INFORMATION TO USERS

This manuscript has been reproduced from the microfilm master. UMI films the text directly from the original or copy submitted. Thus, some thesis and dissertation copies are in typewriter face, while others may be from any type of computer printer.

The quality of this reproduction is dependent upon the quality of the copy submitted. Broken or indistinct print, colored or poor quality illustrations and photographs, print bleedthrough, substandard margins, and improper alignment can adversely affect reproduction.

In the unlikely event that the author did not send UMI a complete manuscript and there are missing pages, these will be noted. Also, if unauthorized copyright material had to be removed, a note will indicate the deletion.

Oversize materials (e.g., maps, drawings, charts) are reproduced by sectioning the original, beginning at the upper left-hand corner and continuing from left to right in equal sections with small overlaps. Each original is also photographed in one exposure and is included in reduced form at the back of the book.

Photographs included in the original manuscript have been reproduced xerographically in this copy. Higher quality 6" x 9" black and white photographic prints are available for any photographs or illustrations appearing in this copy for an additional charge. Contact UMI directly to order.

UMI

A Bell & Howell Information Company
300 North Zeeb Road, Ann Arbor MI 48106-1346 USA
313/761-4700 800/521-0600

PERIPHERAL MECHANISMS OF ADENOSINE AND 5-HYDROXYTRYPTAMINE CONTRIBUTING TO NOCICEPTION AND INFLAMMATION IN THE RAT FORMALIN MODEL

by

Greg J. Doak

Submitted in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

at

Dalhousie University

Halifax, Nova Scotia

July 2, 1997

DALHOUSIE UNIVERSITY

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 "Peripheral Mechanisms of Adenosine and 5-Hydroxytryptamine Contributing to Nociception and Inflammation in the Rat Formalin Model"

by Gregory James Doak

in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

Dated: July 2, 1997

External Examiner

Research Supervisor

Examining Committee

DALHOUSIE UNIVERSITY

DATE: July 2, 1997

AUTHOR: Greg J. Doak

TITLE: Peripheral mechanisms of adenosine and 5-hydroxytryptamine contributing to nociception and inflammation in the rat formalin model

DEPARTMENT: Pharmacology

DEGREE: PhD CONVOCATION: FALL YEAR: 1997

Permission is herewith granted to Dalhousie University to circulate and to have copied for non-commercial purposes, at its discretion, the above title upon the request of individuals or institutions.

Signature of Author

This thesis is dedicated to the memory of

Stuart T. Udovitch

mundus vult decipi et decipiatur

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABSTRACT

When applied peripherally, adenosine and 5-HT have been shown to be pronociceptive in a number of animal and human models. There are no data on possible interactions between adenosine and 5-HT in the context of inflammatory pain. It was hypothesized that nociceptor activation occurred through stimulation of adenosine and 5-HT receptors. The role of adenosine, 5-HT, and possible interactions between these substances was investigated using the rat formalin model. Injection of dilute formalin in the rat hindpaw produces an inflammatory, nociceptive response which is quantifiable and characterized by flinching and swelling of the affected hindpaw. Injection of 2.5% formalin produces a maximal inflammatory and nociceptive response, the mediators of which can be identified by using selective blockers. Injection of 0.5% formalin produces a low level inflammatory response but provides a background of the necessary co-mediators to which may be added putative pronociceptive substances. The role of *endogenously* released adenosine and 5-HT was assessed using subtype selective antagonist analogues of the respective mediators co-injected with 2.5% formalin, and observing for any block of the nociceptive response or paw swelling. The receptor subtypes involved were further defined by attempting to augment the response to 0.5% formalin by the *exogenous* co-injection of adenosine, 5-HT or their respective subtype selective agonist analogues. Interactions between adenosine and 5-HT were studied using co-injection of adenosine and 5-HT and combinations of antagonist analogues with 0.5% formalin. Stimulation of peripheral adenosine A_2 receptors produces hyperalgesia while peripheral A_1 receptor stimulation has an analgesic effect. These receptors appear to have no significant effect on edema formation. Multiple 5-HT receptors, including 5-HT_1, 5-HT_3, and 5-HT_4 receptors, are involved in generating the nociceptive response to formalin injection. 5-HT_1, 5-HT_2, and 5-HT_4 but not 5-HT_3 receptors contribute to edema formation but no single subtype selective 5-HT antagonist is effective at diminishing edema. Combining adenosine and 5-HT with 0.5% formalin revealed a greater than additive response and it appears that 5-HT may sensitize the nociceptor to the effects of adenosine. The data do not support the hypothesis of a peripheral nociceptive effect of 5-HT being mediated through adenosine release.

LIST OF ABBREVIATIONS

5,7-DHT	5,7-dihydroxytryptamine
5-CT	5-carboxamidotryptamine
5-HT	5-hydroxytryptamine, serotonin
5-MeOT	5-methoxytryptamine
8-OH-DPAT	8-hydroxy-2-(di-n-propylamino)tetralin
8-SPT	8-p-sulfophenyltheophylline
ADA	adenosine deaminase
ADN	adenosine
AMP	adenosine monophosphate
APNEA	N^6-2-(4-aminophenyl)ethyladenosine
ATP	adenosine triphosphate
BK	bradykinin
BW-A 522	3-(3-iodo-4-aminobenzyl)-8-(4-oxyacetate)-1-propylxanthine
Ca^{2+}	calcium ion
CGRP	calcitonin gene-related peptide
CGS15943A	9-chloro-2-(2-furanyl)-5,6-dihydro-[1,2,4]-triazolo[1,5]quinazolin-5-imine monomethanesulfonate
CGS21680	2-[p-(2-carbonyl-ethyl)-phenylethylamino]-5'-N-ethylcarboxamido adenosine hydrochloride
CHA	N^6-cyclohexyladenosine
CNS	central nervous system

CPBG	m-chlorophenylbiguanide
CP93129	3-(1,2,5,6,-tetrahydropyrid-4-yl)pyrrolo[3,2-b]pyrid-5-one
CPT	8-cyclopentyltheophylline
CPX	1,3-dipropyl-8-cyclopentylxanthine
CSC	8-(3-chlorostyryl)-caffeine
CV1808	2-phenylaminoadenosine
cAMP	cyclic adenosine monophosphate
CYT	cytokines
DMPX	1,3-dimethyl-7-propylxanthine
DMSO	dimethyl sulfoxide
DOI	1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane
GR113808A	[1-[2-(methylsulphonyl)amino]ethyl-4-piperidinyl]methyl 1-methyl-1H-indole-3-carboxylate
GR127935	N-[4-methoxy-3-(4-methyl-1-piperazinyl)phenyl]-2'-methyl-4'-(5-methyl-1,2,4-oxadiazol-3-yl)[1,1-biphenyl]-4-carboxamide
H^+	hydrogen ion
H_2O_2	hydrogen peroxides
HIST	histamine
ICV	intracerebroventricular
IP_3	inositol trisphoshate
K^+	potassium ion
K_i	binding affinity

KF17837	1,3-dipropyl-8-(3,4-dimethoxystyryl)-7-methylxanthine
L694247	2-[5-[3-(4-methylsulphonylamino)benzyl-1,2,4-oxadiazol-5-yl]-1H-indole-3-yl]ethylamine
MDL72222	1αH,3α,5αH-tropan-3-yl-3,5-dichlorobenzoate
mRNA	messenger ribonucleic acid
NBI	nitrobenzylthioinosine
NECA	5'-N-ethyl-carboxamidoadenosine
NO	nitric oxide
NRD	nucleus raphe dorsalis
NRM	nucleus raphe magnus
NSAIDs	nonsteroidal anti-inflammatory drugs
PG	prostaglandins
PGE_2	prostaglandin E_2
PGI_2	prostaglandin I_2
PLA_2	phospholipase A_2
R-PIA	N^6-(R-phenylisopropyl)-adenosine
RU24969	5-methoxy-3(1,2,3,6-tetrahydro-4-pyridinyl)-1H-indole
SB200646	N-(1-metyl-5-indolyl)-N-(3-pyridyl) urea
SDZ21009	4(3-terbutylamino-2-hydroxypropoxy)indol-2-carbonic acid-isopropylester
S.E.M.	standard error of the mean
SP	substance P

S-PIA	N^6-(S-phenylisopropyl)-adenosine
TNFα	tumor necrosis factor
WAY100135	N-tert-butyl-3-(4-[2-methoxyphenyl]piperazin-1-yl)-2-phenylpropanamide
XAC	xanthine amine congener

ACKNOWLEDGEMENTS

The author wishes to acknowledge his supervisor, Dr. Jana Sawynok, for her wisdom and guidance during the years of experimentation and preparation culminating in this thesis.

The author is grateful to the following groups and organizations for financial support during the preparation of this thesis:

Canadian Anaesthetists' Society

Dalhousie Department of Anaesthesia

Dalhousie Department of Pharmacology

Dalhousie Medical Research Foundation

Janssen Pharmaceutica

PUBLICATIONS

Portions of this thesis have been published elsewhere.

Papers:

Doak, G.J., and Sawynok, J.: Complex role of peripheral adenosine in the genesis of the response to subcutaneous formalin in the rat. Eur. J. Pharmacol. 281: 311-318, 1995.

Doak, G.J., and Sawynok, J.: Formalin-induced nociceptive behavior and edema: involvement of multiple peripheral 5-hydroxytryptamine receptor subtypes. Neuroscience (in press), 1997.

Abstracts:

Doak, G.J., and Sawynok, J: Peripheral 5-HT receptors mediating the flinching response in the rat hindpaw formalin test. Can. J. Anaesth. 42: A36-B, 1995.

Sawynok, J., Reid, A., and **Doak, G.**: Analgesic actions of caffeine in the rat hot plate and formalin tests. 5th International Symposium on Adenosine and Adenosine nucleotides, Haverton, PA. Drug Dev. Res. 31: 314, 1994.

INTRODUCTION

I. Inflammation and nociception

Acute pain of clinical significance is very often associated with some degree of inflammation. From the pain associated with sunburn to that following major surgery, there is some element of associated inflammation. The efficacy of anti-inflammatory agents as analgesics underlines the importance of that inflammatory response in contributing to hyperalgesia (reviewed, Cashman and McAnulty 1995). The acute inflammatory response has been extensively studied and a number of cellular and chemical participants have been identified (reviewed, Treede *et al.* 1992; Dray 1995). These participants in the inflammatory response may arise from a number of different sources including: a) damaged or disrupted cells; b) plasma; c) immune cells, platelets and mast cells; and d) sensory afferent and sympathetic nerve fibres (Fig. 1). Chemical sensitivity is an important characteristic of nociceptive neurons. The relative importance of these cellular and chemical mediators, and interactions among them, in producing a nociceptive signal is less well defined.

Inflammatory mediators from damaged cells

Tissue injury results in ischemia and cell damage and the subsequent release of nonspecific cellular components. These breakdown products and subsequent inflammation can have both direct and indirect effects on primary afferent nociceptors. Elements that directly interact with the nociceptive afferent (*e.g.* hydrogen ions) may activate the neuron resulting in a nociceptive signal (algesia). Others may have direct interaction with the

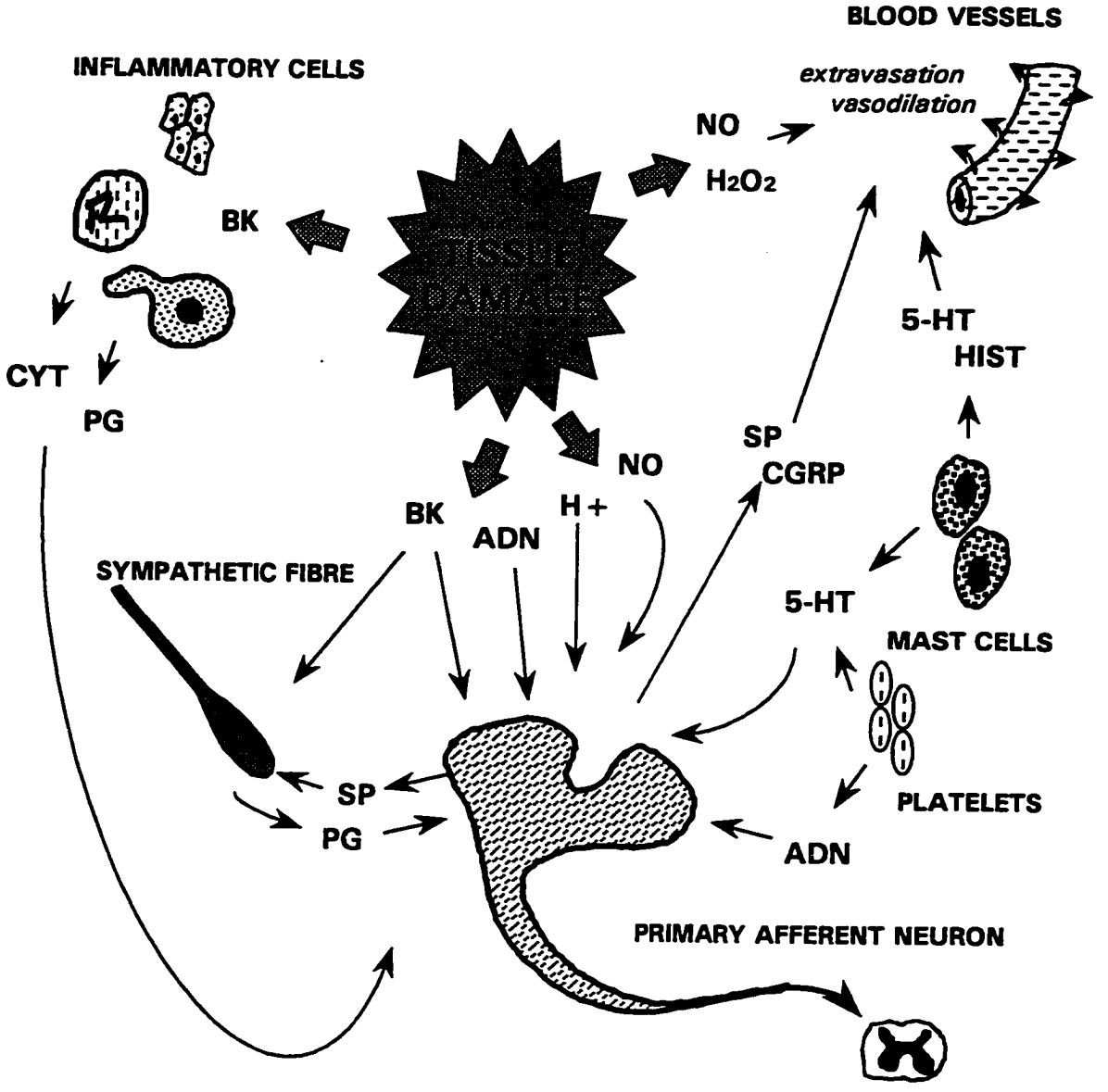


Figure 1. The inflammatory cascade showing interactions between neuronal, cellular, and humoral mediators (see text for details). ADN, adenosine; BK, bradykinin; CGRP, calcitonin gene-related peptide; H^+, hydrogen ion; 5-HT, 5-hydroxytryptamine; H_2O_2, hydrogen peroxides; HIST, histamine; CYT, cytokines; NO, nitric oxide; PG, prostaglandins; SP, substance P.

nociceptor to reduce the threshold for activation (*e.g.* adenosine), thereby sensitizing it to the effects of other mediators (hyperalgesia). Indirectly acting agents exert their effects through the release of other intermediators which in turn may directly activate or sensitize the nociceptor (*e.g.* bradykinin induced release of prostaglandins).

Elevated hydrogen ion concentrations are associated with inflamed and ischemic tissue (reviewed, Steen *et al.* 1995). Recently, it has been demonstrated that acid pH plays a major role in excitation and sensitization of the afferent nociceptor (Steen *et al.* 1992; Bevan and Geppetti 1994; Steen *et al.* 1995). Low pH stimulates sensory nerve terminals to release neuropeptides like calcitonin gene-related peptide (CGRP) and substance P (SP) through a Ca^{2+} dependent process (Bevan and Geppetti 1994). Furthermore, it seems likely that this excitation/stimulation occurs through some interaction with the capsaicin-activated cation channel (Bevan and Geppetti 1994; Fox *et al.* 1995).

Reactive molecules, such as hydrogen peroxide and nitric oxide (NO) are abundant under conditions of ischemia-reperfusion and inflammation and they may interact with other inflammatory mediators to enhance the inflammatory response. Rather than producing direct nociceptor activation, the more likely effect of hydrogen peroxide is to amplify the effects of other inflammatory mediators such as bradykinin and prostaglandin E_2 (Dray 1995). NO is a diffusible free radical molecule released from vascular endothelial cells, polymorphonuclear leucocytes and activated macrophages. Its release may be stimulated by a number of inflammatory mediators, including bradykinin, histamine, 5-hydroxytryptamine (5-HT), cytokines, and leukotrienes (reviewed, Ialenti *et al.* 1992). NO has been suggested to be an important link in chemical induced nociception

(Haley *et al.* 1992) and intracutaneous injection evokes burning pain in human volunteers, suggesting a direct activation of nociceptors (Holthusen and Arndt 1994). NO modulates edema formation during inflammation, possibly through changes in vascular permeability and regional blood flow (Ialenti *et al.* 1992).

Adenine nucleotides are ubiquitous components of cells and therefore likely to be released in large amounts during conditions of ischemia or cell damage. Extracellular adenosine, which is derived from the breakdown of adenosine triphosphate (ATP), has been shown to be abundant under conditions of ischemia and inflammation (Matherne *et al.* 1990; Cronstein 1994). Adenosine (Taiwo and Levine 1990; Karlsten *et al.* 1992) and ATP (Bleehen and Keele 1977) produce hyperalgesia when applied exogenously.

Inflammatory mediators derived from plasma

Increased blood flow, vasodilation and altered capillary permeability contribute to plasma extravasation (with its associated humoral mediators such as the kinins) and entry into the inflamed region of increased numbers of inflammatory cells such as polymorphonuclear leucocytes, monocytes, and platelets. One of the major players in the inflammatory process is bradykinin, formed from the enzymatic degradation of kininogen precursors (reviewed, Dray and Perkins 1993). Once formed, bradykinin may act directly on nociceptive afferents via bradykinin B2 receptors to sensitize or excite the nerve ending (Dray 1995) or it may interact with endothelium, immune cells, mast cells, and sympathetic neurons resulting in further enhancement of the inflammatory process (Dray and Perkins 1993). The bradykinin B1 receptor appears to become involved under

conditions of inflammation and may act indirectly to facilitate the release of other mediators such as prostaglandins.

Inflammatory mediators derived from inflammatory cells

Associated with the inflammatory process is the accumulation and/or activation of a number of cell types capable of releasing inflammatory mediators. Activated platelets can sensitize and excite nociceptors, possibly through the release of 5-HT, histamine, and adenine nucleotides (Ringkamp *et al.* 1994). Degranulation of mast cells results in further release of 5-HT (in rodents) as well as histamine (reviewed, Dray 1995). 5-HT is associated with direct nociceptor excitation (Beck and Handwerker 1974; Richardson *et al.* 1985) and sensitization (Guilbaud *et al.* 1989; Rueff and Dray 1992). Histamine may promote further vasodilation and plasma extravasation through a direct action on postcapillary venules, but it also excites small diameter afferent nerve terminals to evoke the release of vasoactive neuropeptides such as CGRP (Amann *et al.* 1995). Intracutaneous injection of low doses of histamine elicits itching, while higher doses produce pain (Simone *et al.* 1991). How important histamine is, in the development of pain under conditions of tissue injury and inflammation, is not clear.

Polymorphonuclear leucocytes are a source of prostanoids which are released under conditions of inflammation and contribute to hyperalgesia. Prostaglandins may directly activate nociceptors, but more often the result is sensitization (Birrell *et al.* 1991; Levine *et al.* 1993). Leukotrienes and dihydroxyeicosatetraenoic acid are also released from polymorphonuclear leucocytes and can contribute to hyperalgesia through nociceptor

sensitization (Levine *et al.* 1993). Finally, a number of cytokines, including the interleukins and tumor necrosis factor (TNFα), may be released from immune cells, resulting in marked hyperalgesia (Dray 1995).

Inflammatory mediators from sensory afferent and sympathetic nerve terminals

In addition to their afferent function, peripheral nerves are capable of releasing neuropeptides following activation of an axo-axonic loop. Neurokinins (SP, neurokinin A) released from primary afferent nerve terminals following noxious stimulation, promote a number of proinflammatory effects, including vasodilation and plasma extravasation, chemotaxis and activation of inflammatory cells, and release of prostaglandins and cytokines (reviewed, Levine *et al.* 1993). CGRP evokes arteriolar vasodilation, potentiating the edema formation initiated by other mediators such as SP, 5-HT and histamine (Dray and Bevan 1993). The sympathetic nervous system appears to contribute little to the generation of pain in normal tissue. However, activation of sympathetic fibres under conditions of inflammation may result in a number of pronociceptive and proinflammatory effects. Sympathetically mediated alterations in blood flow contribute to plasma extravasation which may augment inflammation (Dray *et al.* 1994). Under conditions of inflammation, norepinephrine, released from sympathetic nerve terminals and acting via α_2-adrenergic receptors, may augment the nociceptive effects of other inflammatory mediators (Sato *et al.* 1993). In addition, norepinephrine may stimulate release of prostaglandins from sympathetic postganglionic nerve terminals (Levine *et al.* 1986; Raja 1995).

Interactions among inflammatory mediators

With so many inflammatory mediators involved, it has been proposed that there may be significant interactions among them. This has given rise to the concept of an "inflammatory soup" (Handwerker and Reeh 1991) with all the essential ingredients contributing to the overall nocieptive response (Fig. 1). The ingredients of this "inflammatory soup" may contribute to hyperalgesia directly, through interaction with receptors on the primary afferent, or indirectly, through interaction with other cell types, to facilitate release of other mediators or enhance plasma extravasation. For example, via stimulation of B2 receptors, bradykinin may directly activate the primary afferent nerve terminal. However, it also has multiple indirect actions through its effects on plasma extravasation and B1 receptor-mediated promotion of prostaglandin synthesis by inflammatory cells (Dray 1995). These released prostaglandins, such as PGE_2 and PGI_2, may sensitize the nociceptor to the effects of bradykinin (Dray and Bevan 1993) or 5-HT (Hong and Abbott 1994).

