent protein kinase II on cardiac excitation-contraction coupling in ferret ventricular myocytes. *Journal of Physiology* **501**, 17-35.

Lindsay, A.R. & Williams, A.J. (1991). Functional characterisation of the ryanodine receptor purified from sheep cardiac muscle sarcoplasmic reticulum. *Biochimia and Biophysical Acta* **1064**, 89-102.

Lipp, P. & Niggli, E. (1994). Sodium current-induced calcium signals in isolated guinea-pig ventricular myocytes. *Journal of Physiology (London)* **474**, 439-446.

Litwin, S.E., Li, J. & Bridge, J.H.B. (1998). Na-Ca exchange and the trigger for sarcoplasmic reticulum Ca release: studies in adult rabbit ventricular myocytes. *Biophysical Journal* **75**, 359-371.

Lokuta, A.J., Rogers, T.B., Lederer, W.J. & Valdivia, H.H. (1995). Modulation of cardiac ryanodine receptors of swine and rabbit by a phosphorylation-dephosphorylation mechanism. *Journal of Physiology* **487.3**, 609-622.

London, B. & Krueger, J.W. (1986). Contraction in voltage-clamped, internally perfused single heart cells. *Journal of General Physiology* **88**, 475-505.

López-López, J.R., Shacklock, P.S., Balke, C.W. & Wier, W.G. (1995). Local calcium transients triggered by single L-type calcium channel currents in cardiac cells. *Science* **268**, 1042-1045.

López-López, J.R. & Wier, W.G. (1994). Gain of CICR in cardiac cells depends on membrane voltage and on SR Ca^{2+} content. *Biophysical Journal* **66**, 132A

Lukyanenko, V., Wiesner, T.F. & Györke, S. (1998). Termination of Ca^{2+} release during Ca^{2+} sparks in rat ventricular myocytes. *Journal of Physiology* **507**, 667-677.

Lynch, C. III. (1991). Pharmacological evidence for two types of myocardial sarcoplasmic reticulum Ca^{2+} release. *American Journal of Physiology* **260**, H785-H795.

Ma, J., Bhat, M.B. and Zhao, J. (1995). Rectification of skeletal muscle ryanodine receptor mediated by FK506 binding protein. *Biophysical Journal* **69**, 2398-2404.

Marban, E. & Wier, W.G. (1985) Ryanodine as a tool to determine the contributions of calcium entry and calcium release to the calcium transient and contraction of cardiac Purkinje fibers. *Circulation Research* **56**, 133-138.

McCall, E., Hryshko, L.V., Stiffel, V.M., Christensen, D.M. & Bers, D.M. (1996). Possible functional linkage between the cardiac dihydropyridine and ryanodine receptor: acceleration of rest decay by Bay K 8644. *Journal of Molecular and Cellular Cardiology* **28**, 79-93.

McDonald, T.F., Pelzer, S., Trautwein, W. & Pelzer, D. (1994). Regulation and modulation of calcium channels in cardiac, skeletal, and smooth muscle cells. *Physiological Reviews* **74**, 365-507.

McGrew, S.G., Wolleben, C., Siegl, P. Inui, M. & Fleischer, S. (1989) Positive cooperativity of ryanodine binding to the calcium release channel of sarcoplasmic reticulum from heart and skeletal muscle. *Biochemistry* **28**, 1686-1691.

Meissner, G. (1986). Ryanodine activation and inhibition of the Ca^{2+} release channel of sarcoplasmic reticulum. *The Journal of Biological Chemistry* **261**, 6300-6306.

Meissner, G. & Henderson, J.S. (1987). Rapid calcium release from cardiac sarcoplasmic reticulum vesicles is dependent on Ca^{2+} and is modulated by Mg^{2+}, adenine nucleotide, and calmodulin. *Journal of Biological Chemistry* **262**, 3065-3073.

Melzer, W., Herrmann-Frank, A. & Lüttgau, H. Ch. (1995). The role of Ca^{2+} ions in excitation-contraction coupling of skeletal muscle fibres. *Biochimica et Biophysica Acta* **1241**, 59-116.