Directly acting mediators may be excitatory or may sensitize the neuron, lowering its threshold for stimulation from another, direct acting agent. This may be agent specific, such as with prostaglandins, which usually sensitize sensory neurons (Levine *et al.* 1993), or as is the case with 5-HT, it may be concentration-related. At higher concentrations of 5-HT, a direct nociceptive effect can be observed. Low concentrations of 5-HT produce no behavioural response, but sensitize the afferent nerve terminal to the excitatory effects of other agents. Thus, in the presence of concentrations of 5-HT having no intrinsic algogenic effect, the addition of other agents, that by themselves are incapable of inducing

a pain response (*e.g.* SP, norepinephrine, histamine), results in nociceptive behaviour (Hong and Abbott 1994). 5-HT receptors coupled to cyclic AMP formation may be responsible for lowering nociceptor thresholds, resulting in hyperalgesia or sensitization (Taiwo and Levine 1992; Taiwo *et al.* 1992). Conversely, through stimulation of 5-HT_3 receptors and subsequent activation of the cation channel, 5-HT can directly activate nociceptors (Richardson *et al.* 1985).

It is important to appreciate that not all inflammatory mediators are exclusively pro-inflammatory. For example, 5-HT_1-like receptor activation may inhibit the release of sensory neuropeptides (Buzzi *et al.* 1991), a possible mechanism of the anti-migraine effects of the 5-HT agonist, sumatripan (Humphrey and Feniuk 1991). Adenosine, acting through A_1 receptor stimulation, produces analgesia (Taiwo and Levine 1990; Karlsten *et al.* 1992) as well as anti-inflammatory effects (Cronstein 1994).

With such a large number of players in the inflammatory process, the potential for interactions is great. It is beyond the scope of this thesis to outline all the known or theorized ways in which the different mediators combine to initiate pain and inflammation. Rather, the intention is to focus on two specific mediators, adenosine and 5-HT, and attempt to clarify their role in the nociceptive process and to explore possible interactions between them.

II. Role of adenosine in inflammation and nociception

To appreciate the potential for adenosine to interact in the process of inflammation and nociception, it is necessary to have an understanding of the pharmacology of adenosine and adenosine receptors.

Pharmacology of adenosine and adenosine receptors

Burnstock (1978) proposed two purinergic receptors, P_1 and P_2, based on four criteria: 1) differential affinities for adenosine and ATP; 2) differential sensitivity to antagonism by methylxanthines; 3) distinct transduction mechanisms; and 4) differing effects on the induction of prostaglandin synthesis. The P_1 receptor corresponded to the putative adenosine receptor, having affinity for adenosine > ATP, being sensitive to antagonism by methylxanthines, modulating adenylate cyclase, and was not associated with prostaglandin synthesis. Since that time, the adenosine receptors have been further characterized and subclassified on the basis of differential binding of selective ligands, affinity states (high vs low), transduction mechanisms, and molecular structure (Collis and Hourani 1993; Dalziel and Westfall 1994; Fredholm *et al.* 1994). The currently accepted classification is summarized in Table I.

Besides differing affinity profiles to the selective adenosine receptor ligands (Table I), the adenosine A_1 receptor is distinguished from the A_2 receptor by its ability to inhibit adenylate cyclase (Van Calker *et al.* 1979). The A_1 receptor appears to be coupled to the G_i/G_o family of G-proteins (Freissmuth *et al.* 1991; Munshi *et al.* 1991) and it is now

Table I. Adenosine receptors: classification, characteristics, and ligands (Gustafsson *et al.* 1990; Dalziel and Westfall 1994; Fredholm *et al.* 1994).

Nomenclature	Agonists	Antagonists	Transduction mechanism
A_{1A} (high affinity) A_{1B} (low affinity)	CHA= R-PIA ≥ NECA > S-PIA	CPX, CPT, 8-SPT	G_i ∘ ↓cAMP ↑IP_3 ↑K^+ ↓Ca^{2+}
A_{2A}	CGS21680 = NECA > R-PIA ≥ S-PIA	XAC, KF17837, CSC, DMPX	G_s ∘ ↑cAMP
A_{2B}	NECA > CGS21680	XAC, CPX	G_s ∘ ↑cAMP
A_3	APNEA > NECA	BW-A 522	G? ∘ ↓cAMP
A_4	CV1808	CGS15943A	? K^+ channel

Abbreviations: APNEA, N^6-2-(4-aminophenyl)ethyladenosine; BW-A 522, 3-(3-iodo-4-aminobenzyl)-8-(4-oxyacetate)-1-propylxanthine; Ca^{2+}, calcium ion; cAMP, cyclic adenosine monophosphate; CGS15943A, 9-chloro-2-(2-furanyl)-5,6-dihydro-[1,2,4]-triazolo[1,5]quinazolin-5-imine monomethanesulfonate; CGS21680, 2-[p-(2-carbonyl-ethyl)-phenylethylamino]-5'-N-ethylcarboxamido-adenosine; CHA, N^6-cyclohexyladenosine; CPT, 8-cyclopentyltheophylline; CPX, 1,3-dipropyl-8-cyclopentylxanthine; CSC, 8-(3-chlorostyryl)-caffeine; CV1808, 2-phenylaminoadenosine; DMPX, 1,3-dimethyl-7-proplxanthine; IP_3, inositol trisphoshate; K^+, potassium ion; KF17837, 1,3-dipropyl-8-(3,4-dimethoxystyryl)-7-methylxanthine; NECA, 5'-N-ethyl-carboxamidoadenosine; R-PIA, N^6-(R-phenylisopropyl)-adenosine; S-PIA, N^6-(S-phenylisopropyl)-adenosine; 8-SPT, 8-p-sulfophenyltheophylline; XAC, xanthine amine congener

clear that, in addition to decreasing cyclic AMP, it may be associated with a range of cellular responses including inhibition of Ca^{2+} conductance (Scholz and Miller 1991), increased K^+ conductance (Trussel and Jackson 1985), and stimulation of phospholipase C (Gerwins and Fredholm 1992). The distinction between adenosine A_1 and A_2 receptor subtypes has been further established with the cloning of both receptors (reviewed, Collis and Hourani 1993). Functionally, the A_1 receptor has been proposed to mediate cardiac depression, vasoconstriction, bronchoconstriction, inhibition of renin secretion, inhibition of lipolysis, and inhibition of neurotransmitter release (Collis and Hourani 1993). It has been suggested that A_1 receptor stimulation may also mediate peripheral analgesia (Karlsten *et al.* 1992). Subclassification of the A_1 receptor has been proposed on the basis of high (A_{1A}) and low (A_{1B}) affinity states (Gustafsson *et al.* 1990). The A_{1A} receptor is proposed to be centrally located while the A_{1B} receptor is found in the periphery.

The adenosine A_2 receptor is positively coupled to adenylate cyclase through a G_s protein. It has been further subclassified into A_{2A} and A_{2B} receptors on the basis of differential affinity for 5'-N-ethyl-carboxamidoadenosine (NECA) and 2-[p-(2-carbonyl-ethyl)-phenylethylamino]-5'-N-ethylcarboxamido-adenosine (CGS21680) (Table I). The A_2 receptor is widely distributed in neuronal and non-neuronal tissue. Physiological effects attributed to A_2 receptor stimulation include decreased locomotion, platelet inhibition, bronchodilation, gluconeogenesis, and inhibition of immune cell function (Collis and Hourani 1993). A pronociceptive effect of A_2 receptor stimulation in the periphery has also been proposed (Karlsten *et al.* 1992).

Less well defined are the adenosine A_3 and A_4 receptor subtypes. The A_3 receptor was first cloned and later characterized (Zhou *et al.* 1992). The A_3 receptor appears to be negatively coupled to adenylate cyclase through a G-protein but may also have effects on IP_3 and Ca^{2+} influx. It is distinguished by its insensitivity to the usual methylxanthine antagonists. Data on the distribution of the A_3 receptor is incomplete but there is evidence of its association with immune and inflammatory cells (Ramkumar *et al.* 1993; Van Schaik *et al.* 1996). The novel adenosine A_4 receptor is distinguished by a high affinity to the agonist 2-phenylaminoadenosine (CV1808). Having been isolated from the rat brain, the A_4 receptor appears to increase K^+ currents through a mechanism not involving a G-protein (Cornfield *et al.* 1992).

Adenosine and nociception

Within the central nervous system (CNS), adenosine and related synthetic analogues exert significant antinociceptive effects which are blocked by methylxanthines, suggesting an adenosine receptor mediated phenomenon (reviewed, Sawynok 1997). There is considerable evidence linking adenosine to nociceptive processing within the CNS.

Immunoreactivity to conjugates of an adenosine derivative is a marker for cells containing high concentrations of adenosine. Immunocytochemistry has been used to identify areas of neural tissue exhibiting high concentrations of adenosine. Although this is not evidence of adenosine receptors, this has provided indirect evidence for a correlation between high concentrations of adenosine and neural tissue known to be associated with nociceptive processing such as the thalamus, central gray region and medulla, and the

substantia gelatinosa in the dorsal spinal cord (Braas *et al.* 1986). Nitrobenzylthioinosine (NBI) binds to the bidirectional nucleoside transporter, a necessary component in the regulation of extracellular adenosine activity. Significant binding of [^{3}H]NBI has been identified in those areas of the brain (Geiger and Nagy 1984) and spinal cord (Geiger and Nagy 1985) involved in nociceptive processing. Adenosine deaminase (ADA), a cytoplasmic enzyme responsible for the breakdown of adenosine to inosine, can be identified using immunohistochemistry and this provides a useful marker for areas of high adenosine turnover. Although ADA activity is widespread within the brain and spinal cord, its presence in those areas known to be involved in pain processing supports the possibility of adenosine playing a role in nociception. Identification of adenosine receptors throughout the brain and spinal cord using autoradiographic and binding techniques has provided further evidence of the link between adenosine and pain.

Despite the anatomical evidence of adenosine activity in those areas of the brain associated with pain processing, behavioural data suggests a limited role for brain adenosine in modulation of pain. Intracerebroventricular (ICV) administration of adenosine and its analogues produces an antinociceptive effect in mice as measured in the hot plate (Yarbrough and McGuffin-Clineschmidt 1981) and acetic acid writhing tests (Herrick-Davis *et al.* 1989). Interpretation of these data is complicated by the fact that significant sedative and motor effects were observed at comparable doses. Furthermore, antinociceptive effects from ICV administration of a number of adenosine analogues were not seen using the tail flick test in either rats (Mantegazza *et al.* 1984; Holmgren *et al.* 1986) or mice (Yarbrough and McGuffin-Clineschmidt 1981).

Antinociception following systemic administration of adenosine agonists appears to be mediated mostly through spinal cord adenosine receptors (Holmgren *et al.* 1986). The spinal administration of a number of adenosine receptor agonists produces antinociception in a variety of animal models of pain and these effects would appear to be mediated largely via adenosine A_1 receptor stimulation (reviewed, Sawynok 1997). As previously alluded to, a number of anatomical techniques have provided indirect evidence for adenosine involvement in the dorsal horn (see above). In particular, the highest concentrations of adenosine A_1 and A_2 receptors are found in the substantia gelatinosa (Choca *et al.* 1987). The majority of these receptors appear to be located postsynaptically, likely on spinal interneurons, since their numbers are not substantially altered by lesions of the primary afferents or descending spinal tracts (Choca *et al.* 1988; Geiger *et al.* 1984). Consistent with this view is the inhibitory effect of intrathecal adenosine analogues on the biting-licking-scratching syndrome induced by intrathecal SP or N-methyl-D-aspartate activation of postsynaptic convergent neurons (Doi *et al.* 1987; Delander and Wahl 1988). Similarly, *in vivo* electrophysiological activation of dorsal horn neurons by L-glutamate is inhibited by adenosine monophosphate (AMP); this inhibition is methylxanthine sensitive, indicating an inhibitory effect mediated through adenosine receptors (Salter and Henry 1985).

Direct evidence of presynaptic adenosine receptors is lacking. Electrophysiological data from cultured dorsal root ganglion cells suggests adenosine may exert presynaptic inhibitory effects (Dolphin *et al.* 1986; MacDonald *et al.* 1986), and electrical field stimulated release of CGRP from capsaicin-sensitive afferent terminals in the spinal cord

is inhibited by activation of adenosine A_1 receptors (Santicioli *et al.* 1993). Presumably such inhibitory effects could translate into an antinociceptive action. Alternatively, there is evidence that primary afferent nerves may be stimulated to release adenosine (Sweeney *et al.* 1987; Sweeney *et al.* 1990). Release of adenosine or co-release of adenosine precursors with excitatory neurotransmitters may serve a neuromodulatory function. Besides having direct antinociceptive effects at the spinal level, adenosine may mediate a component of the analgesic effects of spinal opioids and 5-HT (reviewed, Sawynok 1997).

While CNS adenosine receptors mediate analgesia, manifested largely through activation of spinal receptors, the activation of peripheral adenosine receptors has an algesic or pronociceptive effect. When administered by the intravenous (Sylvén *et al.* 1986, 1988a) or intracoronary route (Lagerqvist *et al.* 1990) in human subjects, adenosine is capable of producing chest pain characteristic of angina pectoris. Similar ischemic type pain occurs in the forearm with injection of adenosine into the brachial artery (Sylvén *et al.* 1988b). Direct application of adenosine to the human blister base produces pain (Bleehen and Keele 1977). These pronociceptive actions of adenosine are blocked by methylxanthines, suggesting an effect mediated by adenosine receptors.

Evidence has emerged for a peripheral hyperalgesic effect of adenosine in two animal models of pain. Taiwo and Levine (1990) have shown a hyperalgesic effect of adenosine in the rat paw-withdrawal pressure threshold reflex. A direct action on the primary afferent nociceptor through an adenosine A_2 receptor was implicated in this action. In mice, the first phase response to injection of dilute formalin into the paw has been shown to be augmented by adenosine A_2 receptor activation (Karlsten *et al.* 1992). The

first phase of the formalin test is generally thought to be the result of direct nociceptor activation (see Section V, below). These data suggest a role for adenosine in the development of hyperalgesia associated with phasic pain paradigms and a direct enhancement of chemically induced pain.

Adenosine receptors are present on inflammatory cells and adenosine appears to play a significant role during the inflammatory process (Cronstein 1994). Tissue adenosine levels have been shown to be enhanced under conditions of ischemia and inflammation (Matherne *et al.* 1990; Cronstein 1994). In view of the inferred presence of adenosine receptors on sensory neurons in mediating hyperalgesia and pronociception (see above), endogenous adenosine may well play a role in the genesis of inflammatory pain.

III. Role of 5-hydroxytryptamine in inflammation and nociception

5-HT is widely distributed in neural tissue and is involved in a variety of physiological events including modulation of smooth muscle tone, neuroendocrine function, and a host of neurological functions (reviewed, Zifa and Fillion 1992; Saxena 1995). To be able to appreciate the role of 5-HT within the context of inflammation and nociception, a brief overview of the pharmacology of 5-HT and 5-HT receptors is necessary.

Pharmacology of 5-hydroxytryptamine and 5-hydroxytryptamine receptors

With the development of more selective ligands, radioligand-labelling, and molecular biology techniques, the 5-HT receptor classification system has become increasingly complex. The most recent classification (Hoyer *et al.* 1994) incorporates data from ligand specificity, second messenger systems, and amino acid homology (Table II).

The 5-HT_1 receptors are, for the most part, well characterized. All have seven transmembrane domains and are negatively linked to adenylate cyclase through a G-protein. Most of the 5-HT_1 subtypes are located in the CNS or on peripheral nerves (5-HT_{1B}) where they have an inhibitory function. An exception is the less well characterized 5-HT_1-like subtype which is found on intracranial vasculature and mediates vasoconstriction. The distribution and function of the 5-HT_{1B} receptor in rodents mirrors that of the 5-HT_{1D} receptor in other species. Since 5-HT_{1B} receptor binding sites have not been identified in non-rodent species, it appears that the 5-HT_{1B} receptor is the rodent homologue of the 5-HT_{1D} receptor found in other species.

The 5-HT_{1C} receptor is structurally homologous to the 5-HT_2 receptors and shares other similarities, hence its renaming as 5-HT_{2C} (Hoyer *et al.* 1994). The classical 5-HT_2 receptor becomes the 5-HT_{2A}. Structurally similar, the 5-HT_2 receptors operate through stimulation of phospholipase C activity. Functionally, the 5-HT_2 class is more diverse. The 5-HT_{2A} receptor mediates smooth muscle contraction, platelet aggregation, increased capillary permeability, neuronal depolarization, and possibly some behavioural and neuroendocrine effects. The 5-HT_{2B} receptor appears to be limited to the rat fundus

Table II. 5-Hydroxytryptamine receptors: classification, characteristics, and ligands (Zifa and Fillion 1992; Hoyer *et al.* 1994).

Nomenclature	Agonists	Antagonists	Transduction mechanism
5-HT_{1A}	5-CT , 8-OH-DPAT, buspirone	WAY100135, methiothepin, propranaolol	$G_i \circ \downarrow cAMP$ $\uparrow IP_3$ $\uparrow K^+$? $G_s \circ \uparrow cAMP$
5-HT_{1B}	RU24969, 5-CT, CP93129	SDZ21009, methiothepin, propranolol	G? $\circ \downarrow cAMP$
5-HT_{1D}	sumatripan, L694247, 5-CT	GR127935, methiothepin	G? $\circ \downarrow cAMP$
5-HT_{1E}	5-HT	methiothepin	G? $\circ \downarrow cAMP$
5-HT_{1F}	5-HT	methiothepin	G? $\circ \downarrow cAMP$
5-HT_1 -like	sumatripan, 5-CT	methiothepin	? $\uparrow cAMP$
5-HT_{2A}	DOI, α-methyl-5-HT	pireperone, ketanserin, cinanserin	G? $\circ \uparrow IP_3$ $\uparrow PLA_2$
5-HT_{2B}	DOI, α-methyl-5-HT	SB200646	G? $\circ \uparrow IP_3$
5-HT_{2C}	DOI, α-methyl-5-HT	mesulergine, methysergide, ketanserin	G? $\circ \uparrow IP_3$
5-HT_3	2-methyl-5-HT, m-chlorophenylbiguanide	tropisetron, MDL72222, ondansetron	cation channel
5-HT_4	Cisapride, 5-MeOT	GR113808A, tropisetron	G? $\circ \uparrow cAMP$ $\downarrow K^+$
5-HT_5	5-HT	methiothepin	?
5-HT_6	5-HT	methiothepin	G? $\circ \uparrow cAMP$
5-HT_7	5-HT	methiothepin	G? $\circ \uparrow cAMP$

Table II. (continued)

Abbreviations: 5-CT, 5-carboxamidotryptamine; 5-HT, 5-hydroxytryptamine (serotonin); 5-MeOT, 5-methoxytryptamine; 8-OH-DPAT, 8-hydroxy-2-(di-n-propylamino)tetralin; CP93129, 3-(1,2,5,6,-tetrahydropyrid-4-yl)pyrrolo[3,2-b]pyrid-5-one; DOI, 1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane; GR113808A, [1-[2-(methylsulphonyl)amino]ethyl-4-piperidinyl]methyl 1-methyl-1H-indole-3-carboxylate; GR127935, N-[4-methoxy-3-(4-methyl-1-piperazinyl)phenyl]-2'-methyl-4'-(5-methyl-1,2,4-oxadiazol-3-yl)[1,1-biphenyl]-4-carboxamide; L694247, 2-[5-[3-(4-methylsulphonylamino)benzyl-1,2,4-oxadiazol-5-yl]-1H-indole-3-yl]ethylamine; MDL72222, 1αH,3α,5αH-tropan-3-yl-3,5-dichlorobenzoate; PLA_2, phospholipase A_2; RU24969, 5-methoxy-3(1,2,3,6-tetrahydro-4-pyridinyl)-1H-indole; SB200646, N-(1-metyl-5-indolyl)-N-(3-pyridyl) urea; SDZ21009, 4(3-terbutylamino-2-hydroxypropoxy)indol-2-carbonic acid-isopropylester; WAY100135, N-tert-butyl-3-(4-[2-methoxyphenyl]piperazin-1-yl)-2-phenylpropanamide

where it stimulates smooth muscle contraction. The 5-HT_{2C} receptor is found throughout the CNS and is highly concentrated in the choroid plexus.

The 5-HT_3 receptor is exclusively neuronal, being found on peripheral nerves as well as within the CNS. The receptor is linked to a cation channel whose activation triggers a rapid depolarization and subsequent increase in cytosolic Ca^{2+}. Functionally, 5-HT_3 receptors mediate peripheral nociception (see below), control of gastrointestinal tone, and a number of animal behaviours.

The 5-HT_4 receptor has only recently been characterized (reviewed, Ford and Clarke 1993). It is widely distributed in neuronal tissue and is also found in gut, bladder and vascular tissue. The stimulatory action of 5-HT_4 receptor activation is mediated through a G-protein dependent activation of adenylate cyclase. A direct G-protein coupling to mediate closure of K^+ channels has also been suggested (Ford and Clarke 1993). Within the CNS, 5-HT_4 receptor activation mediates depolarization but the functional correlates are not well described. In the gut, the 5-HT_4 receptor appears to modulate motility and secretory activity. Atrial 5-HT_4 receptors mediate tachycardia and a positive inotropic effect. To date, no peripheral nociceptive effect has been ascribed to 5-HT_4 receptor activation.

The remaining classes of 5-HT receptors (Table II) are cloned receptors that are provisionally classified as 5-HT_5, 5-HT_6, and 5-HT_7. They are only just beginning to be characterized since selective ligands are as yet not available. All have been located in the CNS and at least two (5-HT_6 and 5-HT_7) are positively coupled to adenyl cyclase. To date, none have been linked to any nociceptive function, however, mRNA for 5-HT_6 and

5-HT_7 receptors have been identified in rat peripheral sensory and sympathetic ganglia (Pierce *et al.* 1996).

5-hydroxytryptamine and nociception

Since the proposal of a descending pain modulatory system containing 5-HT, investigations into the role of 5-HT in nociceptive transmission have focused on nerve fibres originating in the various nuclei of the brainstem. 5-HT projections originate in discrete cell groups within the nulcei of the brainstem and project rostrally as well as to the spinal cord. 5-HT immunoreactive neurons have been identified in the rostroventromedial medulla and caudal pons, mainly in the nucleus raphe magnus (NRM), nucleus raphe dorsalis (NRD), nucleus gigantocellularis and nucleus paragigantocellularis (Kwiat and Basbaum 1992). The NRD, in particular, has rostral serotonergic projections to the parafascicular nucleus, medial thalamus, amygdala, nucleus accumbens, and cerebral cortex (Matsuzaki *et al.* 1993; Kazakov *et al.* 1993; Wang and Nakai 1994), which may be involved in nociceptive processing. However, although many of the serotonergic neurons of these nuclei project rostrally, almost 90% can be labelled retrogradely from the spinal cord (Bowker and Abbott 1990).

The importance of the descending serotonergic pathways in modifying nociceptive input is now well established (reviewed, Cesselin *et al.* 1994; Stamford 1995). Functional blockade of these raphe-spinal serotonergic tracts using inhibitors of 5-HT synthesis (eg. *p*-chlorophenylalanine), electrolytic lesions of the NRM, or serotonergic neurotoxins (eg. 5,7-dihydroxytryptamine, 5,7-DHT) abolishes analgesia induced by stimulation of higher

centres (Sawynok 1991; Stamford 1995). It is likely that much of the analgesic effect of centrally applied opioids is derived through subsequent activation of descending serotonergic pathways, as evidenced by the increased release and metabolism of spinal 5-HT in the spinal cord (Rivot *et al.* 1984). Lesions of the descending serotonergic pathways significantly reduce the analgesic effects of systemic and supraspinal morphine (Sawynok 1991).

Using anti-5-HT antibody techniques, serotonergic fibres and nerve endings have been identified in the dorsal horn, with particularly high densities in the superficial laminae (Ruda *et al.* 1986). It is likely that most, if not all of the serotonergic terminals in the dorsal horn have a supraspinal origin since transection of the thoracic cord completely eliminates 5-HT immunoreactivity in the more distal segments (Ruda *et al.* 1986). Autoradiographic and immunohistochemical studies have provided much information on the location and relative densities of 5-HT receptor types throughout the spinal cord (reviewed, Cesselin *et al.* 1994). 5-HT_{1A}, 5-HT_{1B}, and 5-HT_3 receptor binding sites are especially concentrated in the superficial laminae of the dorsal horn while 5-HT_{2A} sites are of very low density (Cesselin *et al.* 1994). There is little data on 5-HT_4 binding sites. A significant proportion of 5-HT binding sites in the spinal cord belong to the yet to be characterized "5-HT_{1S}" receptor (Huang and Peroutka 1987; Zemlan *et al.* 1990). Although not yet specifically localized in the spinal cord, mRNA of the more recently characterized 5-HT_6 and 5-HT_7 receptors have been identified in dorsal root ganglion cells (Pierce *et al.* 1996).

It would appear that most dorsal horn 5-HT receptors are not located presynaptically on descending serotonergic nerve terminals since selective lesions of the tracts with 5,7-DHT do not significantly affect the density of binding sites (Brown *et al.* 1989). A significant number of 5-HT_{1A} and 5-HT_{1B} receptors (20-25%) and 5-HT_3 receptors (50-80%) are located on the primary afferent nerve terminal, as their numbers are significantly reduced following dorsal rhizotomy or neonatal capsaicin (Cesselin *et al.* 1994). Nevertheless, 5-HT receptors located postsynaptically also play an important role in nociceptive modulation.

Intrathecal 5-HT has an antinociceptive effect in a number of behavioural test models (Cesselin *et al.* 1994). Much of the antinociceptive effect of intrathecal 5-HT may be mediated by 5-HT_3 receptors (Glaum *et al.* 1990). Furthermore, there is evidence that only those 5-HT_3 receptors located postsynaptically to the primary afferents mediate an antinociceptive effect (Alhaider *et al.* 1991). The role of the 5-HT_{1A} receptor is not clear since both pro- and antinociceptive actions have been reported (Fasmer *et al.* 1986; Archer *et al.* 1987; Eide and Tjolsen 1988) while 5-HT_{1B} receptor stimulation seems to mediate antinociception (Alhaider and Wilcox 1993). Again, the role of spinal 5-HT_2 receptors has not been clearly defined since antinociceptive effects associated with 5-HT_2 receptor stimulation (Solomon and Gebhart 1988) and pronociceptive effects (Eide and Hole 1991) have been reported. A significant nociceptive effect attributable to 5-HT_{1S} receptor stimulation has not been identified (Alhaider *et al.* 1993).

An important role for 5-HT in the peripheral activation of sensory neurons has clearly been identified. Peripherally applied 5-HT has been shown to evoke pain in

humans when applied to the blister base (Richardson *et al.* 1985) or injected subcutaneously (Jensen *et al.* 1990) and has been shown to enhance nociceptive behaviour in a variety of animal models (Vinegar *et al.* 1989; Sufka *et al.* 1991; Taiwo and Levine 1992). Exogenously applied 5-HT stimulates an inflammatory reaction consisting of edema and flaring in humans (Jensen *et al.* 1990) and rats (Maling *et al.* 1974; Sufka *et al.* 1991). As an endogenous mediator of pain and inflammation, there are ample stores of 5-HT to be found in platelets (Hourani and Cusack 1991) and in rodent mast cells (Church *et al.* 1986), which may be released under conditions of injury.

A number of 5-HT receptor subtypes have been identified in peripheral neuronal and non-neuronal tissues. In rats, 5-HT_1 receptors may function as autoreceptors inhibiting neurotransmitter release (Göthert *et al.* 1986) and in some tissues mediate vascular reactivity (reviewed, Martin 1994). 5-HT_2 receptors may be found on platelets (Drummond and Gordon 1975) and vascular tissues (reviewed, Martin 1994). 5-HT_3 receptors seem to be exclusively neuronal, both centrally (Yakel and Jackson 1988) and peripherally (Fozard 1984), while the 5-HT_4 receptor subtype has been associated with central (Grossman *et al.* 1993) and peripheral neurones (Rhodes *et al.* 1992) as well as non-neuronal tissues (reviewed, Hoyer *et al.* 1994).

Although there are data on the identity of 5-HT receptor subtypes involved in peripheral inflammation and nociception, there are inconsistencies in this body of information. Richardson *et al.* (1985) initially identified the 5-HT_3 receptor as responsible for mediating the pain producing effects of serotonin in humans. Since that time, 5-HT_3 receptors have been implicated in a number of animal models of inflammatory pain

(Giordano and Rogers 1989; Guilbaud *et al.* 1989; Sufka *et al.* 1991). However in other studies, 5-HT_{1A} receptors (Taiwo and Levine 1992) or 5-HT_2 receptors (Grubb *et al.* 1988; Abbott *et al.* 1996) seem to be important in mediating pronociceptive actions of 5-HT. The role of the more recently characterized 5-HT_4 receptor in inflammatory pain remains largely unexplored.

IV. Potential interactions between adenosine and 5-hydroxytryptamine

The potential exists for adenosine and 5-HT to interact in the modulation of pain transmission at several levels. Both substances mediate analgesia at the spinal level (see above) and a component of 5-HT induced analgesia is sensitive to methylxanthine blockade, indicating the involvement of adenosine receptors (Delander and Hopkins 1987). 5-HT stimulated release of adenosine from spinal cord synaptosomes (Sweeney *et al.* 1988) and from spinal cord perfusate *in vivo* (Sweeney *et al.* 1990) supports this. The mechanism of this release is unclear but much of the adenosine is released as cAMP and then further broken down to adenosine (Sweeney *et al.* 1988; Sweeney *et al.* 1990). 5-HT induced elevation of intracellular cAMP and subsequent release provides an attractive hypothesis.

There are no data on interactions of adenosine and 5-HT to modulate nociception at the peripheral nerve terminal, however there are data on interactions between these agents at other sites in the periphery. Nordström and Delbro (1986) reported that adenosine induced contraction of isolated rat trachea could be inhibited by ketanserin and concluded that the release of 5-HT was involved. In human studies, adenosine induced

bronchoconstriction could also be inhibited by ketanserin, even though ketanserin itself had no bronchodilating properties (Cazzola *et al.* 1992). Furthermore, this adenosine/5-HT mediated bronchoconstriction has been linked to capsaicin sensitive sensory nerves. Using an *in vivo* guinea-pig model, Manzini and Ballati (1990) were able to show that 2-chloroadenosine induced bronchoconstriction involved a neuronal reflex arc that included capsaicin-sensitive afferent vagal fibres. The source of the 5-HT remains speculative, but mast cells, platelets, or other inflammatory cells are potential candidates.

In the rat, mast cells contain significant amounts of 5-HT and mast cell degranulation can be provoked by adenosine, via activation of adenosine A_3 receptors (Hannon *et al.* 1995; Fozard *et al.* 1996). Conversely, the release of 5-HT from platelets is inhibited by adenosine, an adenosine A_2 receptor mediated effect (Cooper *et al.* 1995). Interestingly, in rat basophils, adenosine has a biphasic effect on 5-HT release, being inhibitory (A_1-mediated) at low levels and stimulatory (A_2-mediated) at higher concentrations (Abbracchio *et al.* 1992).

Interactions between 5-HT and adenosine are not limited to adenosine-induced release of 5-HT. Indeed, it is possible that 5-HT may release adenosine in association with platelet activation or from peripheral nerve endings (see above). Finally, the potential exists for additive or synergistic effects on stimulation of peripheral nerve endings directly or through other cellular components of inflammation.

V. A model for acute inflammatory pain

Different models for different kinds of pain

Much of the accumulated data on the study of pain is derived from animal models based on acute nociceptive stimuli such as heat, electric shock, or pressure, as seen in the hot-plate test, tail-flick test, and paw-pressure withdrawal test (reviewed, Franklin and Abbott 1989). In such animal models, the noxious stimulus is transient (*i.e.* phasic) and a nociceptive threshold is measured. The stimuli in these so-called "threshold tests" is of an intensity intended to produce avoidance behaviour and withdrawal occurs before tissue damage occurs. In contrast, much of the pain experienced clinically is sustained (*i.e.* tonic) and results from some degree of tissue injury and subsequent inflammation. It is possible that these threshold models may not be an accurate representation of all forms of clinical pain.

In order to be useful, a nociceptive test must be able to reliably discriminate those analgesic drugs which are clinically efficacious from those which are not. Most of the more than 50 animal pain tests are able to identify opioids as effective analgesics, however many other drugs, including nonsteroidal anti-inflammatory drugs (NSAIDs) and opioid partial agonists are excluded by some of these tests, particularly the phasic tests (reviewed, Franklin and Abbott 1989). This was the impetus for development of tests which incorporated an inflammatory component. Injection of yeast into a rat's paw results in inflammation and measurable hypersensitivity to applied pressure. Injected carrageenan also results in an inflammatory reaction and hyperalgesia to radiant heat. These inflammatory models have proved sensitive to the effects of non-opioid analgesics such as

NSAIDs, which are clinically effective for mild to moderate pain (Franklin and Abbott 1989).

Over the past few decades it has become evident that not all clinical pain is the same. The fundamental mechanisms may differ and the clinical syndromes presented may differ (eg. inflammatory arthritis versus phantom limb pain). Not surprisingly, the responsiveness of different pain syndromes to different analgesic modalities is quite variable. As a result, it has become evident that one must consider the clinical pain syndrome and its underlying mechanisms in choosing a nociceptive model.

The formalin test is one of the few animal models of acute, tissue injury-induced cutaneous pain (Franklin and Abbott 1989). It is distinguished from other nociceptive tests by a number of characteristics: (a) *Duration.* The pain stimulus in the formalin test is a chemical tissue injury producing an initial response lasting 5 to 10 minutes (phase 1) and, following a brief quiescent period, a sustained stimulus lasting up to 60 minutes or more (phase 2). This second phase is a tonic stimulus as opposed to the brief (phasic) stimulus of thermal pain threshold tests such as the tail-flick, hot plate, and tail dip tests. (b) *Stimulus.* Threshold tests are based on measurement of the latency of a response to injury avoidance whereas the formalin test attempts to quantify the response to an injury sustained (*i.e.* the chemical irritant). (c) *Inflammation.* The chemical injury in the formalin test results in an inflammatory response and associated peripheral sensitization (Dubuisson and Dennis 1977; Hunskaar and Hole 1987; Shibata *et al.* 1989; reviewed, Tjølsen *et al.* 1992). (d) *Plasticity.* Perhaps most importantly, because the stimulus in the formalin test is tonic, the system may adapt in response to that stimulus (*i.e.* neuroplasticity can occur;

Coderre *et al.* 1990), something which cannot be expected from brief phasic stimuli. Interestingly, the threshold tests reliably identify opioid analgesics but are relatively insensitive to non-opioid analgesics such as non-steroidal anti-inflammatory drugs or opioid agonist-antagonists, whereas the formalin test (and other tests with an inflammatory component) are quite sensitive to these "milder" analgesics (Franklin and Abbott 1989).

Pain associated with subcutaneous injection of formalin in humans can be described as an initial, intensely burning pain, not unlike a bee sting, followed by a less well localized, less intense aching feeling which subsides over 60 to 90 minutes. Others have described the sensation in similar terms (Dubuisson and Dennis 1977; Franklin and Abbott 1989). Qualitatively these properties, especially of the second phase, resemble clinical post-surgical pain.

Identifying mediators of hyperalgesia in the presence of inflammation

As previously alluded to, whether or not a substance is likely to stimulate a nociceptive response in the setting of tissue injury and inflammation may be dependent on several conditions including: (a) *Concentration.* At low concentrations a substance may sensitize peripheral nerve terminals while at higher concentrations it may stimulate (*e.g.* 5-HT). (b) *Other mediators.* Some substances may not produce pain by themselves but in the presence of other "sensitizing" agents may be capable of eliciting hyperalgesia (*e.g.* adenosine). (c) *Indirect effects.* While not directly activating the nociceptor, a substance may be capable of releasing another, directly acting mediator (*e.g.* norepinephrine promotes release of prostaglandins). (d) *Neuroplasticity.* The sensitivity of the system to

a particular stimulus may be dependent on more long term peripheral and central changes in neuronal thresholds.

To investigate the role of any putative substance in mediating a pronociceptive effect in the setting of tissue injury/inflammation, it may be necessary to observe its effects within the context of other mediators (Fig. 1), rather than in isolation. There have been a number of electrophysiological and behavioural models developed to investigate the actions of individual mediators within the context of an inflammatory milieu (reviewed, Treede *et al.* 1992). Some examples are discussed below.

An *in vitro* rat skin-saphenous nerve preparation has been developed which allows the electrophysiological detection of nociceptor activation following application of any number of inflammatory mediators, alone or in combination (Lang *et al.* 1990; Kessler *et al.* 1992). This model excludes any potential contribution from blood-borne inflammatory cells or plasma derived factors and while it allows detection of changes in threshold of the peripheral nociceptor in response to different chemical agents, any neuroplastic effects at the level of the dorsal horn cannot be studied. Herbert and Schmidt (1992) measured activation of articular afferent nerves following induction of an experimental arthritis. Electrical activity of previously identified afferent units was recorded following mechanical stimulation or intra-arterial injection of putative inflammatory mediators. Although no behavioural effects could be observed since the experiment occurred under anaesthesia, a nociceptive effect was inferred from the increase in electrical activity of the nociceptive fibres.

Hong and Abbott (1994) have developed a behavioural test based on scoring of the spontaneous pain behaviours associated with the formalin test. Hindpaw injection of various inflammatory mediators (5-HT, prostaglandin E_2, bradykinin, SP, histamine, and norepinephrine), alone or in combinations of two, were found to produce a quantifiable behavioural response in rats. Such a paradigm does not permit investigation of the activity of antagonists to endogenous mediators of pain and inflammation. Karlsten *et al.* (1992) used peripheral formalin injection to study the effects of adenosine analogues on nociceptive behaviour, but only reported on phase 1.

The second phase of the formalin test is most interesting as a model of acute, peripheral pain, since it is associated with tissue injury/inflammation and some element of central sensitization. The behavioural response is quantifiable and reproducible (Dubuisson and Dennis 1977; Wheeler-Aceto *et al.* 1990; Tjølsen *et al.* 1992). It is proposed that through the use of selective antagonists, co-injected with a concentration of formalin producing a near maximal behavioural response, important mediators of the nociceptive/inflammatory response may be inhibited and therefore identified from the diminished behavioural response. Alternatively, injection of a reduced concentration of formalin should result in formation of all the necessary components of the nociceptive/inflammatory response (including sensitization of the nociceptor), but result in a submaximal behavioural response. Co-injection of agonist analogues of candidate inflammatory mediators should result in an enhanced behavioural response, confirming the mediators role in the "inflammatory soup".

VI. Rationale

The formalin test, with particular reference to the second phase of the response, provides a model of spontaneous pain behaviour in the setting of a tonic, low level inflammatory stimulus. Because of the tonic nature of the stimulus and the associated tissue injury and inflammation, the formalin model may be more representative than phasic or threshold tests, of some of the more commonly encountered clinical pain syndromes such as postoperative pain. While adenosine analogues have been demonstrated to enhance the first phase of this response (Karlsten *et al.* 1992), there is no information available on its effects during the second, tonic phase. The data on 5-HT involvement in peripheral nociception has some inconsistencies and there are no data on the role of 5-HT in the second phase of the formalin test. Although 5-HT clearly plays a role in inflammatory pain, the identity of the specific receptors involved is controversial and there are no data on the possible role of the more recently characterized 5-HT_4 receptor. Finally, interactions between adenosine and 5-HT in the periphery have been identified (see above) and the possibility of similar interactions with respect to modulation of peripheral nociceptive input has not been explored. Identification of such interactions could, for example, lead to novel strategies for development of peripherally acting analgesics.

VII. Hypotheses and objectives

It was hypothesized that adenosine was a peripheral mediator of the nociceptive and inflammatory response to subcutaneous formalin injection in the rat and that pro- and antinociceptive effects were mediated through peripheral adenosine A_2 and A_1 receptors,

respectively. It was hypothesized that 5-HT was a peripheral mediator of the nociceptive and inflammatory response to subcutaneous formalin injection in the rat and that multiple peripheral 5-HT receptor subtypes were involved, including 5-HT_1, 5-HT_2, 5-HT_3, and 5-HT_4 receptors. Finally, it was hypothesized that peripheral adenosine and 5-HT interacted, either as co-mediators stimulating the peripheral nociceptor, or in a sequential way with one mediator stimulating the release of the other.

To test the hypotheses, the following objectives were laid out:

1) To establish an animal model of tissue injury associated with a tonic, inflammatory stimulus that would allow pro- and antinociceptive characteristics of endogenous and exogenous mediators to be distinquished. Using the formalin test, establish a dose-response relationship to determine the optimal "low" and "high" concentrations of formalin which produce a graded inflammatory response. This would facilitate identification of pronociceptive and antinociceptive substances, respectively.

2) To quantify the inflammatory response induced by formalin injection, based on paw swelling as measured by plethysmometry.

3) To determine the effect on nociceptive behaviour and inflammation, of exogenously administered adenosine and receptor subtype selective adenosine analogues when co-administered with low dose formalin.

4) To determine the role of endogenous adenosine and the adenosine receptor subtypes involved in the nociceptive and inflammatory response to high dose formalin injection using non-selective and selective adenosine receptor antagonists.

5) Determine the effect on nociceptive behaviour and inflammation, of exogenously administered 5-HT and receptor subtype selective 5-HT analogues when co-administered with low dose formalin.

6) Determine the role of endogenous 5-HT and the 5-HT receptor subtypes involved in the nociceptive response to high dose formalin injection using selective 5-HT receptor antagonists.

7) Identify and characterize the receptor subtypes involved in any interactions between adenosine and 5-HT in the nociceptive and inflammatory response to subcutaneous formalin injection. Additive or synergistic relationships will be tested by combining adenosine and 5-HT in the presence of low dose formalin. The possibility of adenosine mediated activity being a function of 5-HT release and receptor activation (or *vice versa*) will be tested by injecting one mediator (*e.g.* adenosine) in the presence of low dose formalin and an antagonist analogue of the other mediator (*e.g.* 5-HT).

MATERIALS

I. Animals

All experiments were conducted on male Sprague Dawley rats (Charles River, Quebec, Canada) weighing between 100 and 150 g. The animals were housed in groups of 2-4 in the animal care facility and maintained on a 12 h light/dark cycle with rat chow and water available *ad libitum*. A minimum 24 h acclimatization period was allowed after shipment to the facility. On the day of testing, rats were removed from the animal care facility to the testing area at least 1 h prior to testing. Each experiment utilized 3-8 rats per group, as indicated in individual figure legends.

All procedures were reviewed by the University Committee on Laboratory Animals and deemed to be in accordance with the Canadian Council on Animal Care Guidelines and IASP guidelines on the use of animals in pain research. Rats were used once only.

II. Drugs and chemicals

A complete list of adenosine and 5-HT analogs used, as well as sources, may be found in Tables III and IV. Drugs to be tested were initially dissolved in either saline or 100% dimethyl sulfoxide (DMSO; Sigma Chemical Co., St. Louis, MO). Formalin (Sigma Chemical Co., St. Louis, MO) for subcutaneous injection into the rat hindpaw was prepared freshly, just prior to testing. Stock formalin solution (37% formaldehyde) was diluted in 0.9% saline and then added to the test drug solution to produce the final concentration of formalin and test drug. Hence, formalin and the test drugs were always

TABLE III. Adenosine ligands and receptor selectivity (Bruns *et al.* 1986; Seale *et al.* 1988; Collis and Hourani 1993; Fredholm *et al.* 1994).

DRUG	SOURCE	SOLVENT	RECEPTOR AFFINITY
adenosine	Sigma Chemical Co., St. Louis, MO	saline	A_1: K_i = 3-30 nM A_2: K_i = 1-20 nM
N^6-cyclohexyladenosine (CHA)	Research Biochemicals Inc., Natick, MA	10% DMSO/saline	A_1: K_i = 1.3 nM A_2: K_i = 514 nM
2-[p-(2-carboxyethyl) phenethylamino]-5'-N-ethylcarboxamido adenosine (CGS21680)	Research Biochemicals Inc., Natick, MA	10% DMSO/saline	A_1: K_i = 2600 nM A_2: K_i = 15 nM
8-cyclopentyltheophylline (CPT)	Research Biochemicals Inc., Natick, MA	20% DMSO/saline	A_1: K_i = 11 nM A_2: K_i = 1400 nM
1,3-dimethyl-7-propylxanthine (DMPX)	Research Biochemicals Inc., Natick, MA	saline	A_1: K_i = 45 μM A_2: K_i = 11 μM
caffeine	Sigma Chemical Co., St. Louis, MO	saline	A_1: K_i = 55 μM A_2: K_i = 32 μM

Table IV. 5-hydroxytryptamine ligands and receptor selectivity (Zifa and Fillion 1992; Hoyer *et al.* 1994).