Mitchell, M.R., Powell, T., Terrar, D.A. & Twist, V.W. (1984) Ryanodine prolongs Ca-currents while suppressing contraction in rat ventricular muscle cells. *British Journal of Pharmacology* **81**, 13-15

Mitchell, M. R., Powell, T., Terrar, D. A. & Twist, V. W. (1987). Electrical activity and contraction in cells isolated from rat and guinea-pig ventricular muscle: a comparative study. *Journal of Physiology (London)* **391**, 527-544.

Mitra, R. & Morad, M. (1986). Two types of calcium channels in guinea pig ventricular myocytes. *Procedings of the National Academy of Sciences* **83**, 5340-44.

Moore, H.M, Chartier, K., Leblanc, N., Howlett, S.E. & Ferrier, G.R. (1999). Isoproterenol modulates a voltage sensitive release mechanism for contraction independently of TTX-sensitive inward current. *Biophysical Journal* **76**, A458.

Morad, M. & Cleeman, L. (1987). Role of Ca^{2+} channel in development of tension in heart muscle. *Journal of Molecular and Cellular Cardiology* **19**, 527-53.

Mullins, L.J. (1979). The generation of electric currents in cardiac fibers by Na/Ca exchange. *American Journal of Physiology* **236**, C103-C110.

Muto, Y., Kudo, S. & Nozawa, Y. (1983). Effects of local anesthetics on calmodulin-dependent guanylate cyclase in the plasma membrane of Tetrahymena pyriformis. *Biochemical Pharmacology* **32**, 3559-63.

Näbauer, M., Callewaert, G., Cleemann, L. & Morad, M. (1989). Regulation of calcium release is gated by calcium current, not gating charge, in cardiac myocytes. *Science* **244**, 800-803.

Niggli, E. & Lederer, W.J. (1990). Voltage-independent calcium release in heart muscle. *Science* **250**, 565-568.

Niggli, E. & Lipp, P. (1995). Subcellular features of calcium signalling in heart muscle: what do we learn? *Cardiovascular Research* **29**, 441-448.

Nilius, B., Hess, P. Lansman, J.B. & Tsien, R.W. (1985). A novel type of cardiac calcium channel in ventricular cells. *Nature* **316**, 443-446.

Nuss, H.B. & Houser, S.R. (1992). Sodium-calcium exchange-mediated contractions in feline ventricular myocytes. *American Journal of Physiology* **263**, H1161-H1169.

Nuss, H.B. & Houser, S.R. (1993). T-type Ca^{2+} current is expressed in hypertrophied adult feline left ventricular myocytes. *Circulation Research* **73**, 777-782.

O'Brien, J., Valdivia, H.H. & Block, B.A. (1995). Physiological differences between alpha and beta ryanodine receptors of fish skeletal muscle. *Biophysical Journal* **68**, 471-482.

O'Neill, S. C., Donoso, P. & Eisner, D. A. (1990). The role of $[Ca^{2+}]_i$ and $[Ca^{2+}]$ sensitization in the caffeine contracture of rat myocytes: measurement of $[Ca^{2+}]_i$ and $[caffeine]_i$. *Journal of Physiology (London)* **425**, 55-70.

Ochi, R. & Trautwein, W. (1971). The dependence of cardiac contraction on depolarization and slow inward current. *Pflügers Archives* **323**, 187-203.

Overend, C. L., Eisner, D. A. & O'Neill, S. C. (1997). The effect of tetracaine on spontaneous Ca^{2+} release and sarcoplasmic reticulum calcium content in rat ventricular myocytes. *Journal of Physiology (London)* **502**, 471-479.

Overend, C. L., O'Neill, S. C. & Eisner, D. A. (1998). The effect of tetracaine on stimulated contractions, sarcoplasmic reticulum Ca^{2+} content and membrane current in isolated rat ventricular myocytes. *Journal of Physiology (London)* **507**, 759-769.

Pabbathi, V.K., Patel, K.C.R, Hinde, A.K., Arberry, L.A., Jones, J.V. & Levi, A.J. (1999). Repolarisation terminates SR Ca release in guinea-pig ventricular myocytes dialysed with cAMP at 37 °C. *Biophysical Journal* **76**, A457.

Peachey, L.D. (1965). The sarcoplasmic reticulum and transverse tubules of the frog's sartorius. *Journal of Cell Biology* **25**, 209-231.

Pessah, I.N., Francini, A.O., Scales, D.J., Waterhouse, A.L. & Casida, J.E. (1986). *Journal of Biological Chemistry* **261**, 8643-8648.

Pessah, I.N., Stambeck, R.A. & Casida, J.E. (1987). *Molecular Pharmacology* **31**, 231-238.

Pessah, I.N., Waterhouse, A.L. & Casida, J. E. (1985). The calcium-ryanodine receptor complex of skeletal and cardiac muscle. *Biochemical and Biophysical Research Communications* **128**, 449-456

Penefsky, Z. J. (1974). Studies on the mechanism of inhibition of cardiac muscle contractile tension by ryanodine. *Pflugers Archiv* **347**, 173-184.

Pizarró, G., Csernoch, L., Uribe, I. & Ríos, E. (1989). Tetracaine and pathways of Ca release in skeletal muscle. *Biophysical Journal* **55**, 237A.

Pizarró, G., Csernoch, L., Uribe, I. & Ríos, E. (1992). Differential effects of tetracaine on two kinetic components of calcium release in frog skeletal muscle fibres. *Journal of Physiology (London)* **457**, 525-538.

Pizarró, G., Fitts, R., Uribe, I. & Ríos, E. (1989). The voltage sensor of excitation-contraction coupling in skeletal muscle. Ion dependence and selectivity. *Journal of General Physiology* **94**, 405-428.

Rardon, D.P., Cefali, D.C., Mitchell, R.D., Seiler, S.M. & Jones, L.R. (1989). High molecular weight proteins purified from cardiac junctional sarcoplasmic reticulum vesicles are ryanodine-sensitive calcium channels. *Circulation Research* **64**, 779-89.

Reimer, K.A. & Jennings, R.B. (1986). Myocardial ischemia, hypoxia, and infarction. In: The heart and cardiovascular system, H.A. Fozzard *et al.*, eds., Raven Press, New York. pp. 1133-1201.

Reiter, M. (1988). Calcium mobilization and cardiac inotropic mechanisms. *Pharmacological Reviews* **40**, 189-217.

Reuter, H. & Seitz, N. (1968). The dependence of calcium efflux from cardiac muscle on temperature and external ion composition. *Journal of Physiology* **195**, 451-70.

Richard, M.J. & Ferrier, G.R. (1999). Modulation of activation of the cardiac voltage sensitive release mechanism by conditioning pulses. *Biophysical Journal* **76**, A458.

Ringer S. (1883). A further contribution regarding the influence of the different constituents of the blood on the contraction of the heart. *Journal of Physiology* **4**, 29-42.

Ríos, E. & Brum, G. (1987). Involvement of dihydropyridine receptors in excitation-contraction coupling in skeletal muscle. *Nature* **325**, 717-720.

Ríos, E. & Pizarró, G. (1988). Voltage sensors and calcium channels of excitation-contraction coupling. *NIPS* **3**, 223-227.

Ríos, E. & Pizarró, G. (1991). Voltage sensor of excitation-contraction coupling in skeletal muscle. *Physiological Reviews* **71**, 849-908.

Ríos, E. & Pizarró, G. & Stefani, E. (1992). Charge movement and the nature of signal transduction in skeletal muscle excitation-contraction coupling. *Annual Review of Physiology* **54**, 109-133.

Ritchie, J.M. & Greene, N.M. (1980). Local anesthetics. In Goodman and Gilman's The Pharmacological Basis of Therapeutics. 6th edition, A.G. Gilman, et al., eds., MacMillan Publishing Co., New York. pp. 300-320.

Ritchie, J.M. & Greengard, P. (1965). On the mode of action of local anesthetics. *Annual Review of Pharmacology* **6**, 405-30.

Rothschuh, K.E. (1973). History of Physiology. Translated and edited by G.B. Risse. Robert E.Krieger Publishing Co. Huntington, New York.