DRUG	SOURCE	SOLVENT	RECEPTOR AFFINITY*
5-hydroxytryptamine hydrochloride (5-HT)	Sigma Chemical Co., St. Louis, MO	saline	5-HT_{1A} : K_i = 2 nM 5-HT_{1B} : K_i = 11 nM 5-HT_{2A} : K_i = 7.8 nM 5-HT_{2C} : K_i = 11 nM 5-HT_{3} : K_i = 130 nM 5-HT_{4} : K_i = 92 nM
5-carboxamidotryptamine (5-CT)	Research Biochemicals Inc., Natick, MA	saline	5-HT_{1A} : K_i = 0.3 nM 5-HT_{1B} : K_i = 5.1 nM 5-HT_{2C} : K_i = 630 nM
1-(2,5-dimethoxy-4-iodophenyl)-2-amino-propane (DOI)	Research Biochemicals Inc., Natick, MA	saline	5-HT_{2A} : K_i = 1.3 nM 5-HT_{2C} : K_i = 6.5 nM
m-chlorophenylbiguanide (CPBG)	Research Biochemicals Inc., Natick, MA	saline	5-HT_{3} : K_i = 0.3 nM
5-methoxytryptamine (5-MeOT)	Sigma Chemical Co., St. Louis, MO	saline	5-HT_{1B} : K_i = 398 nM 5-HT_{2A} : K_i = 305 nM 5-HT_{2C} : K_i = 25 nM 5-HT_{4} : K_i = 100 nM
S(-)propranolol	Research Biochemicals Inc., Natick, MA	saline	5-HT_{1A} : K_i = 46 nM 5-HT_{1B} : K_i = 50 nM 5-HT_{2A} : K_i = 590 nM 5-HT_{2C} : K_i = 588 nM
ketanserin	Research Biochemicals Inc., Natick, MA	saline	5-HT_{2A} : K_i = 1.2 nM 5-HT_{2C} : K_i = 28 nM

Table IV. (continued)

tropisetron	Research Biochemicals Inc., Natick, MA	saline	5-HT_3 : K_i = 0.4 nM 5-HT_4 : K_i = 710 nM
[1-[2-(methylsulphonyl) amino]ethyl-4-piperidinyl]methyl 1-methyl-1H-indole-3-carboxylate (GR113808)	Glaxo Group Research Ltd., Greenford, Middlesex, UK	saline	5-HT_3 : K_i =1000 nM 5-HT_4 : K_i = 0.2 nM
* very low affinity values (K_i > 1000 nM excluded)			

co-injected. Final formalin concentrations ranged from 0.5% (1:200 dilution of stock formalin solution) to 5.0% (1:20 dilution of stock formalin solution), depending on the particular experiment. Once the behavioural response to the full range of formalin concentrations was established, two concentrations of formalin were selected for testing in specific circumstances (see Results). Where the test drug was anticipated to augment the response, 0.5% formalin was used for injection. Where antagonism of the response was anticipated, 2.5% formalin was co-injected with the test drug.

The adenosine analogues tested (Table III) were selected on the basis of relevant receptor selectivity and efficacy (Bruns *et al.* 1986; Seale *et al.* 1988; Collis and Hourani 1993), and solubility in the solution containing formalin. Wherever possible, drugs were dissolved in saline but N^6-cyclohexyladenosine (CHA) and 2-[*p*-(2-carboxyethyl)phenethylamino]-5'-*N*-ethylcarboxamido adenosine (CGS21680) required 10% DMSO, while 8-cyclopentyltheophylline (CPT) required 20% DMSO. Appropriate concentrations of DMSO were added to control formalin injections.

The 5-HT analogues tested (Table IV) were selected on the basis of relevant receptor selectivity and efficacy (Zifa and Fillion 1992; Hoyer *et al.* 1994), and solubility in the solution containing formalin. All 5-HT analogues were freshly prepared in saline and then added to the formalin solution to achieve appropriate concentrations.

III. Miscellaneous equipment

Observation chambers

Rats were observed in transparent 30 x 30 x 30 cm plexiglass chambers. The chambers had an open bottom and a removable cover that could be left ajar for ventilation. The chambers were placed side by side on a paper covered table in a quiet area of the laboratory. A mirror was placed behind each chamber to facilitate observation of the injected limb regardless of position.

Anaesthetizing apparatus

For those experiments where formalin injection was carried out under halothane anaesthesia, rats were placed in a plexiglass box (12.5 x 12.5 x 25 cm) with a tight fitting lid. Approximately 0.5 ml of halothane was added to the box. Rats were removed for injection once the righting reflex was lost.

Plethysmometer

Hindpaw volume was determined using a commercial plethysmometry device (Ugo Basile, Varese, Italy). The device consists of two, connected, transparent plastic cylinders. One chamber accommodated the rat paw while the other housed two parallel electrodes. Measurement of paw volume occurred via displacement of a weak electrolyte solution within the chambers. This resulted in a proportional change in the length of the

parallel electrodes immersed in the electrolyte solution. The resulting change in electrical resistance was then transformed to a digital readout expressed as mls displaced.

pH tester

Formalin (diluted in saline) pH was tested utilizing a Beckman ø12 pH/ISE meter (Beckman Instruments Inc., Fullerton, CA).

Statistical software

Data was analyzed using *SigmaStat for Windows* (Ver. 1.0, Jandel Corporation, San Rafael, CA).

METHODS

I. Formalin test

The laboratory temperature was maintained at 17-21 °C during behavioural experiments. Rats were placed separately in the observation chamber for 10 - 15 minutes to accommodate to their surroundings, then removed for formalin administration. In the earlier experiments (see Results), rats were first briefly anaesthetized with halothane (Wyeth-Ayerst Canada Inc., Montreal, PQ), then the dorsum of the hindpaw was injected with 50 μl of dilute formalin/test drug solution through a 30 gauge needle. The rat was immediately returned to the observation chamber and had fully recovered from anaesthesia within 1-2 minutes. Later experiments were conducted without halothane anaesthesia for injection. Rats were gently restrained by hand while being injected and then immediately returned to the observation chamber.

Two animals were observed per trial, in adjacent observation chambers. The first rat was injected at time zero and placed in the left hand observation chamber. During the next 2 minutes the second rat was prepared for injection. At time 2 minutes the second rat was injected and placed in the right hand observation chamber. Recording of nociceptive behaviour of the first rat began at 2 minutes post injection and lasted 2 minutes before switching to observation and recording of the second rat (now 2 minutes post injection) for the following 2 minutes, and so on. Thus rats were observed for nociceptive behaviour in alternate 2 minute bins, commencing 2 minutes post injection until 60 minutes post injection (total of 15 bins). A behavioural score collected in any given bin was assumed to be similar to that in the preceding bin, but no correction for this was

made. Thus the cumulative value for any given phase represents about half of the real incidence of behaviours. Nociceptive behaviour was quantified as the number of lifts or flinches of the affected limb during the observation bin. Such behaviour could vary between a simple lift of the paw (not associated with locomotion) to a vigorous shaking of the limb, or it could be a rippling of back muscles associated with limb movement. Lifts or flinches were discrete and easily quantifiable. Formalin induced flinching behaviour has been shown to be more robust than the paw licking response and less affected by other behavioural influences (Wheeler-Aceto and Cowan 1991).

II. Plethysmometry

Using the plethysmometer, hindpaw volume was determined by immersion to the junction of hairy and glabrous skin. Values recorded were an average of three successive measurements. The injected and non-injected hindpaw of each rat were measured prior to formalin injection and at the conclusion of behavioural observations (60 minutes post-injection). Previous work has indicated that most of the swelling associated with formalin injection occurs within 60 minutes of injection (Wheeler-Aceto *et al.* 1990).

III. Statistical analysis

Data collected over the 60 minutes of observation were divided into phase 1 (2-12 min, 3 bins) and phase 2 (14-60 min, 12 bins) as previously described (Dubuisson and Dennis 1977; Wheeler-Aceto and Cowan 1991). For group comparisons, the cumulative response in a given phase was analyzed using analysis of variance followed by Dunnett's

test (when comparisons were made to a single control group) or the Student-Newman-Keuls test (pairwise comparisons). Plethysmometry results were analyzed by two-way repeated measures analysis of variance followed by the Student-Newman-Keuls test. All results are expressed as mean $\pm$ SEM.

RESULTS

I. Establishment of the formalin model

Formalin was available as a 37% solution of formaldehyde gas (HCHO). Dilutions of 0.5 to 5% stock formalin solution in saline were prepared for establishment of a dose response relationship upon which subsequent experiments would be based. The pH of these formalin preparations ranged from 3.33 ± 0.01 (5%) to 4.15 ± 0.09 (0.5%) compared to 5.39 ± 0.12 for saline (Table V).

Formalin dose-response relationship

The initial protocol required injection of the formalin in anaesthetized animals. Anaesthesia could be achieved within 30 - 60 seconds using halothane vapour. It was found that injection could be accomplished within a few seconds of removing the animal from the anaesthetic box and the animal had completely recovered from anaesthesia before the beginning of the first observation bin (*i.e.* < 2 minutes). Using this protocol, the first dose-response curves were derived (Fig. 2). All concentrations of formalin resulted in flinching behaviour during phase 2 (Fig. 2B) that was significantly greater than that seen with saline injection ($P < 0.05$). Flinching behaviour during phase 1 (Fig. 2A) was much more variable and was not significantly different from that elicited by saline injection. In order to test potentially algogenic substances which might augment, or require the permissive effect of the established inflammatory reaction, a stimulus which would result in a minimal but significant nociceptive response was desired. Conversely, testing of potential antagonist substances would require a stimulus producing a robust, near maximal

Table V. Comparison of pH values for saline and formalin concentrations.

Solution	pH
saline	5.39
formalin 0.5%	4.15
formalin 1.0%	3.85
formalin 2.5%	3.55
formalin 5.0%	3.33

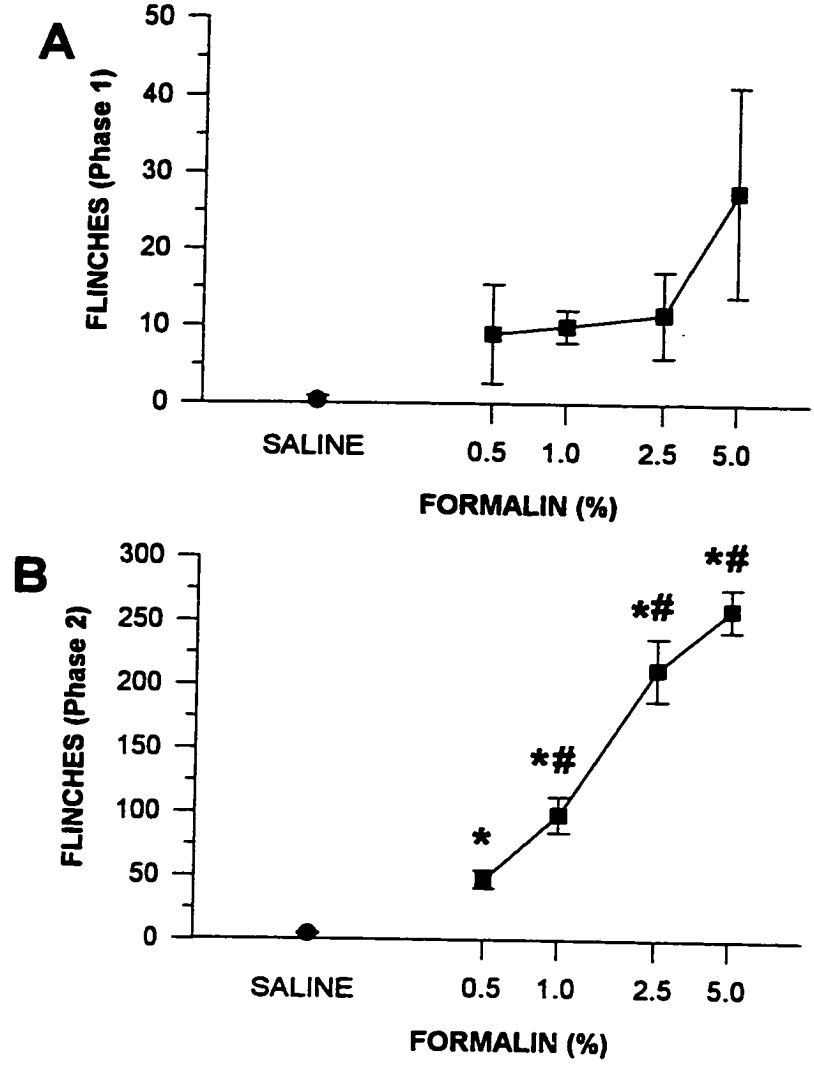


Figure 2. Flinching response in rats following subcutaneous injections of formalin 0.5% to 5% (n=5-7 rats/group, * P <0.05 vs saline, # P <0.05 vs 0.5%). Injections were made under brief halothane anaesthesia. Values are mean ± S.E.M. Where S.E.M. bars are not shown they are within the symbol. **A:** Phase 1 response (cumulative flinches in alternate 2 min bins between 2 and 12 min). **B:** Phase 2 response (cumulative flinches in alternate 2 min bins between 14 and 60 min).

nociceptive response. Inhibition of such a response would then be easily identified. These stimulus criteria were satisfied by formalin concentrations of 0.5% and 2.5%, respectively (Fig. 2B - 3). These formalin concentrations were then used in subsequent experiments (see Methods).

II. Adenosine and the formalin test

Formalin injections for all experiments in this section were carried out under brief halothane anaesthesia.

Effects of exogenous adenosine

Adenosine was co-injected with 0.5% formalin to determine if there was an augmentation of the flinching response. Adenosine produced no effect until a dose of 50 nmol was injected. This significantly augmented the response in both phases ($P < 0.05$, Fig. 4A-B). To determine if this response to exogenous adenosine also was seen with higher concentrations of formalin, a second formalin dose-response was constructed in the presence of adenosine 50 nmol. Although adenosine augmented the response to 0.5% formalin injection during both phases, this effect was no longer significant at higher formalin concentrations (1.0% and 2.5%). Adenosine injection in the absence of formalin elicited no behavioural response (cumulative Phase 2 response for adenosine = 4.7 ± 3.3 vs saline = 4.7 ± 0.7, n = 3/group, NS).

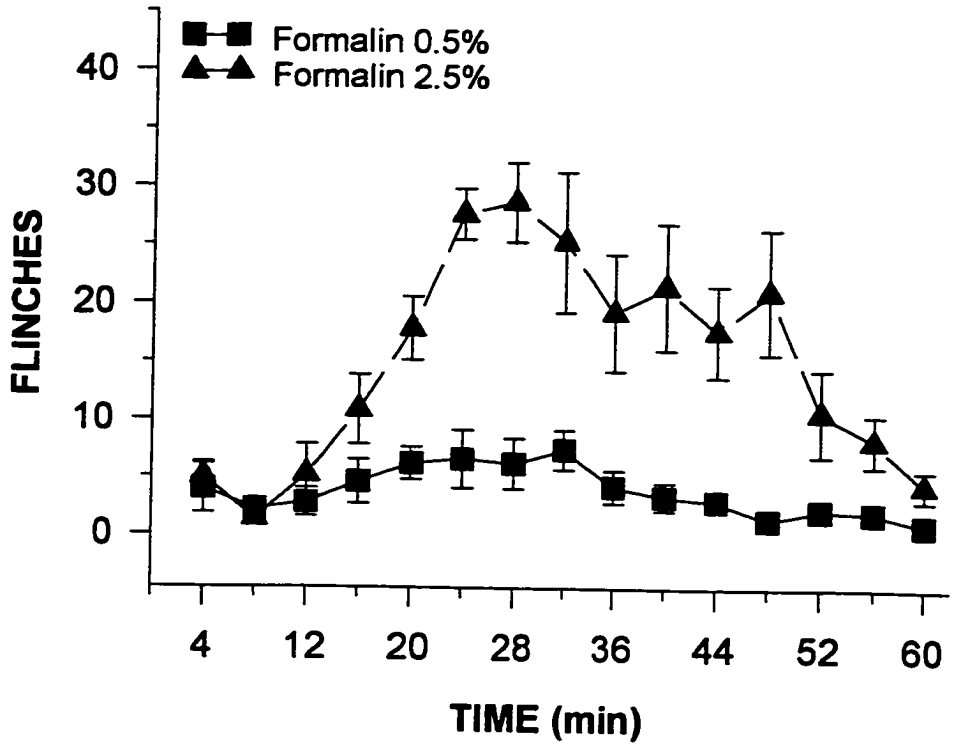


Figure 3. Time course of flinching following subcutaneous injections of formalin 0.5% and 2.5% at time zero. Injection occurred under brief halothane anaesthesia. Each point represents cumulative flinches over the preceding 2 min bin. Each individual rat was observed on alternate 2 min bins beginning 2 min post-injection (n=5-7 rats/group). Values are mean ± S.E.M. Where S.E.M. bars are not shown they are within the symbol.

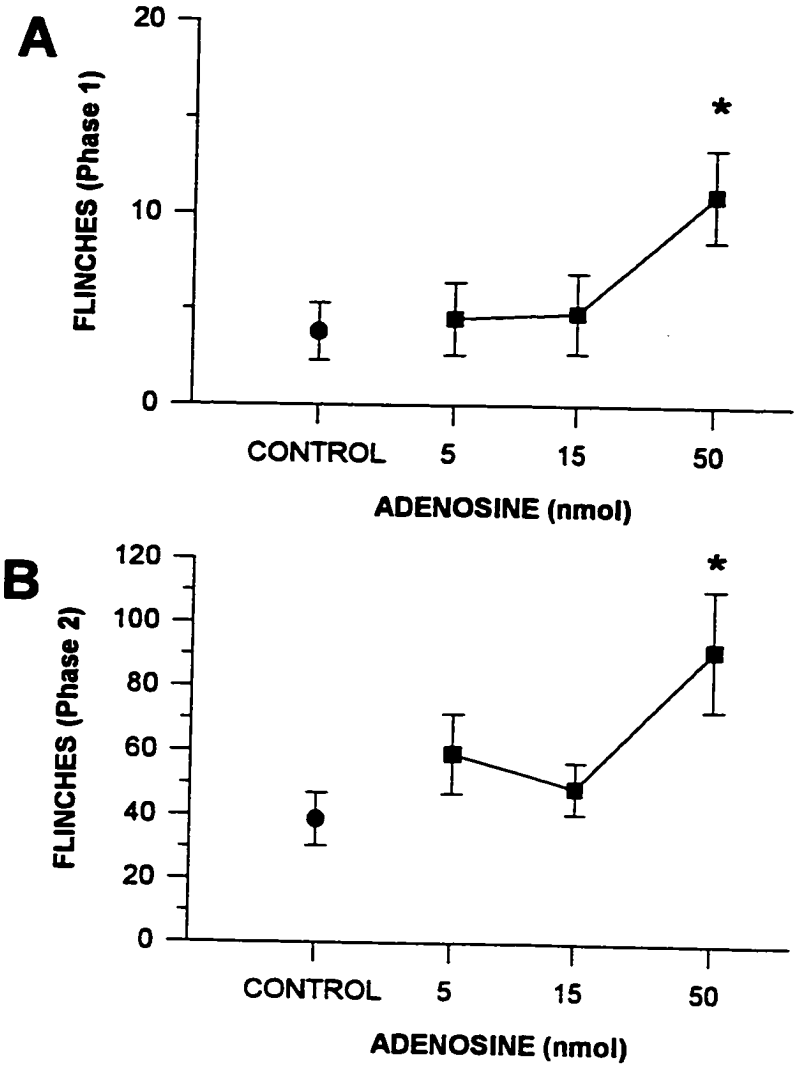


Figure 4. Flinching response in rats following subcutaneous injections of 0.5% formalin alone (control) and co-injected with increasing doses of adenosine (n=5-7 rats/group, * P <0.05 vs control). Injections were made under brief halothane anaesthesia. Values are mean ± S.E.M. **A:** Phase 1 response (cumulative flinches in alternate 2 min bins between 2 and 12 min). **B:** Phase 2 response (cumulative flinches in alternate 2 min bins between 14 and 60 min).

Effects of selective adenosine receptor agonists

When co-injected with 0.5% formalin, the adenosine A_2 receptor agonist CGS21680 augmented the flinching response during both phases ($P < 0.05$, Fig. 5). Augmentation of phase 1 flinching was maximal at the lowest dose tested (0.5 nmol), while phase 2 flinching peaked at a dose of 1.5 nmol. At progressively higher doses of CGS21680, flinching in both phases returned to control levels and then below this level (Fig. 5). The highest dose of CGS21680 (50 nmol) was associated with marked motor and behavioural changes, including reduced exploration, reduced grooming, drooping eyelids, and flattened body posture. These behavioural effects, and the associated reduction in flinching at the highest dose (50 nmol), were due to a systemic effect of the adenosine A_2 receptor agonist since injection of 50 nmol CGS21680 into the paw contralateral to the formalin injection site produced identical behavioural changes and reduction in flinching.

Observations in the first phase of the formalin test in mice (Karlsten *et al.* 1992) suggested a possible peripheral antinociceptive effect for adenosine A_1 receptor agonists. Because of this, the adenosine A_1 receptor agonist CHA was tested in the presence of 2.5% formalin. The effect on phase 1 flinching was insignificant but CHA produced a dose related reduction in phase 2 activity ($P < 0.05$, Fig. 6). However, this reduction in flinching was associated with changes in behaviour and motor activity similar to those seen with the highest dose of CGS21680. Contralateral injection of the same doses of CHA produced identical behavioural changes and suppression of flinching (Fig. 6), therefore a centrally mediated behavioural effect cannot be excluded.

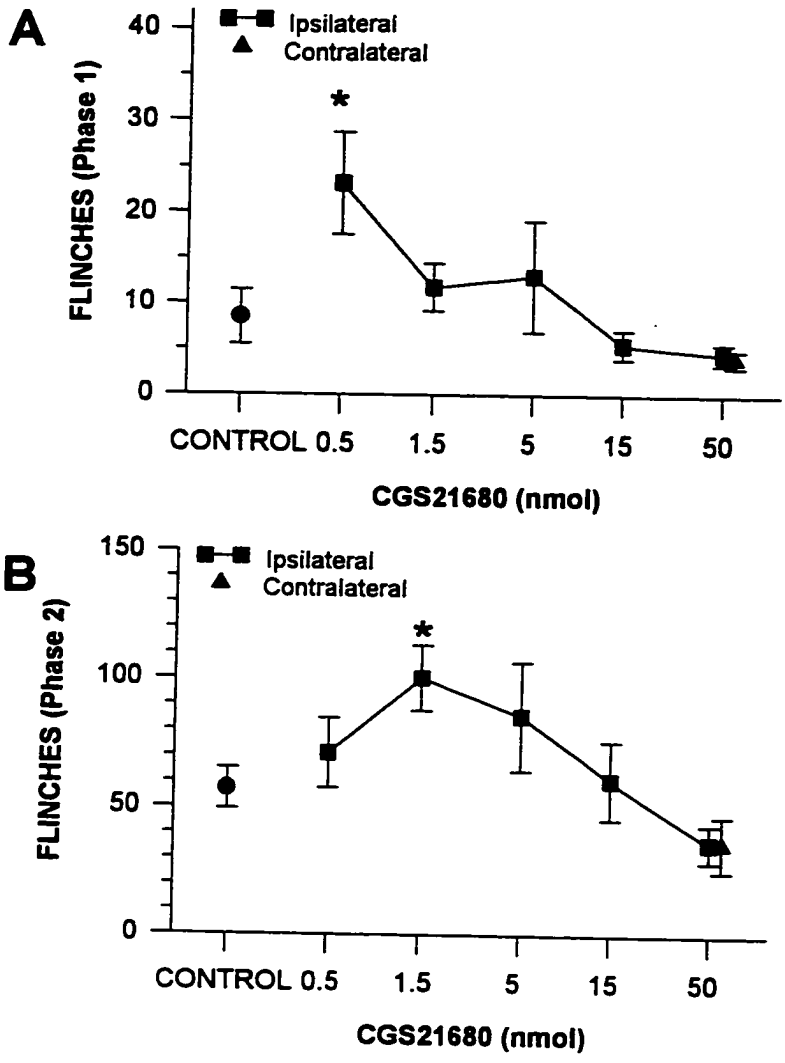


Figure 5. Flinching response in rats following subcutaneous injections of 0.5% formalin alone (control) and co-injected with increasing doses of CGS21680 (n=5-7 rats/group, * P <0.05 vs control). The response to injection of the highest dose of CGS21680 contralateral to 0.5% formalin also shown. Injections were made under brief halothane anaesthesia. Values are mean ± S.E.M. **A:** Phase 1 response (cumulative flinches in alternate 2 min bins between 2 and 12 min). **B:** Phase 2 response (cumulative flinches in alternate 2 min bins between 14 and 60 min).

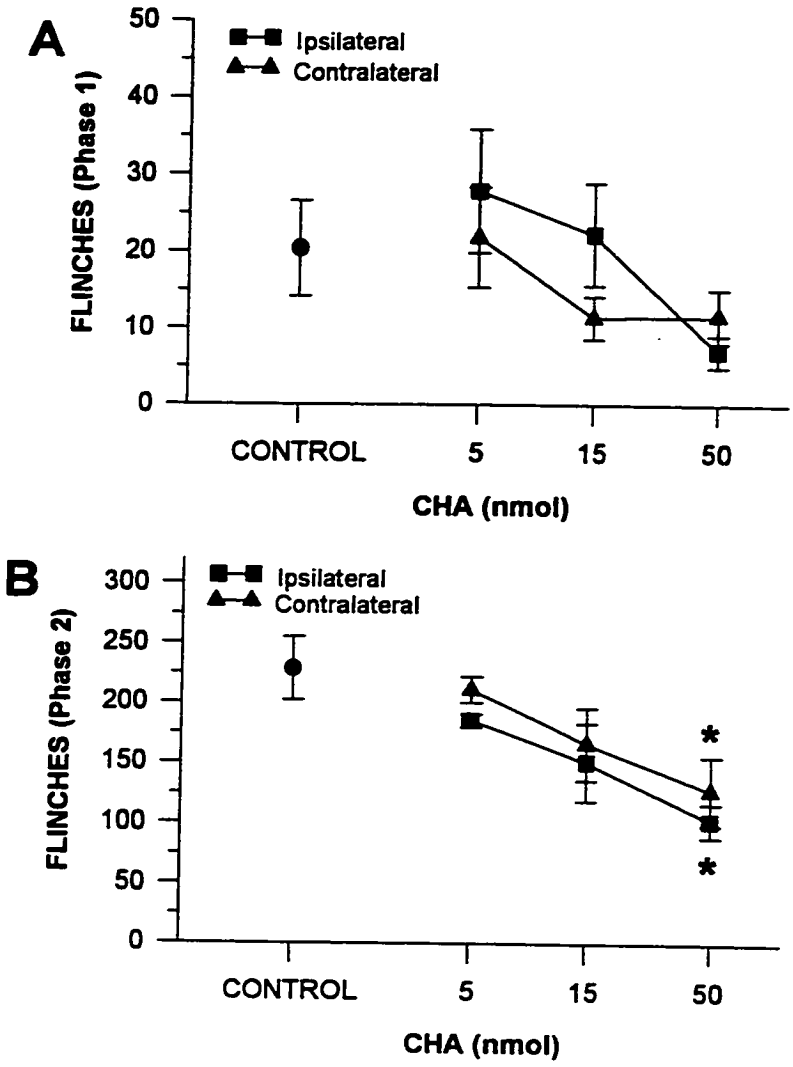


Figure 6. Flinching response in rats following subcutaneous injections of 2.5% formalin alone (control) and co-injected with increasing doses of CHA (n=5-7 rats/group, * P <0.05 vs control). The response to injection of the highest dose of CHA contralateral to 2.5% formalin also shown. Injections were made under brief halothane anaesthesia. Values are mean ± S.E.M. **A:** Phase 1 response (cumulative flinches in alternate 2 min bins between 2 and 12 min). **B:** Phase 2 response (cumulative flinches in alternate 2 min bins between 14 and 60 min).

Effects of selective adenosine antagonists

The role of endogenous adenosine in formalin induced flinching was investigated by co-injection of selective adenosine antagonists with a near maximally effective concentration of formalin (2.5%). The adenosine A_2 receptor antagonist, DMPX , was tested at doses of 5 - 50 nmol. Examination of the time course of flinching in the presence of DMPX 50 nmol revealed that most of the reduction in phase 2 flinching occurred in the first half of phase 2, from 14 to 36 minutes (Fig. 7), designated phase 2A. At doses of 15 and 50 nmol, DMPX significantly reduced flinching in phase 1 and phase 2A (Fig. 8). Co-injection of the adenosine A_1 receptor antagonist, CPT, with 2.5% formalin had no effect on phase 1 flinching at any dose tested (Fig. 9A). However, flinching in phase 2A was increased ($P < 0.05$) in the presence of 15 nmol CPT (Fig. 9B). This increase was not sustained in the latter half of phase 2. Injection of the same CPT doses was repeated in the presence of the minimally effective concentration of formalin (0.5%), but no drug effect was observed at any dose tested.

Effects of caffeine

Systemically administered caffeine produces antinociception in the rat formalin test (Sawynok *et al.* 1995), and this could potentially result from a peripheral block of the pronociceptive effects of adenosine A_2 receptor activation. In the presence of 2.5% formalin, locally administered caffeine (5 - 500 nmol) had no significant effect on nociceptive behaviour.

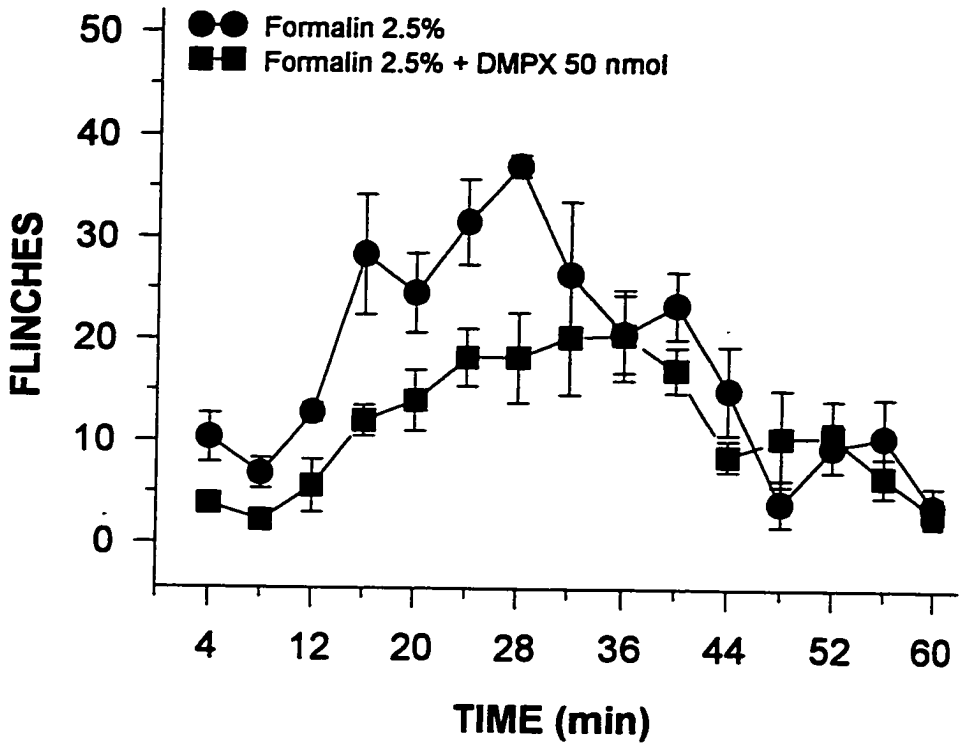


Figure 7. Effects of co-injection of DMPX 50 nmol on the time course of flinching following 2.5% formalin injection. Injections were made under brief halothane anaesthesia. Each point represents cumulative flinches over the preceding 2 min bin. Each individual rat was observed on alternate 2 min bins beginning 2 min post-injection (n=5 rats/group). Values are mean ± S.E.M. Where S.E.M. bars are not shown they are within the symbol.

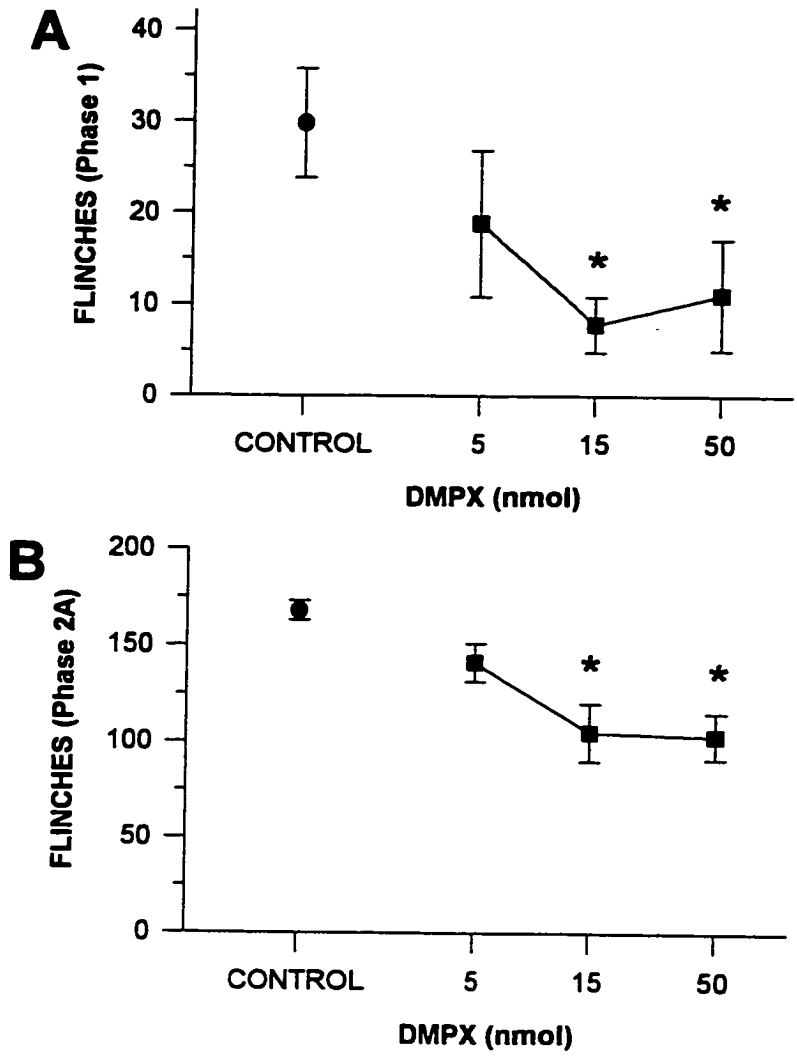


Figure 8. Flinching response in rats following subcutaneous injections of 2.5% formalin alone (control) and co-injected with increasing doses of DMPX (n=5 rats/group, * P <0.05 vs control). Injections were made under brief halothane anaesthesia. Values are mean ± S.E.M. **A:** Phase 1 response (cumulative flinches in alternate 2 min bins between 2 and 12 min). **B:** Phase 2A response (cumulative flinches in alternate 2 min bins between 14 and 36 min).

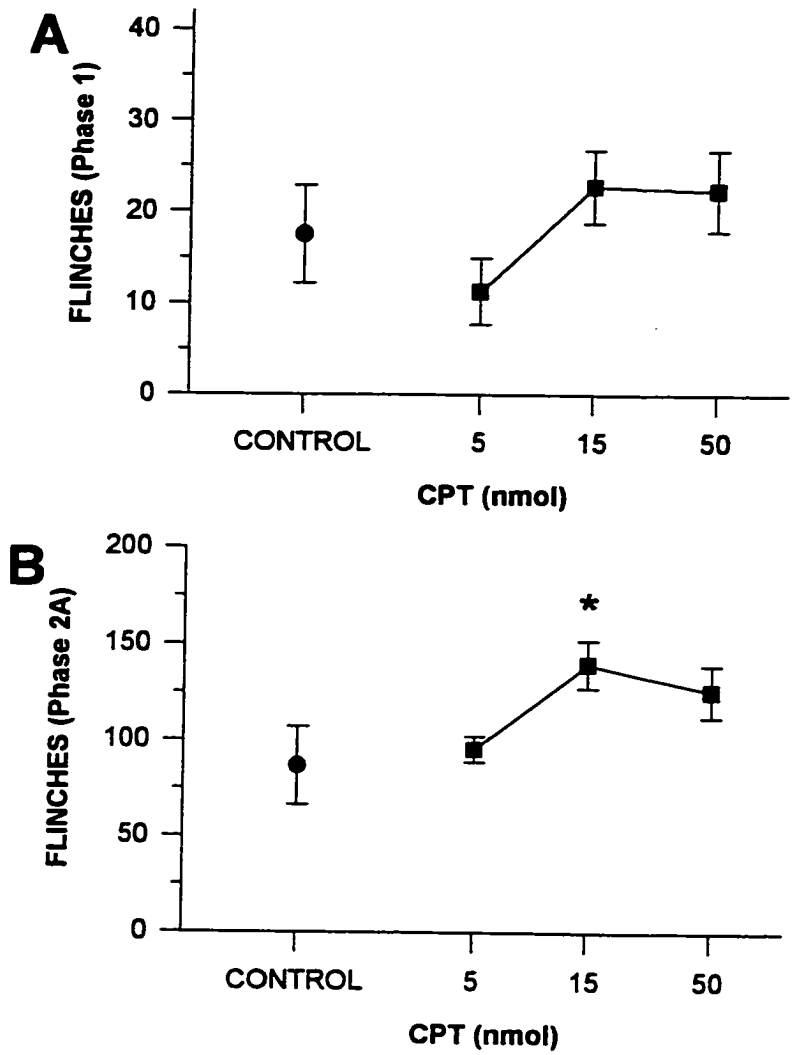


Figure 9. Flinching response in rats following subcutaneous injections of 2.5% formalin alone (control) and co-injected with increasing doses of CPT (n=5-7 rats/group, * P <0.05 vs control). Injections were made under brief halothane anaesthesia. Values are mean $\pm$ S.E.M. **A:** Phase 1 response (cumulative flinches in alternate 2 min bins between 2 and 12 min). **B:** Phase 2A response (cumulative flinches in alternate 2 min bins between 14 and 36 min).

III. Further modifications of the formalin model

Effect of anaesthesia

From the initial group of experiments it was evident that, although the phase 2 flinching response was robust, flinching in phase 1 was highly variable (Fig. 2) and appeared overall to be less robust than has been reported in the literature. This may have been due to a subclinical effect of the halothane anaesthesia. A change in protocol was approved by the University Committee on Laboratory Animals, and an experiment was designed to test this hypothesis. Two groups of rats were tested (n = 5 rats/group). One group received a hindpaw injection of 2.5% formalin while under brief halothane anaesthesia whereas the second group was injected while being gently restrained without anaesthesia. This was achieved with minimal stress to the animal. The rats were then observed for flinching behaviour in the usual manner. There was a decrease in phase 1 flinching (27 $\pm$ 5, anaesthesia vs 49 $\pm$ 6, no anaesthesia, $P < 0.05$) associated with anaesthesia. No difference was observed in phase 2 behaviours (228 $\pm$ 40, anaesthesia vs 238 $\pm$ 11, no anaesthesia, NS). As a result, formalin injections for all subsequent experiments were undertaken without prior halothane anaesthesia.

Training effect

It was observed that occasionally rats would exhibit a "freeze" response following formalin injection. This response was readily identifiable and could be distinguished from sedative effects of drugs or anaesthesia. When manifested, the rat would remain upright, standing, or resting on its haunches, but completely motionless. Often one paw would be

elevated as if "frozen" in mid-stride. The behaviour might last as long as 20 minutes and during this time flinching activity was significantly reduced. It has been suggested that such behaviour may be stress related and may be reduced or eliminated by frequent handling of the animals in the test situation prior to actual experimentation (Tjølsen *et al.* 1992). To test the practical significance of such training, two groups of rats were compared (n = 5 rats/group). One group of rats received 5 consecutive days of training consisting of transfer from the animal holding facility, handling and restraint as if for injection, and placement in the observation chamber for 30 minutes. The second group of rats spent the same time period in the animal holding facility. After the fifth day of training, animals were allowed 1-2 days rest before formalin testing using 2.5% formalin. Trained animals were tested along side of their untrained cohorts. The results of testing revealed no significant effect on behaviour in either phase. As a result, a specific training regimen was not instituted for subsequent formalin experiments.

Plethysmometry

Varying amounts of paw swelling were noticed following formalin injection in the earlier experiments considered, but this was not quantified. The potential for obtaining useful information was recognized and subsequent experiments included measurement of paw swelling by plethysmometry.

IV. 5-HT and the formalin test

Formalin dose-response relationship (without anaesthesia)

Flinching behaviour following formalin injection, in the absence of anaesthesia, was determined for formalin concentrations of 0.5% to 5.0% and compared to saline. Only the higher concentrations of formalin (2.5% and 5.0%) produced flinching in phase 1 that was significantly greater ($P < 0.05$) than a saline injection (Fig. 10A), whereas the flinching response to formalin was significantly greater than saline control in phase 2 ($P < 0.05$) at all formalin concentrations tested (Fig. 10B). The lowest concentration of formalin tested resulted in a minimal, but significant ($P < 0.05$) phase 2 flinching response while flinching responses to 1.0%, 2.5% and 5.0% formalin injection was significantly greater ($P < 0.05$) than the response to 0.5% formalin (Fig. 10B). Since 2.5% formalin produced a near maximal flinching response, this concentration was selected for testing with 5-HT antagonists while 0.5% formalin was used for co-injection with 5-HT and selective agonists.

Paw swelling, measured 60 minutes after formalin injection, was significant ($P < 0.05$) following injection of formalin concentrations of 1% or greater while saline injection did not result in significant paw swelling after 1 hour (Fig. 11).

Effects of selective 5-HT antagonists on the response to 2.5% formalin

Formalin induced flinching. The role of endogenous 5-HT in the behavioural response to subcutaneous injection of formalin was investigated by co-injection of selective 5-HT antagonists with 2.5% formalin. The 5-HT_1 receptor antagonist S(-) propranolol

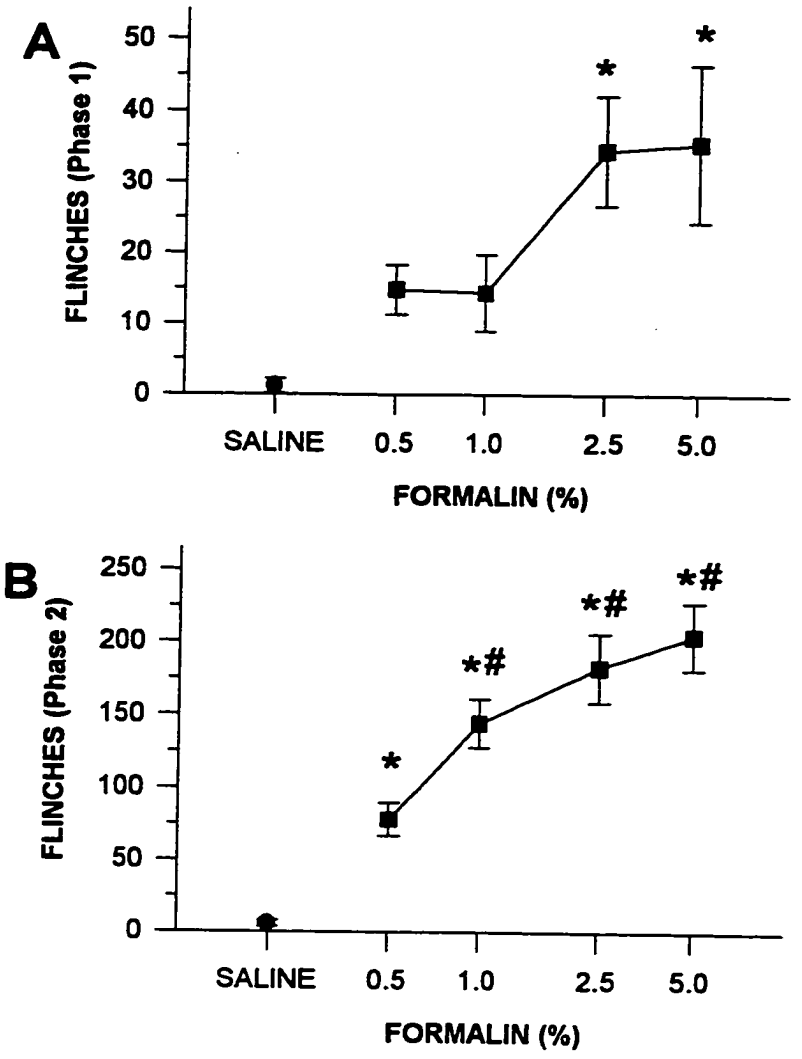


Figure 10. Flinching response in rats following subcutaneous injections of formalin concentrations of 0.5% to 5% (n=5-6 rats/group, * P <0.05 vs saline, # P <0.05 vs 0.5%). Values are mean ± S.E.M. Where S.E.M. bars are not shown they are within the symbol. **A:** Phase 1 response (cumulative flinches in alternate 2 min bins between 2 and 12 min). **B:** Phase 2 response (cumulative flinches in alternate 2 min bins between 14 and 60 min).