Rousseau, E. & Meissner, G. (1989). Single cardiac sarcoplasmic reticulum Ca^{2+}-release channel: activation by caffeine. *American Journal of Physiology* **256**, H328-H333.

Rousseau, E., Smith, J.S., Henderson, J.S. & Meissner, G. (1986). Single channel and Ca^{2+} influx measurements of the cardiac sarcoplasmic reticulum calcium channel. *Biophysical Journal* **50**, 1009-14.

Rousseau, E., Smith, J. S. & Meissner, G. (1987). Ryanodine modifies conductance and gating behavior of single Ca^{2+} release channels. *American Journal of Physiology* **253**, C364-368.

Santana, L.F., Cheng, H., Gómez, A.M., Cannell, M.B. & Lederer, W.J. (1996). Relation between the sarcolemmal Ca^{2+} current and Ca^{2+} sparks and local control theories for cardiac excitation-contraction coupling. *Circulation Research* **78**, 166-171.

Santana, L. E., Gómez, A. M. & Lederer, W. J. (1998). Ca^{2+} flux through promiscuous cardiac Na^{+} channels: slip-mode conductance. *Science* **279**, 1027-1033.

Schatzmann, H.J. (1989). The calcium pump of the surface membrane and of the sarcoplasmic reticulum. *Annual Review of Physiology* **51**, 473-485.

Schmidt-Nielsen, K. (1990). Animal physiology, adaptation and environment, fourth edition. Cambridge University Press, New York, USA.

Schuttler, K., Wang, S.Y., Pfeifer, T. & Meyer, R. (1991). Late contraction in guinea pig ventricular myocytes activated by the Na^{+}-Ca^{2+} exchange during the action potential. *Annals of the New York Academy of Sciences* **639**, 475-477.

Seiler, S., Wegener, A.D., Whang, D.D., Hathaway, D.R. & Jones, L.R. (1984) High molecular weight proteins in cardiac and skeletal muscle junctional sarcoplasmic reticulum vesicles bind calmodulin, are phosphorylated, and are degraded by Ca2+-activated protease. *Journal of Biological Chemistry* **259**, 8550-8557.

Sen, L & Smith, T.W. (1994). T-type Ca^{2+} channels are abnormal in genetically determined cardiomyopathic hamster hearts. *Circulation Research* **75**, 149-155.

Sham, J. S. K., Cleemann, L. & Morad, M. (1992). Gating of the cardiac Ca^{2+} release channel: The role of Na^{+} current and Na^{+}-Ca^{2+} exchange. *Science* **255**, 850-853.

Sham, J.S.K., Cleeman, L. & Morad, M. (1995). Functional coupling of Ca^{2+} channels and ryanodine receptors in cardiac myocytes. *Procedings of the National Academy of Sciences USA* **92**, 121-125.

Sham, J.S.K., Song, L.S., Chem, Y., Deng, L.H., Stern, M.D., Lakatta, E.G. & Cheng, H. (1998). Termination of Ca^{2+} release by a local inactivation of ryanodine receptors in cardiac myocytes. *Procedings of the National Academy of Sciences USA* **95**, 15096-15101.

Shattock, M.J. & Bers, D.M. (1987). Inotropic response to hypothermia and the temperature-dependence of ryanodine action in isolated rabbit and rat ventricular muscle: implications for excitation-contraction coupling. *Circulation Research* **61**, 761-71.

Sheu, S-S. & Fozzard, H.A. (1982). Transmembrane Na^+ and Ca^{2+} electrochemical gradients in cardiac muscle and their relationship to force development. *Journal of General Physiology* **80**, 325-351.

Shoshan-Barmatz, V. & Zchut, S. (1993). The interaction of local anesthetics with the ryanodine receptor of the sarcoplasmic reticulum. *Journal of Membrane Biology* **133**, 171-181.

Sipido, K.R., Carmeliet,E. & Pappano, A. (1995). Na^+ current and Ca^{2+} release from the sarcoplasmic reticulum during action potentials in guinea-pig ventricular myocytes. *Journal of Physiology* **489**, 1-17.

Sipido,K.R., Carmeliet, E. & Van de Werf, F. (1998). T-type Ca^{2+} current as a trigger for Ca^{2+} release from the sarcoplasmic reticulum in guinea-pig ventricular myocytes. *Journal of Physiology* **508**, 439-451.

Sipido, K.R., Maes, M. & Van de Werf, F. (1997). Low efficiency of Ca^{2+} entry through the Na^+ -Ca^{2+} exchanger as trigger for Ca^{2+} release from the sarcoplasmic reticulum. *Circulation Research* **81**, 1034-1044.

Smith, J.S., Coronado, R. & Meissner, G. (1986). Single channel measurements of the calcium release channel from skeletal muscle sarcoplasmic reticulum: activation by Ca^{2+}, ATP and modulation by Mg^{2+}. *Journal of General Physiology* **88**, 573-88.

Smith, J.S., Rousseau, E. & Meissner, G. (1989). Calmodulin modulation of single sarcoplasmic reticulum Ca^{2+} -release channels from cardiac and skeletal muscle. *Circulation Research* **64**, 352-59.

Sommer, J.R. (1995). Comparative anatomy: in praise of a powerful approach to elucidate mechanisms translating cardiac excitation into purposeful contraction. *Journal of Molecular and Cellular Cardiology* **27**, 19-35.

Stephenson, D.G. & Wendt, I.R. (1986). Effects of procaine on calcium accumulation by the sarcoplasmic reticulum of mechanically disrupted rat cardiac myocytes. *Journal of Physiology* **373**, 195-207.

Stern, M.D. (1992). Theory of excitation-contraction coupling in cardiac muscle. *Biophysical Journal* **63**, 497-517.

Stern, M.D. & Lakatta, E. (1992). Excitation-contraction in the heart: the state of the question. *FASEB Journal* **6**, 3092-3100.

Sutko, J.L., Ito, K. & Kenyon, J.L. (1985). Ryanodine: a modifier of sarcoplasmic reticulum calcium release in striated muscle. Federation Proceedings **44**, 2984-2988

Sutko, J.L. & Kenyon, J.L. (1983). Ryanodine modification of cardiac muscle responses to potassium-free solutions. *Journal of General Physiology* **82**, 385-404.

Sutko, J.L. & Willerson, J.T. (1980). Ryanodine alters the contractile state of rat ventricular myocardium. *Circulation Research* **46**, 332-343.

Takasago, T., Imagawa, T., Furukawa, K. Ogurusu, T. & Shigekawa, M. (1991). Regulation of the cardiac ryanodine receptor by protein kinase-dependent phosphorylation. *Journal of Biochemistry* **109**, 163-170.

Takeshima, H., Iino, M., Takekura, H., Nishi, M., Kuno, J. & Minowa, O. (1994). Excitation-contraction uncoupling and muscular degeneration in mice lacking functional skeletal muscle ryanodine-receptor gene. *Nature* **369**, 556-559.

Takeshima, H., Ikemoto, T., Nishi, M., Nishiyama, N., Shimuta, M., Sugitani, Y., Kuno, J., Saito, I., Endo, M., Endo., M. & Noda, T. (1996). Generation and characterization of mutant mice lacking ryanodine receptor type 3. *Journal of Biological Chemistry* **271**, 19649-19652.

Takeshima, H., Yamazawa, T., Ikemoto, T., Takekura, H., Nishi, M., Noda, T. & Endo, M. (1995). Ca(2+)-induced Ca2+ release in myocytes from dyspedic mice lacking the type-1 ryanodine receptor. *Embryology Journal* **14**, 2999-3006.

Talo, A., Stern, M.D., Spurgeon, H.A., Isenberg, G. & Lakatta, E.G. (1990). Sustained subthreshold-for-twitch depolarization in rat single ventricular myocytes causes sustained calcium channel activation and sarcoplasmic reticulum calcium release. *Journal of General Physiology* **96**, 1085-103.

Tanaka, T. & Hidaka, H. (1981). Interaction of local anesthetics with calmodulin. *Biochemical and Biophysical Research Communications* **101**, 447-53.