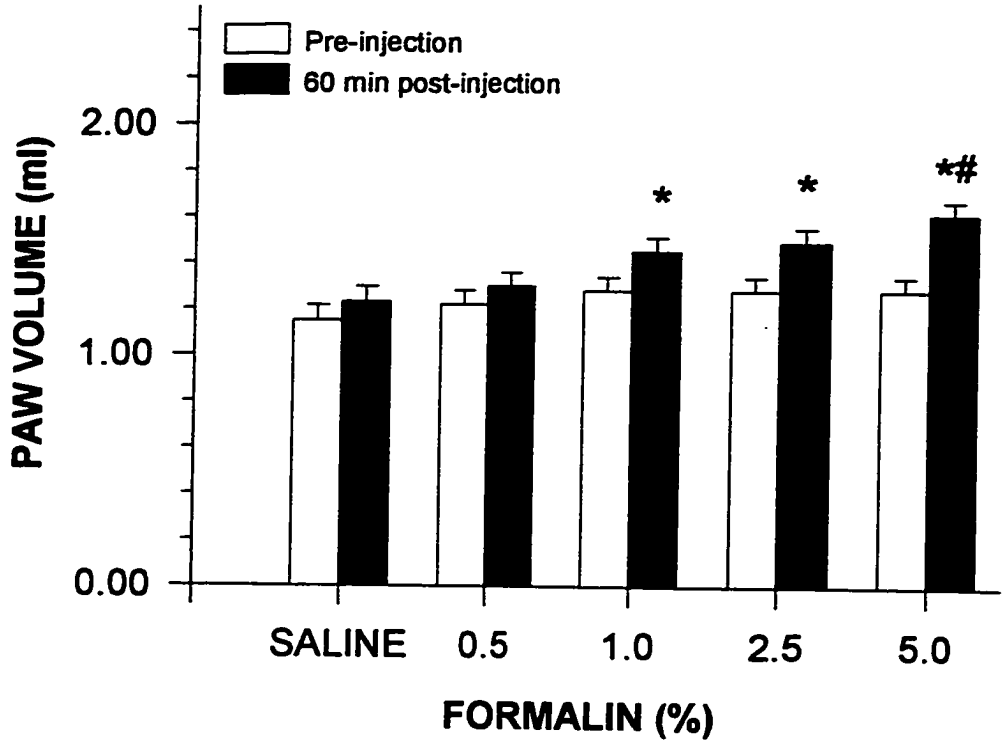


Figure 11. Rat hindpaw volumes, as measured by plethysmometry, before and 60 min after saline or formalin injection (n=5-6 rats/group, * $P < 0.05$ vs pre-injection, # $P < 0.05$ vs saline or 0.5% post-injection). Values are mean $\pm$ S.E.M.

had no effect on phase 1 flinching up to a dose of 150 nmol (Fig. 12A) but at a dose of 50 nmol markedly reduced phase 2 flinching ($P < 0.05$, Fig. 12B). Ketanserin, a 5-HT_2 receptor antagonist, had no effect on either phase 1 or phase 2 flinching even at the highest dose of 500 nmol (Fig. 13). Tropisetron, a 5-HT_3 receptor antagonist, at a dose of 150 nmol significantly reduced flinching in both phases ($P < 0.05$, Fig. 14). The highly selective 5-HT_4 receptor antagonist GR113808A was also effective in reducing flinching in both phases (Fig. 15). Phase 1 was reduced by 84% ($P < 0.05$, Fig. 15A) and phase 2 by 59% ($P < 0.05$, Fig. 15B) at a dose of 500 nmol. Although the reduction in flinching was significant for the cumulative phase 2 score, there was a tendency for the control and treatment time courses to merge in the latter half of phase 2 (Fig. 16). A similar pattern was also noted with S(-) propranolol and tropisetron.

Formalin induced paw swelling. Paw swelling was measured before and 60 minutes after injection of 2.5% formalin alone or co-injected with the selective 5-HT receptor antagonists. Measurements were undertaken for all doses of 5-HT receptor antagonists tested in the behavioural paradigm. Figure 17 shows the effects of co-injection of the 5-HT receptor antagonists on paw swelling induced by 2.5% formalin. For simplicity, only the highest dose tested or the most effective dose from the behavioural tests are illustrated. None of the 5-HT receptor antagonists (at any dose tested) had any effect on paw swelling induced by 2.5% formalin.

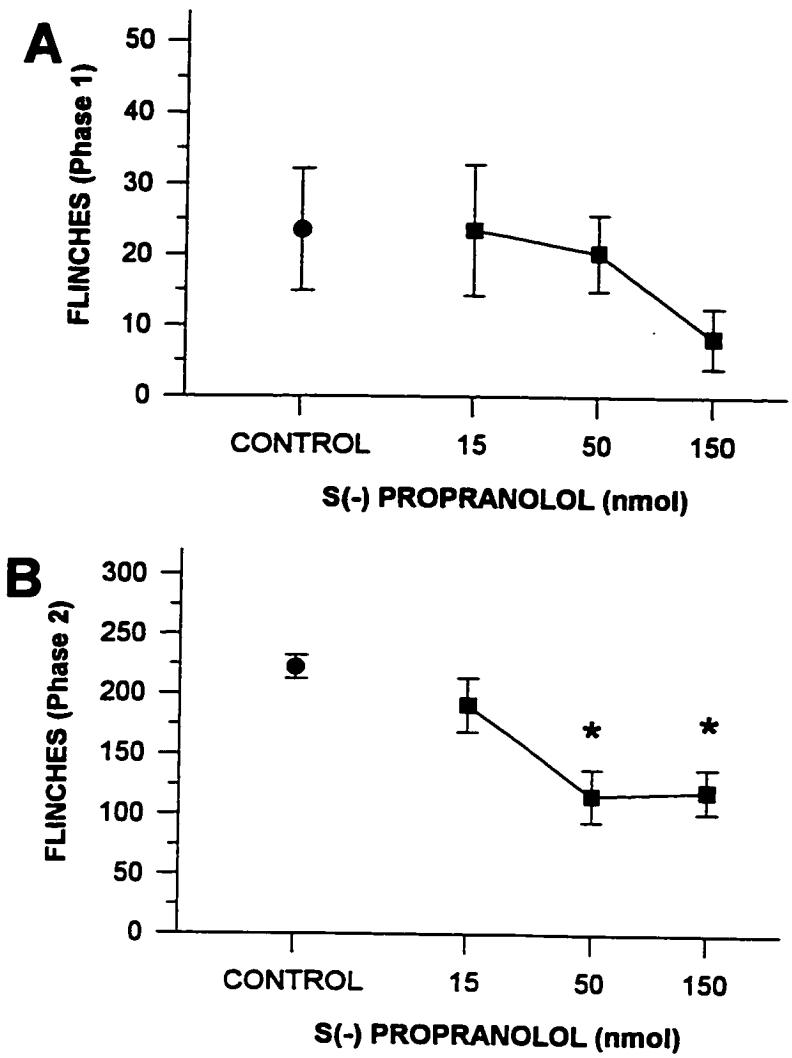


Figure 12. Flinching response in rats following subcutaneous injections of 2.5% formalin alone (control) and co-injected with increasing doses of S(-) propranolol (n=5-6 rats/group, * P <0.05 vs control). Values are mean ± S.E.M. **A:** Phase 1 response (cumulative flinches in alternate 2 min bins between 2 and 12 min). **B:** Phase 2 response (cumulative flinches in alternate 2 min bins between 14 and 60 min).

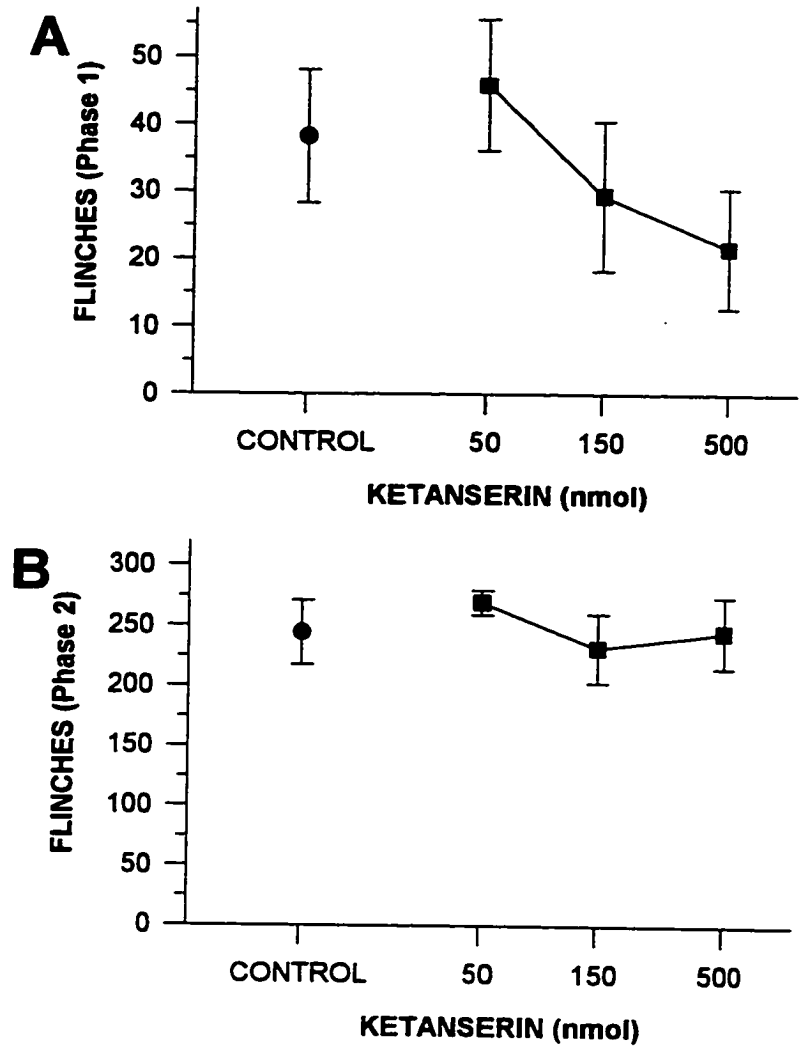


Figure 13. Flinching response in rats following subcutaneous injections of 2.5% formalin alone (control) and co-injected with increasing doses of ketanserin (n=5-6 rats/group, P = NS). Values are mean ± S.E.M. **A:** Phase 1 response (cumulative flinches in alternate 2 min bins between 2 and 12 min). **B:** Phase 2 response (cumulative flinches in alternate 2 min bins between 14 and 60 min).

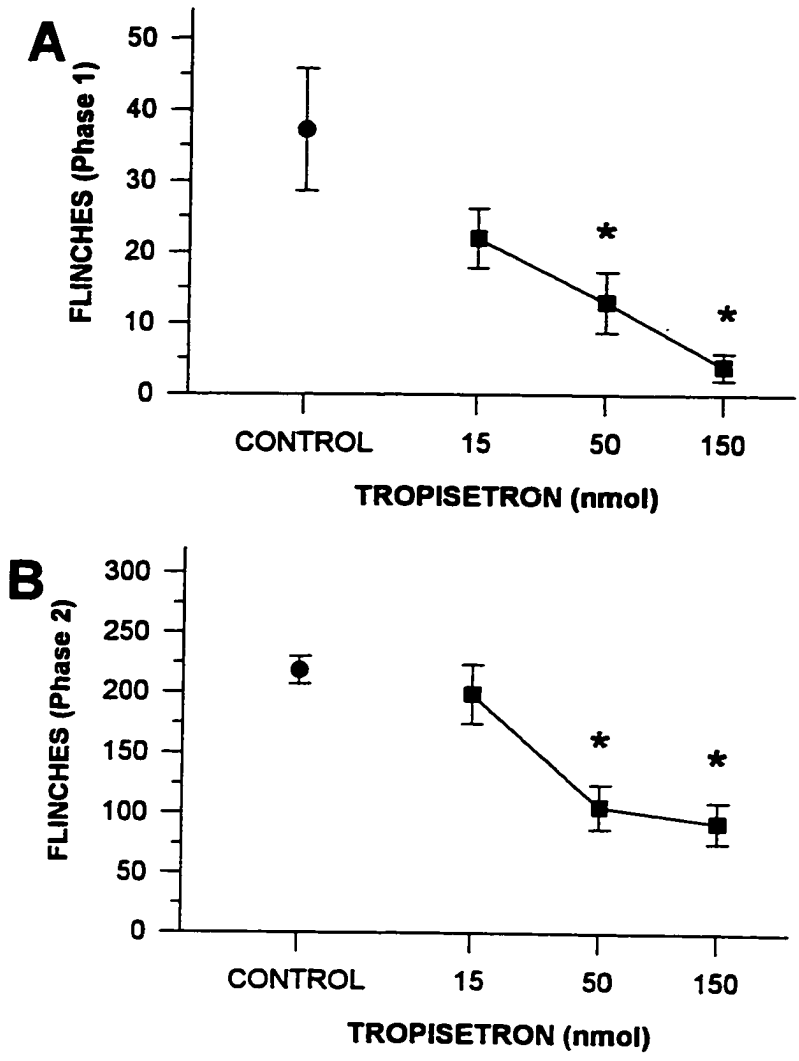


Figure 14. Flinching response in rats following subcutaneous injections of 2.5% formalin alone (control) and co-injected with increasing doses of tropisetron (n=5-6 rats/group, * $P < 0.05$ vs control). Values are mean $\pm$ S.E.M. **A:** Phase 1 response (cumulative flinches in alternate 2 min bins between 2 and 12 min). **B:** Phase 2 response (cumulative flinches in alternate 2 min bins between 14 and 60 min).

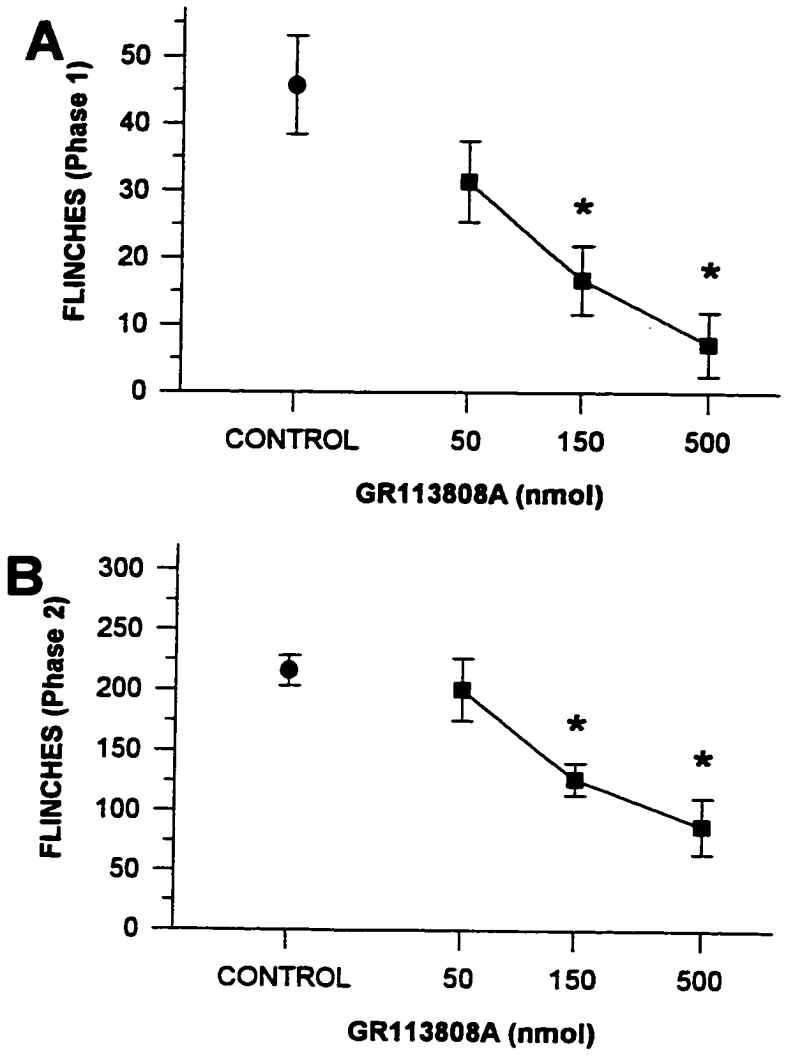


Figure 15. Flinching response in rats following subcutaneous injections of 2.5% formalin alone (control) and co-injected with increasing doses of GR113808A (n=5-6 rats/group, * P <0.05 vs control). Values are mean $\pm$ S.E.M. **A:** Phase 1 response (cumulative flinches in alternate 2 min bins between 2 and 12 min). **B:** Phase 2 response (cumulative flinches in alternate 2 min bins between 14 and 60 min).

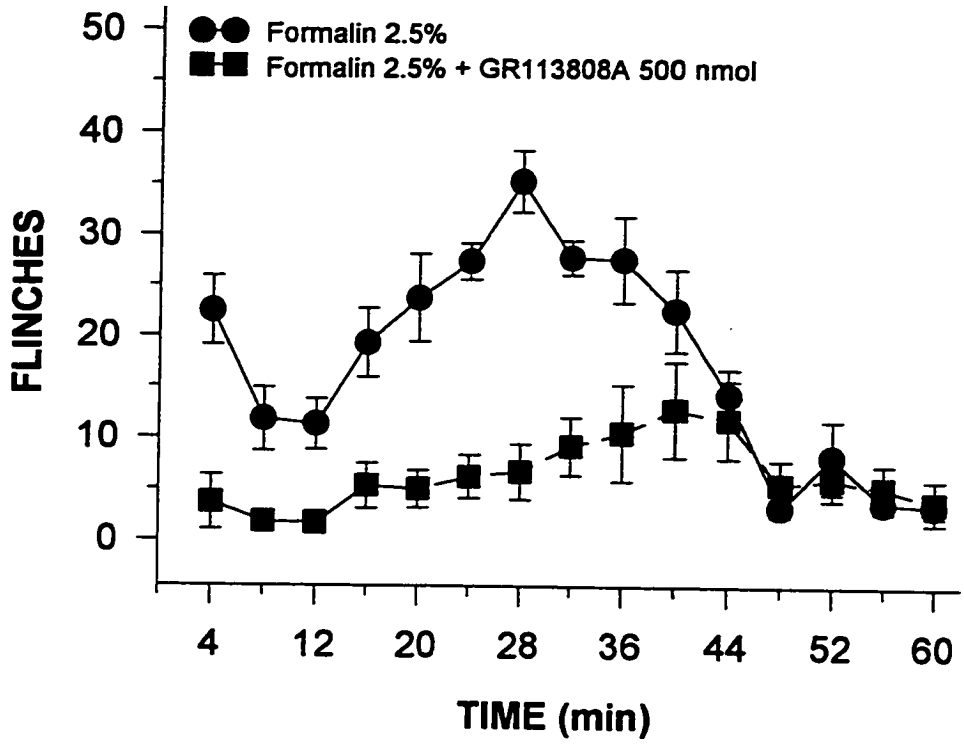


Figure 16. Effects of co-injection of GR113808A 500 nmol on the time course of flinching in response to 2.5% formalin. Each point represents cumulative flinches over the preceding 2 min bin. Each individual rat was observed on alternate 2 min bins beginning 2 min post-injection (n=5-6 rats/group). Values are mean ± S.E.M. Where S.E.M. bars are not shown they are within the symbol.

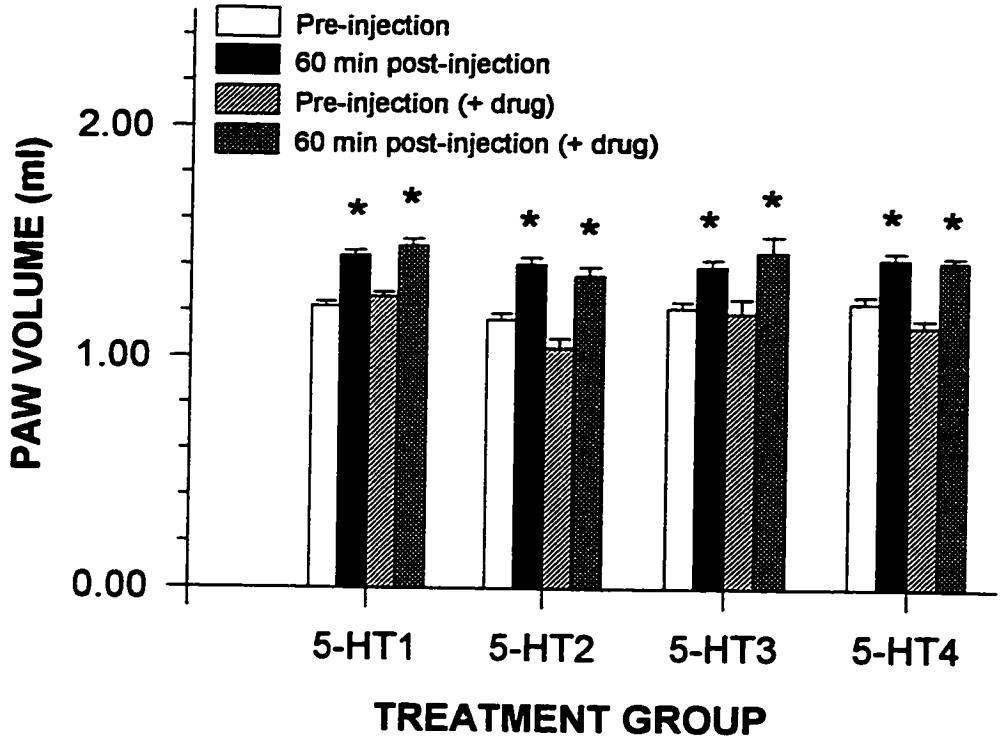


Figure 17. Rat hindpaw volumes, as measured by plethysmometry, before and 60 min after 2.5% formalin injection, alone or co-injected with selective 5-HT antagonists (n=5-6 rats/group, * $P < 0.05$ vs pre-injection). 5-HT1 = S(-) propranolol 50 nmol, 5-HT2 = ketanserin 500 nmol, 5-HT3 = tropisetron 150 nmol, and 5-HT4 = GR113808A 500 nmol. Values are mean $\pm$ S.E.M.