Terracciano, C. M. N. & MacLeod, K. T. (1997). Measurements of Ca^{2+} entry and sarcoplasmic reticulum Ca^{2+} content during the cardiac cycle in guinea pig and rat ventricular myocytes. *Biophysical Journal* **72**, 1319-1326.

Terrar, D.A. & White, E. (1989). Mechanisms and significance of calcium entry at positive membrane potentials in guinea-pig ventricular muscle cells. *Quarterly Journal of Experimental Physiology* **74**, 121-139.

Tokumitsu, H., Chijiwa, T., Hagiwara, M., Mizutani, A., Terasawa, M. & Hidaka, H. (1990). KN-62, 1-[N,O-Bis(5-isoquinolinesulfonyl)-N-methyl-L-tyrosyl]-4-phenylpiperazine, a specific inhibitor of Ca^{2+} /calmodulin-dependent protein kinase II. *Journal of Biological Chemistry* **265**, 4315-4320.

Trautwein, W., McDonald, T.F. & Tripathi, O. (1975). Calcium conductance and tension in mammalian ventricular muscle. *Pflügers Archives* **354**, 55-74.

Tseng, G.-N. & Boyden, P.A. (1989). Multiple types of Ca^{2+} currents in single canine Purkinje cells. *Circulation Research* **65**, 1735-1750.

Tytgat, J., Vereecke, J. & Carmeliet, E. (1990). A combined study of sodium current and T-type calcium current in isolated cardiac cells. *Pflugers Archives* **417**, 142-8.

Valdeolmillos, M., O'Neill, S.C., Smith, G.L. & Eisner, D.A. (1989). Calcium-induced calcium release activates contraction in intact cardiac cells. *Pflügers Archives* **413**, 676-678.

Valdivia, H.H., Kaplan, J.H., Ellis-Davies, G.C.R. & Lederer, W.J. (1995). Rapid adaptation of cardiac ryanodine receptors: modulation by Mg^{2+} and phosphorylation. *Science* **267**, 1997-2000.

Vander, A.J., Sherman, J.H. & Luciano, D.S. (1994). Human Physiology. The Mechanisms of Body Function, sixth edition. (McGraw-Hill, Inc. USA).

Van Wagoner, D.R., Kirian, M., Arakaki, B & Lamorgese, M. (1999). Voltage sensitive calcium release in human atrial myocytes. *Biophysical Journal* **76**, A459.

Vassort, G. & Alvarez, J. (1994). Cardiac T-type calcium current: pharmacology and roles in cardiac tissues. *Journal of Cardiovascular Electrophysiology* **5**, 376-393.

Velez, P., Gyorke, S., Escobar, A.L., Vergara, J. & Fill, M. (1997). Adaptation of single cardiac ryanodine receptor channels. *Biophysical Journal* **72**, 691-697.

Volpe, P., Palade, P., Costello, B., Mitchell, R. D. & Fleischer, S. (1983). Spontaneous calcium release from sarcoplasmic reticulum. Effect of local anesthetics. *Journal of Biological Chemistry* **258**, 12434-12442.

Volpi, M., Sha'afi, R.I., Epstein, P.M., Andrenyak, D.M. & Feinstein, M.B. (1981). Local anesthetics, mepacrine, and propranolol are antagonists of calmodulin. *Procedings of the National Academy of Sciences USA* **78**, 795-9.

Vornanen, M., Shepherd, N. & Isenberg, G. (1994). Tension-voltage relations of single myocytes reflect Ca release triggered by Na/Ca exchange at 35 °C but not at 23 °C. *American Journal of Physiology* **267**, C623-C632.

Wagenknecht, T., Grassucci, R., Frank, J., Saito, A., Inui, M. & Fleischer, S. (1989). Three-dimensional architecture of the calcium channel/foot structure of sarcoplasmic reticulum. *Nature* **338**, 167-170.

Wang, S. Y., Winka, L. & Langer, G. A. (1993). Role of calcium current and sarcoplasmic reticulum calcium release in control of myocardial contraction in rat and rabbit myocytes. *Journal of Molecular and Cellular Cardiology* **25**, 1339-1347.