Effects of exogenous 5-HT and 5-HT agonists on the response to 0.5% formalin

Formalin induced flinching. The effect of exogenous 5-HT on a minimally stimulated model of acute inflammation was tested by co-injection of increasing doses of 5-HT with 0.5% formalin. The addition of 5-HT resulted in a dose related increase in flinching behaviour in both phases (Fig. 18). Phase 1 flinching was significantly increased with 5-HT doses of 5 nmol or greater ($P < 0.05$, Fig. 18A) while phase 2 flinching increased maximally with a dose of 15 nmol of 5-HT ($P < 0.05$, Fig. 18B). The time course of the flinching response to injection of 0.5% formalin plus 5-HT 15 nmol was revealing (Fig. 19). Not only did the addition of 5-HT augment formalin induced flinching behaviour in both phases, but the quiescent period or interphase, characteristic of the formalin test, was apparently obliterated. It should be noted that injection of 5-HT 15 nmol in saline had no behavioural effects on its own.

To further characterize the effects of exogenous 5-HT on formalin induced flinching, a number of selective 5-HT agonists were co-injected with 0.5% formalin. These results are summarized in Figure 20. When co-injected with 0.5% formalin, the 5-HT_1 receptor agonist 5-CT produced a dose related increase in flinching in both phases. Phase 1 flinching was not increased at a dose of 0.5 nmol whereas phase 2 flinching was significantly increased ($P < 0.05$, Fig. 20). At a higher dose of 5-CT (5 nmol), an effect on phase 1 was also seen but erythema of all four paws, the ears, and tail was noted by 8-10 minutes post injection, suggesting a systemic effect. The lowest effective dose of 5-CT (0.5 nmol) resulted in erythema and swelling (see below) confined to the injected paw.

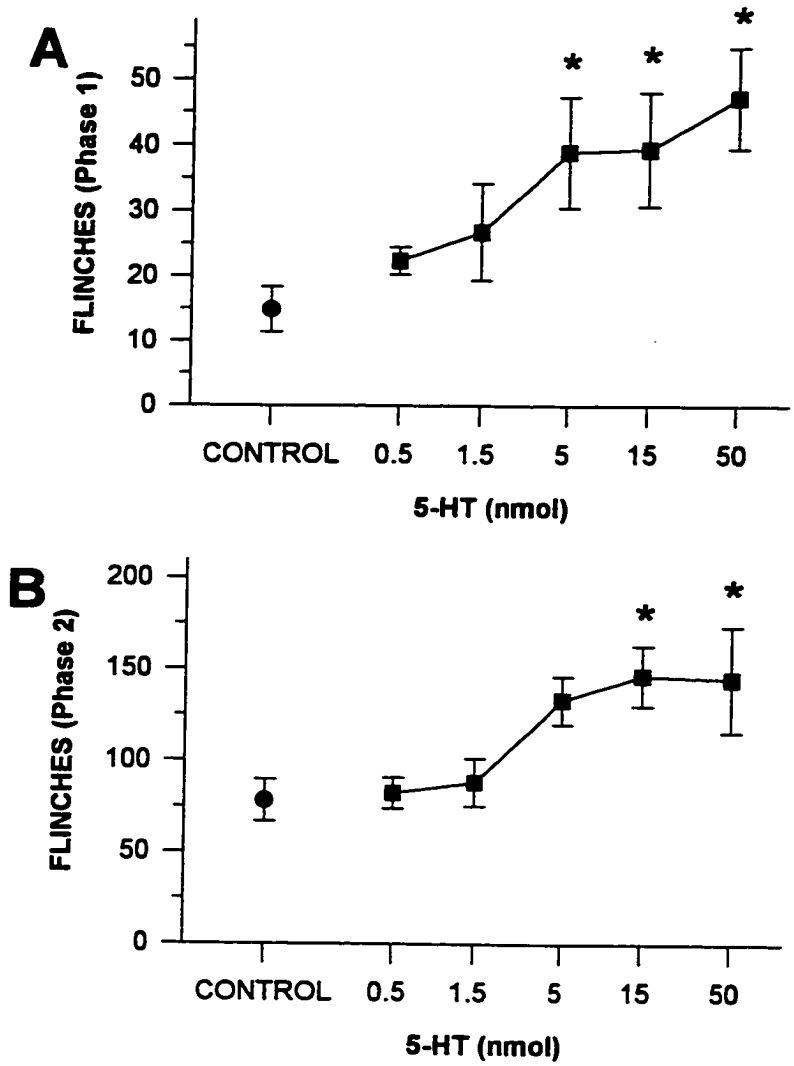


Figure 18. Flinching response in rats following subcutaneous injections of 0.5% formalin alone (control) and co-injected with increasing doses of 5-HT (n=5-6 rats/group, * P <0.05 vs control). Values are mean $\pm$ S.E.M. **A:** Phase 1 response (cumulative flinches in alternate 2 min bins between 2 and 12 min). **B:** Phase 2 response (cumulative flinches in alternate 2 min bins between 14 and 60 min).

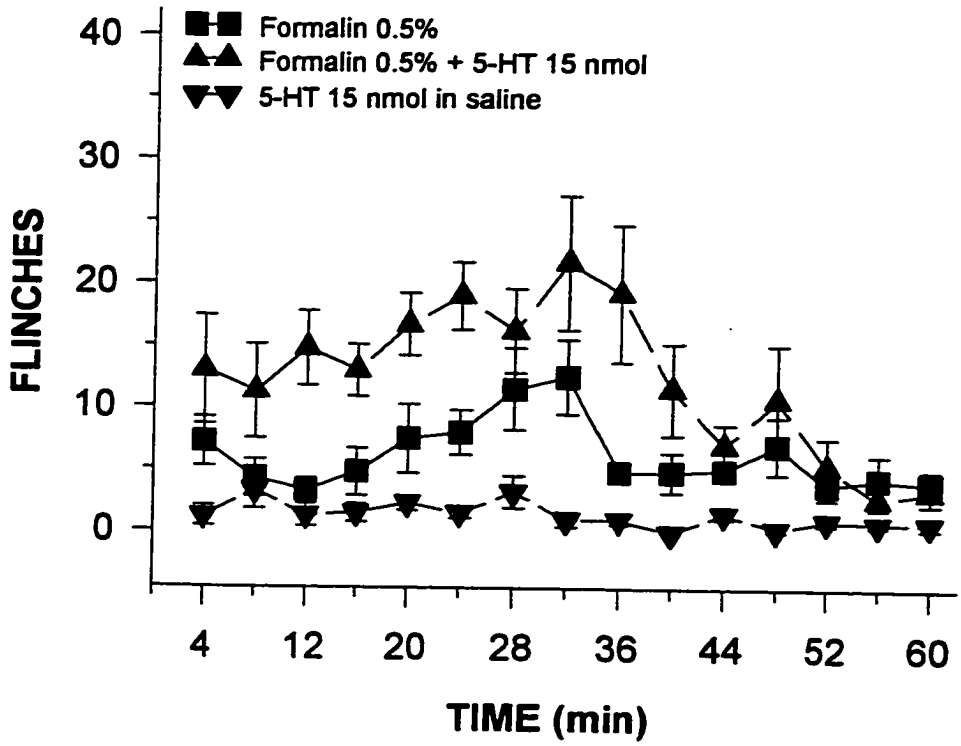


Figure 19. Time course of flinching in response to hindpaw injection of 0.5% formalin with or without co-injection of 5-HT 15 nmol, or 5-HT alone. Each point represents cummulative flinches over the preceding 2 min bin. Each individual rat was observed on alternate 2 min bins beginning 2 min post-injection (n=5-6 rats/group). Values are mean ± S.E.M. Where S.E.M. bars are not shown they are within the symbol.

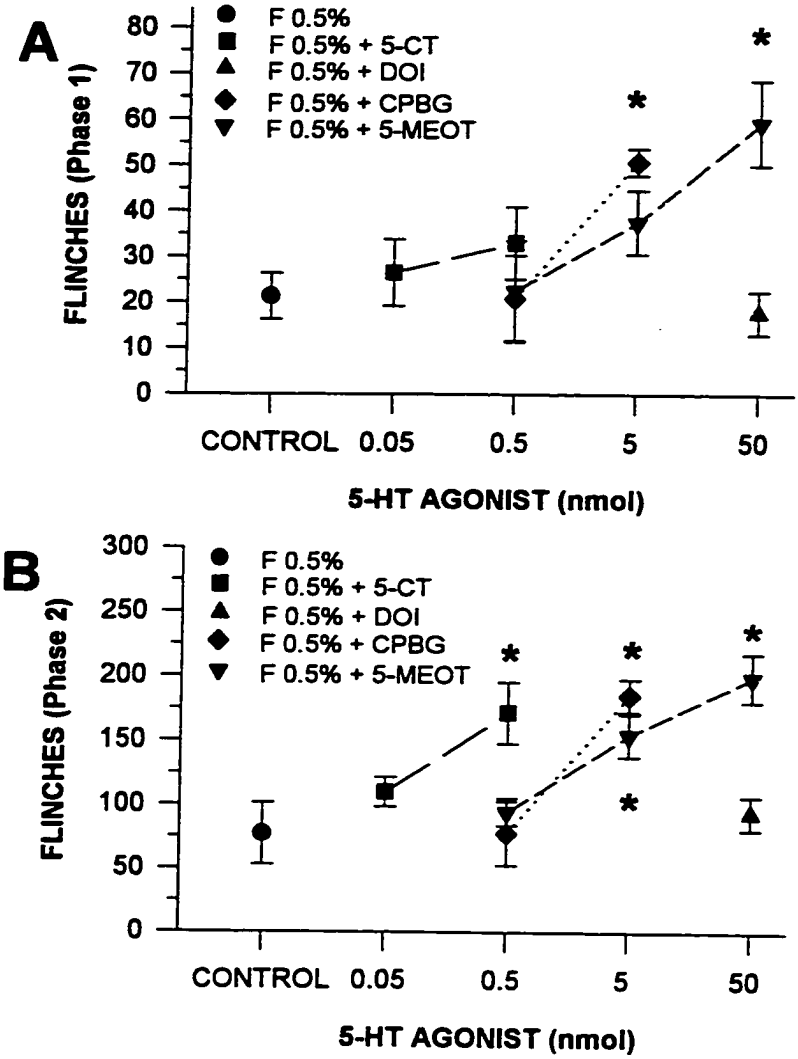


Figure 20. Flinching response in rats following subcutaneous injections of 0.5% formalin alone (control) and co-injected with increasing doses of selective 5-HT agonists (n=5-6 rats/group, * P <0.05 vs control). Values are mean ± S.E.M. **A:** Phase 1 response (cumulative flinches in alternate 2 min bins between 2 and 12 min). **B:** Phase 2 response (cumulative flinches in alternate 2 min bins between 14 and 60 min).

Because the 5-HT receptor antagonist data suggested no involvement of 5-HT_2 receptors in the formalin response, the 5-HT_2 receptor agonist DOI was tested only at a single dose (50 nmol), near its limit of solubility. DOI had no effect on either phase of 0.5% formalin induced flinching (Fig. 20).

The 5-HT_3 receptor agonist CPBG augmented both phases of formalin induced flinching maximally at a dose of 5 nmol ($P < 0.05$, Fig. 20). At a dose of 50 nmol of CPBG flinching in both phases dropped to control levels or lower and in 2 rats receiving 50 nmol, marked salivation was noted within 5 minutes of injection, suggesting possible systemic effects.

To test the effects of 5-HT_4 receptor stimulation, 5-MeOT was co-injected with 0.5% formalin. This resulted in a dose dependent increase in both phases of flinching ($P < 0.05$, Fig. 20). No evidence of systemic effects was seen even at the highest dose tested (50 nmol).

Examination of the time course for flinching in the presence of the selective 5-HT agonists again suggests an effect predominantly in the early part of phase 2. For example, the augmentation of phase 2 flinching by CPBG 5 nmol has largely subsided by 36 - 40 minutes (Fig. 21). The time courses for the other active agonists (5-CT and 5-MeOT) are qualitatively similar.

Formalin induced paw swelling. Co-injection of 5-HT with 0.5% formalin resulted in significant paw swelling at all doses tested (Fig. 22). The lowest dose of 5-HT tested (0.5 nmol) had no effect in the behavioural paradigm but modestly increased paw swelling ($P < 0.05$). The highest dose of 5-HT tested (50 nmol) maximally increased

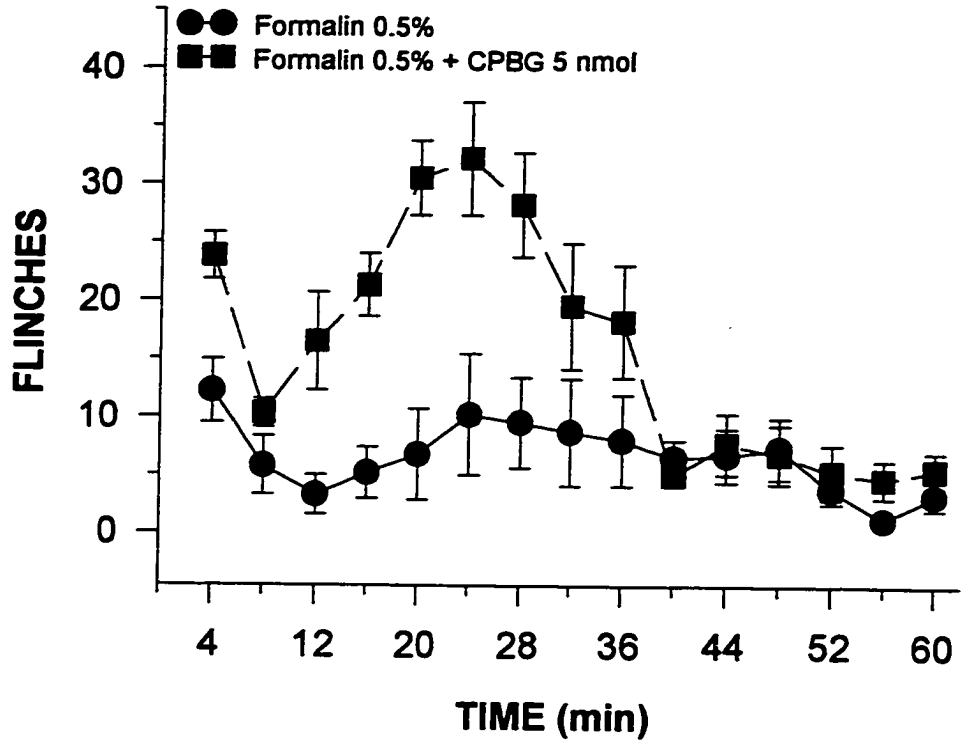


Figure 21. Effects of co-injection of CPBG 5 nmol on the time course of flinching in response to 0.5% formalin. Each point represents cumulative flinches over the preceding 2 min bin. Each individual rat was observed on alternate 2 min bins beginning 2 min post-injection (n=5-6 rats/group). Values are mean ± S.E.M. Where S.E.M. bars are not shown they are within the symbol.

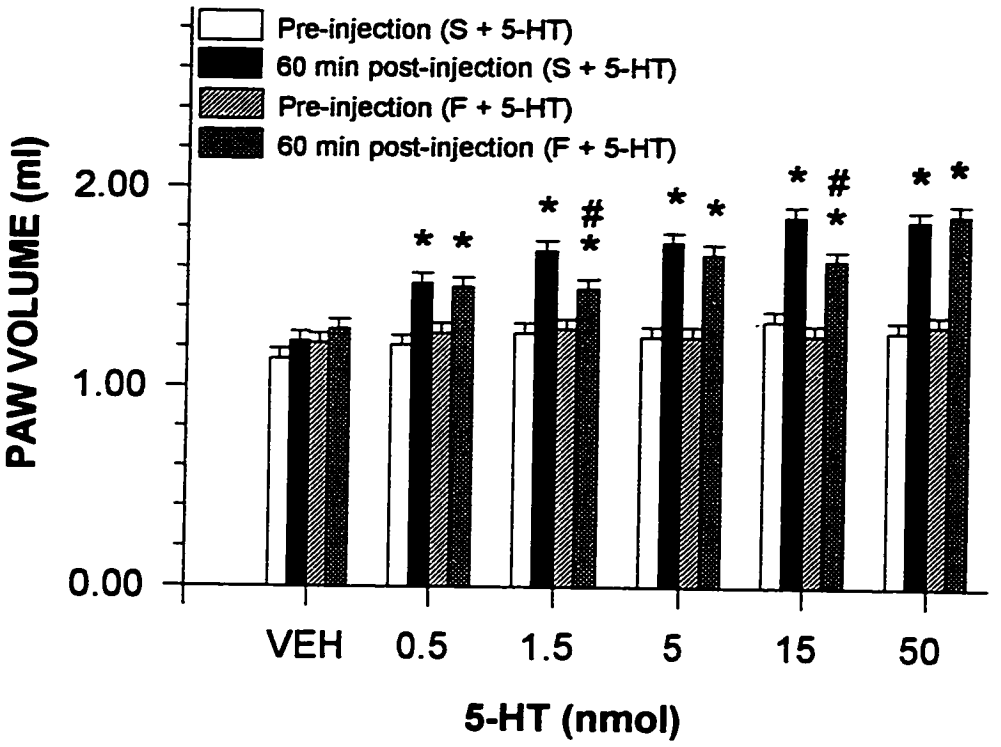


Figure 22. Rat hindpaw volumes, as measured by plethysmometry, before and 60 min after subcutaneous injection of increasing doses of 5-HT in saline (S+5-HT) or co-injected with 0.5% formalin (n=5-6 rats/group, * $P < 0.05$ vs pre-injection, # $P < 0.05$ vs post-injection S+5-HT). VEH = Saline or formalin injection without 5-HT. Values are mean $\pm$ S.E.M.

paw swelling ($P < 0.05$). Injection of 5-HT in saline (*i.e.* without formalin) resulted in paw swelling of similar magnitude (Fig. 22), suggesting no additional effect due to the presence of formalin. In fact, at 5-HT doses of 1.5 and 15 nmol, the presence of formalin resulted in significantly less swelling ($P < 0.05$) than was seen with corresponding 5-HT doses in saline (Fig. 22), a result that was inconsistent with the overall pattern and may have been an artefact.

Plethysmometry was carried out for all doses of the selective 5-HT receptor agonists co-injected with 0.5% formalin. Because the behavioural data suggested a possible systemic effect with higher doses of some of the drugs (see above), only the results of the highest or maximally effective doses without evidence of systemic effects are shown (Fig. 23). When co-injected with 0.5% formalin, 5-CT (0.5 nmol), DOI (50 nmol), and 5-MeOT (50 nmol) each resulted in increased paw swelling ($P < 0.05$) that was also significantly greater than that seen with 0.5% formalin alone ($P < 0.05$, Fig. 23). Interestingly, co-injection of 0.5% formalin and the 5-HT agonist CPBG (5 nmol), which produced markedly increased flinching behaviour, resulted in paw swelling that was no different than injection of 0.5% formalin by itself (Fig. 23).

V. Adenosine and 5-HT interactions in the formalin test

Effects of exogenous adenosine (without anaesthesia)

Formalin induced flinching. Since the effect of exogenous adenosine on the formalin response had been defined using the technique of injection of formalin under halothane anaesthesia, the adenosine dose-response relationship was redefined under

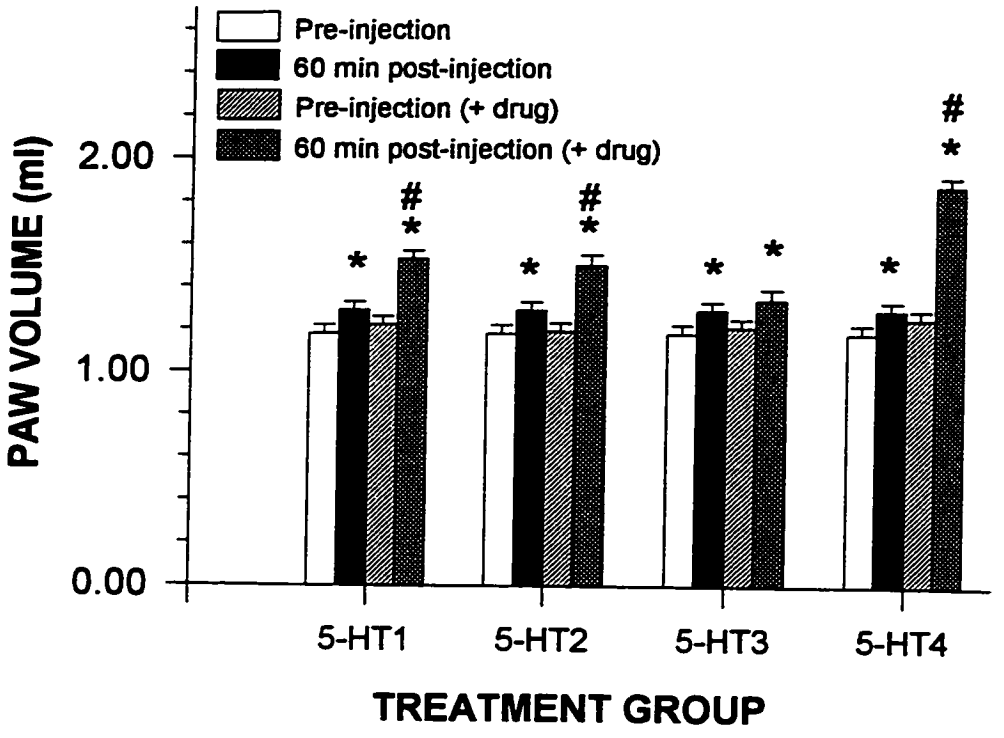


Figure 23. Rat hindpaw volumes, as measured by plethysmometry, before and 60 min after 0.5% formalin injection, alone or co-injected with selective 5-HT agonists (n=5-6 rats/group, * $P < 0.05$ vs pre-injection, # $P < 0.05$ vs post-injection formalin alone). 5-HT1 = 5-CT 0.5 nmol, 5-HT2 = DOI 50 nmol, 5-HT3 = CPBG 5 nmol, and 5-HT4 = 5-MeOT 50 nmol. Values are mean ± S.E.M.

conditions of no anaesthesia. Increasing doses of adenosine (15 - 500 nmol) were co-injected with 0.5% formalin. Phase 1 flinching was progressively augmented by increasing doses of adenosine although only the highest dose tested (500 nmol) resulted in flinching significantly greater than formalin alone ($P < 0.05$, Fig. 24A). Phase 2 flinching was maximally augmented by the addition of adenosine 50 nmol ($P < 0.05$), an effect which appeared to plateau at higher doses (Fig. 24B).