Wasserstrom, J.A. (1998). New evidence for similarities in excitation-contraction coupling in skeletal and cardiac muscle. *Acta Physiology Scandinavia* **162**, 247-252.

Wasserstrom, J.A. & Vites, A.-M. (1996). The role of Na-Ca^{2+} exchange in activation of excitation-contraction coupling in rat ventricular myocytes. *Journal of Physiology* **493**, 529-542.

Wier, W. G. (1991). Intracellular calcium during excitation-contraction coupling in mammalian ventricle. *Med. Sci. Sports and Exercise.* **23**, 1149-1156.

Wier, W.G., Terrance, M.E., López-López, J.R. & Balke, C.W. (1994). Local control of excitation-contraction coupling in rat heart cells. *Journal of Physiology* **474**, 463-471.

Wibo, M. & Godfraind, T. (1991). Stoichiometric ratio of calcium entry to calcium release channels in rat ventricle in relation to the mechanism of sarcoplasmic reticulum calcium release in cardiac tissue. (abstract) *Pflügers Archives* **419**, R13.

Wilson, R. A., Soei, L. K., Bezstarosti, K., Lamers, J. M. J. & Verdouw, P. D. (1993). Negative inotropy of lidocaine: possible biochemical mechanisms. *European Heart Journal* **14**, 2847-289.

Witcher, D.R., Kovacs, R.J., Schulman, H., Cefali, D.C. & Jones, L.R. (1991). Unique phosphorylation site on the cardiac ryanodine receptor regulates calcium channel activity. *The Journal of Biological Chemistry* **266**, 11144-11152.

Wohlfart, B. & Noble, M.I.M. (1982). The cardiac excitation-contraction cycle. *Pharmacology & Therapeutics* **16**, 1-43.

Wu, J. & Lipsius, S.L. (1990). Effects of extracellular Mg^{2+} on T- and L-type Ca^{2+} currents in single atrial myocytes. *American Journal of Physiology* **259**, H1842-H1850.

Wu, G., Jeyakumar, L., Barnett, J.V. & Fleischer, S. (1998). Immunolocalization of RyR3 in heart. *Biophysical Journal* **74**, A56.

Xu, X. & Best, P.M. (1992). Postnatal changes in T-type calcium current density in rat atrial myocytes. *Journal of Physiology* **454**, 657-672.

Xu, L., Jones, R. & Meissner, G. (1993). Effects of local anesthetics on single channel behavior of skeletal muscle calcium release channel. *Journal of General Physiology* **101**, 207-233.

Yasui, K., Palade, P. & Györke, S. (1994). Negative control mechanism with features of adaptation controls Ca^{2+} release in cardiac myocytes. *Biophysical Journal* **67**, 457-460.

Zahradnidova, A. & Palade, P. (1993). Procaine effects on single sarcoplasmic reticulum Ca^{2+} release channels. *Biophysical Journal* **64**, 991-1003.

Zhou, Z. & January, C.T. (1998). Both T- and L-type Ca^{2+} channels can contribute to excitation-contraction coupling in cardiac purkinje cells. *Biophysical Journal* **74**, 1830-1839.

Zhuo, Z. & Lipsius, S.L. (1994). T-type calcium current in latent pacemaker cells isolated from cat right atrium. *Journal of Molecular and Cellular Cardiology* **26**, 1211-1219.

Zhu, J.Q. & Ferrier, G.R. (1996). Steady-state inactivation of the voltage-sensitive release mechanism for cardiac contraction. *Journal of Molecular and Cellular Cardiology* **28**, A180.

Zhu, J-Q. & Ferrier, G.R. (1998). Activation of the voltage-sensitive release mechanism for contraction in isolated guinea-pig ventricular myocytes by Ca-calmodulin dependent protein kinase II. *Biophysical Journal* **74**, A54.

Zhu, J-Q. & Ferrier, G.R. (1999). Role of the voltage sensitive release mechanism in force-interval relations and staircases in cardiac ventricular myocytes. *Biophysical Journal* **76**, A458.

Zipes, D.P. & Jalife, J. (1990). Cardiac electrophysiology From cell to bedside. W.B Saunders Company, Harcourt Brace Jovanovich, Inc. USA.