Formalin induced paw swelling. The effect of adenosine on formalin induced paw swelling had not previously been measured. Modest swelling of the injected paw occurred with injection of 0.5% formalin ($P < 0.05$) but this was not further influenced by the addition of adenosine at any dose tested.

Interaction of exogenous adenosine and 5-HT

Formalin induced flinching. From the data on 5-HT interactions with the formalin test (Fig. 18), a dose of 5-HT (1.5 nmol) that was by itself ineffective in augmenting 0.5% formalin was selected to be added to adenosine 15 and 50 nmol, in the presence of 0.5% formalin. The addition of 1.5 nmol of 5-HT had no effect on phase 1 but significantly augmented phase 2 of 0.5% formalin induced flinching in the presence of adenosine 15 nmol ($P < 0.05$, Fig. 25). This effect of 5-HT appeared to have a ceiling as it was no longer significant when added to 0.5% formalin and adenosine 50 nmol. From the time course of flinching associated with 0.5% formalin plus adenosine 15 nmol with or without 5-HT 1.5 nmol (Fig. 26), it is apparent that most of the augmentation of phase 2 flinching

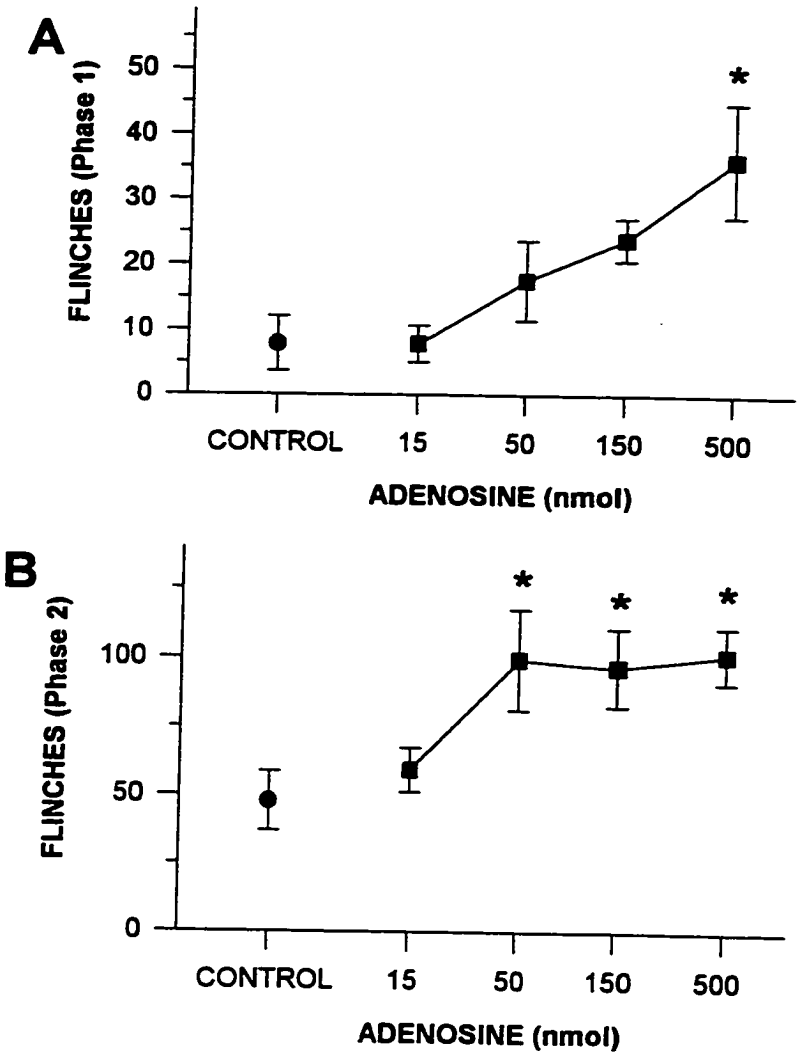


Figure 24. Flinching response in rats following subcutaneous injections of 0.5% formalin alone (control) and co-injected with increasing doses of adenosine (n=6 rats/group, * P <0.05 vs control). Values are mean $\pm$ S.E.M. **A:** Phase 1 response (cumulative flinches in alternate 2 min bins between 2 and 12 min). **B:** Phase 2 response (cumulative flinches in alternate 2 min bins between 14 and 60 min).

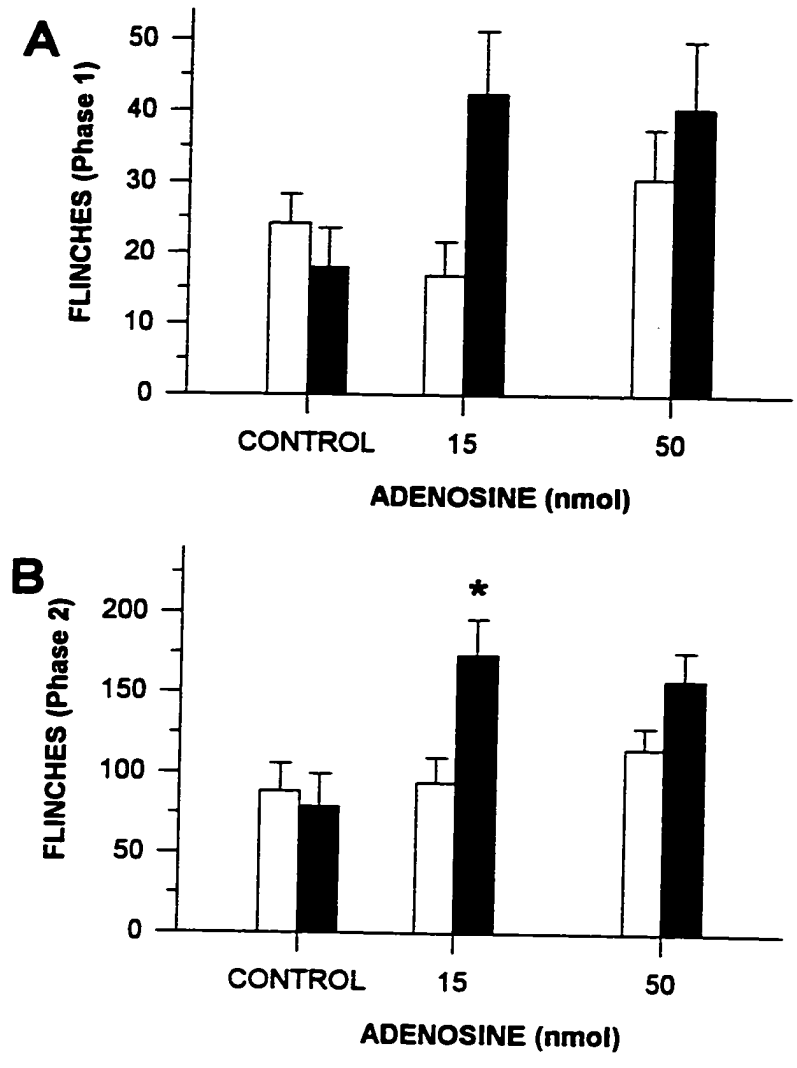


Figure 25. Flinching response in rats following subcutaneous injections of 0.5% formalin alone (control) and co-injected with adenosine 15 and 50 nmol with (■) and without (□) 5-HT 1.5 nmol (n=6 rats/group, * P <0.05 vs control). Values are mean ± S.E.M. **A:** Phase 1 response (cumulative flinches in alternate 2 min bins between 2 and 12 min). **B:** Phase 2 response (cumulative flinches in alternate 2 min bins between 14 and 60 min).

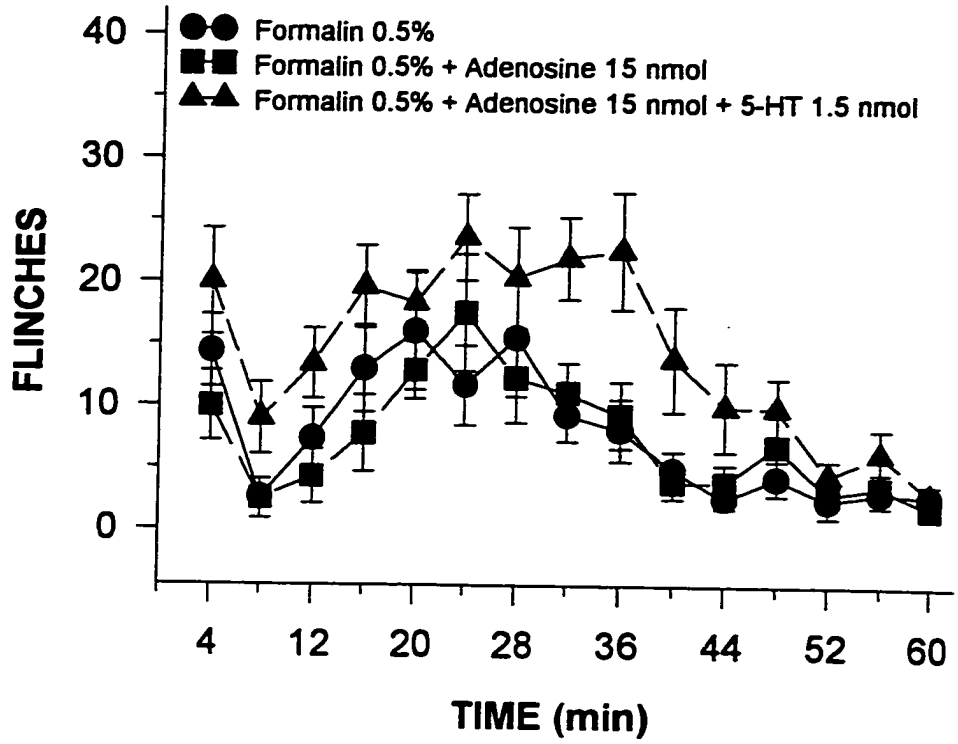


Figure 26. Time course of flinching in response to hindpaw injection of 0.5% formalin alone or with co-injection of adenosine 15 nmol, or adenosine 15 nmol plus 5-HT 1.5 nmol. Each point represents cummulative flinches over the preceding 2 min bin. Each individual rat was observed on alternate 2 min bins beginning 2 min post-injection (n=6 rats/group). Values are mean ± S.E.M. Where S.E.M. bars are not shown they are within the symbol.

associated with the addition of 5-HT results from a prolongation of augmented flinching (*i.e.* into phase 2B), rather than an increase in flinching frequency.

Formalin induced paw swelling. As before, the addition of adenosine (15 - 50 nmol) had no effect on the paw swelling associated with 0.5% formalin. In contrast to the effect on flinching, the combination of 5-HT 1.5 nmol with adenosine, in the presence of 0.5% formalin resulted in no augmentation of paw swelling.

Effect of the A_2 receptor antagonist DMPX on 5-HT augmentation of the formalin response

The hypothesis that a component of the effect of 5-HT in augmenting the response to 0.5% formalin might result from release of adenosine from endogenous stores was tested using the A_2 receptor antagonist DMPX in an attempt to block the 5-HT induced augmentation. A dose of DMPX (15 nmol) previously demonstrated to be effective in blocking 2.5% formalin induced flinching (Fig. 8) was co-injected with 0.5% formalin and 5-HT 15 nmol.

Formalin induced flinching. While no effect on phase 1 was observed, 5-HT 15 nmol significantly augmented phase 2 flinching when co-injected with 0.5% formalin (Fig. 27). The addition of DMPX had no effect on flinching associated with 0.5% formalin alone or the 5-HT augmented flinching (Fig. 27).

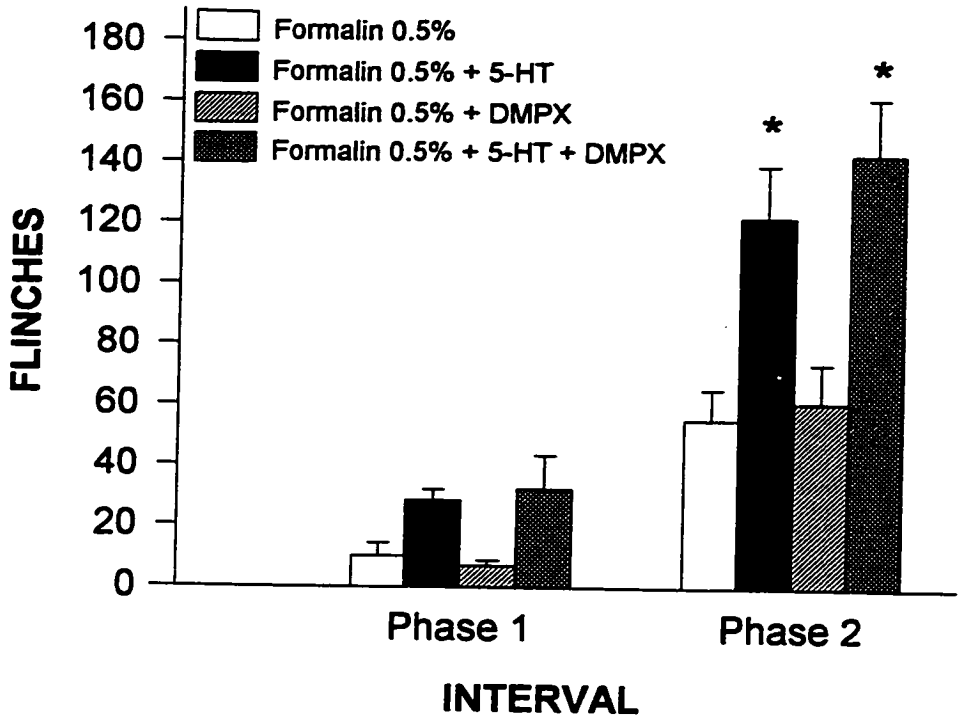


Figure 27. Flinching response in rats following subcutaneous injections of 0.5% formalin alone, formalin plus 5-HT 15 nmol, formalin plus DMPX 15 nmol, or formalin plus 5-HT and DMPX (n=6 rats/group, * P <0.05 vs formalin alone). Values are mean ± S.E.M. Phase 1 response (cumulative flinches in alternate 2 min bins between 2 and 12 min). Phase 2 response (cumulative flinches in alternate 2 min bins between 14 and 60 min).

Formalin induced paw swelling. 5-HT 15 nmol increased paw swelling above that associated with 0.5% formalin alone ($P < 0.05$, Fig. 28). DMPX 15 nmol had no effect on the paw swelling associated with 0.5% formalin, nor did it block the augmented paw swelling associated with 0.5% formalin and 5-HT (Fig. 28).

Effect of 5-HT receptor subtype selective antagonists on adenosine augmentation of the formalin response

To test the hypothesis that adenosine induced augmentation of the formalin response might be mediated through release of endogenous 5-HT, the 5-HT receptor antagonists previously shown to be effective in blocking 2.5% formalin induced flinching (Fig. 12-15) were tested in the presence of 0.5% formalin and adenosine 50 nmol.

Formalin induced flinching. Adenosine 50 nmol significantly augmented flinching associated with 0.5% formalin in both phases ($P < 0.05$, Fig. 29-32). The addition of the 5-HT_1 receptor antagonist S(-) propranolol (50 nmol) eliminated this augmentation in both phases ($P < 0.05$, Fig. 29). S(-) propranolol also significantly reduced the flinching associated with 0.5% formalin alone in phase 2 ($P < 0.05$), but not phase 1 (Fig. 29).

Ketanserin, a 5-HT_2 receptor antagonist, at a dose of 500 nmol had no effect on either phase 1 or phase 2 flinching induced by 2.5% (Fig. 13) or 0.5% formalin (Fig. 30). It is interesting to note however, that the augmentation of flinching associated with adenosine co-injected with 0.5% formalin was abolished in both phases by the presence of ketanserin without any concomitant intrinsic effect ($P < 0.05$, Fig. 30).

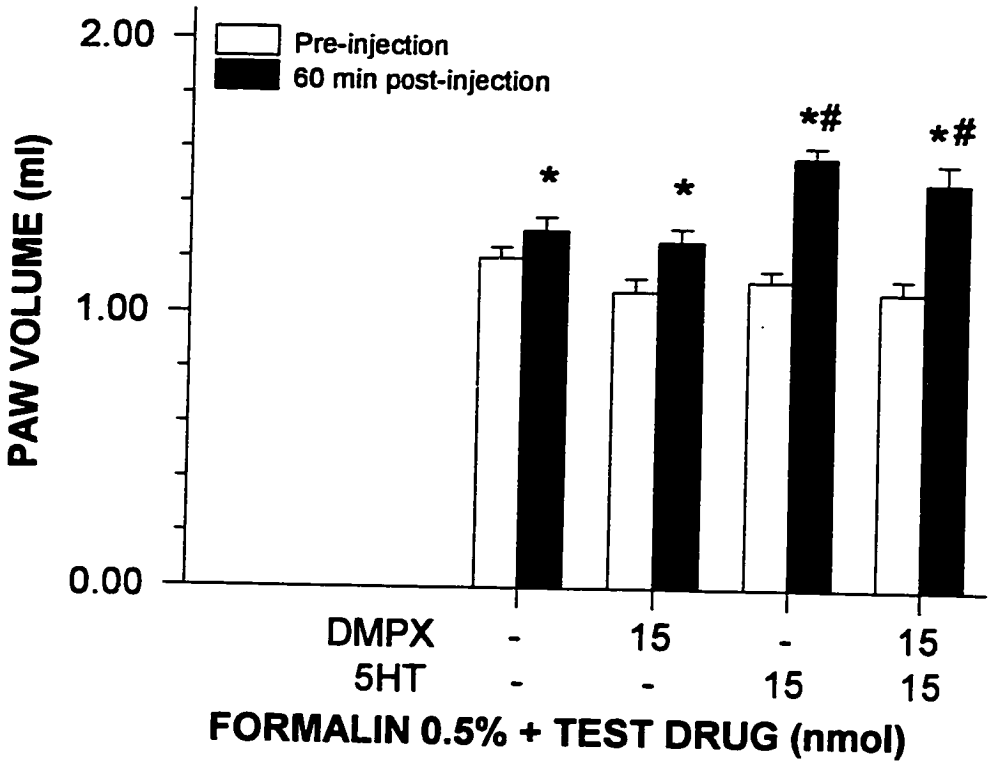


Figure 28. Rat hindpaw volumes, as measured by plethysmometry, before and 60 min after 0.5% formalin injection, alone or co-injected with combinations of DMPX 15 nmol and 5-HT 15 nmol (n=6 rats/group, * $P < 0.05$ vs pre-injection, # $P < 0.05$ vs post-injection formalin alone). Values are mean ± S.E.M.

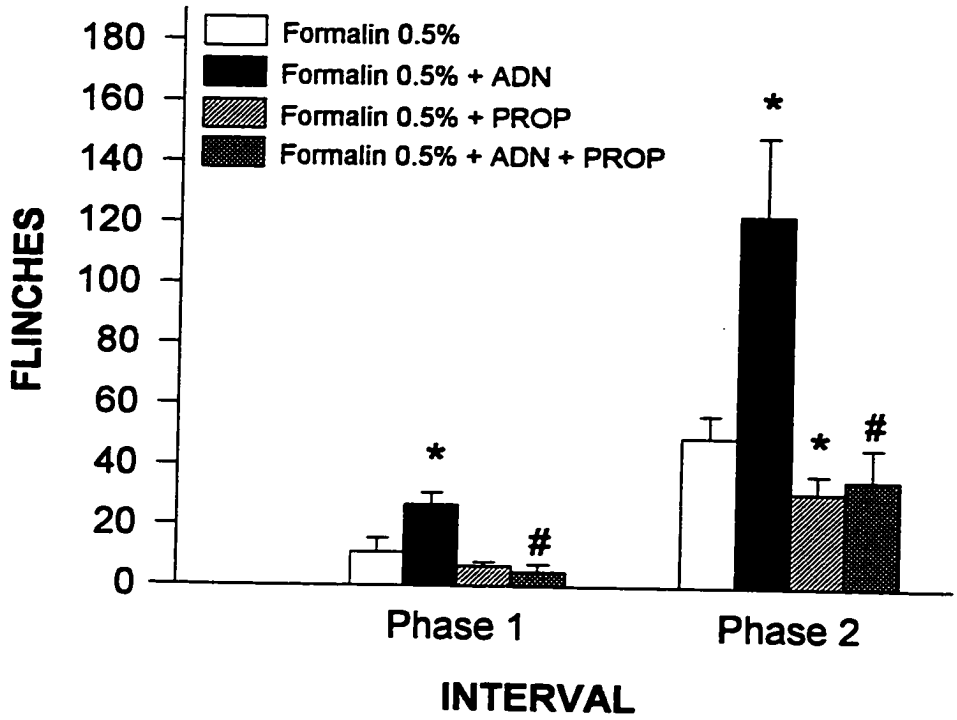


Figure 29. Flinching response in rats following subcutaneous injections of 0.5% formalin alone, formalin plus adenosine (ADN) 50 nmol, formalin plus S(-) propranolol (PROP) 50 nmol, or formalin plus adenosine and S(-) propranolol (n=6 rats/group, * P <0.05 vs formalin alone, # P < 0.05 vs formalin + ADN). Values are mean ± S.E.M. Phase 1 response (cumulative flinches in alternate 2 min bins between 2 and 12 min). Phase 2 response (cumulative flinches in alternate 2 min bins between 14 and 60 min).

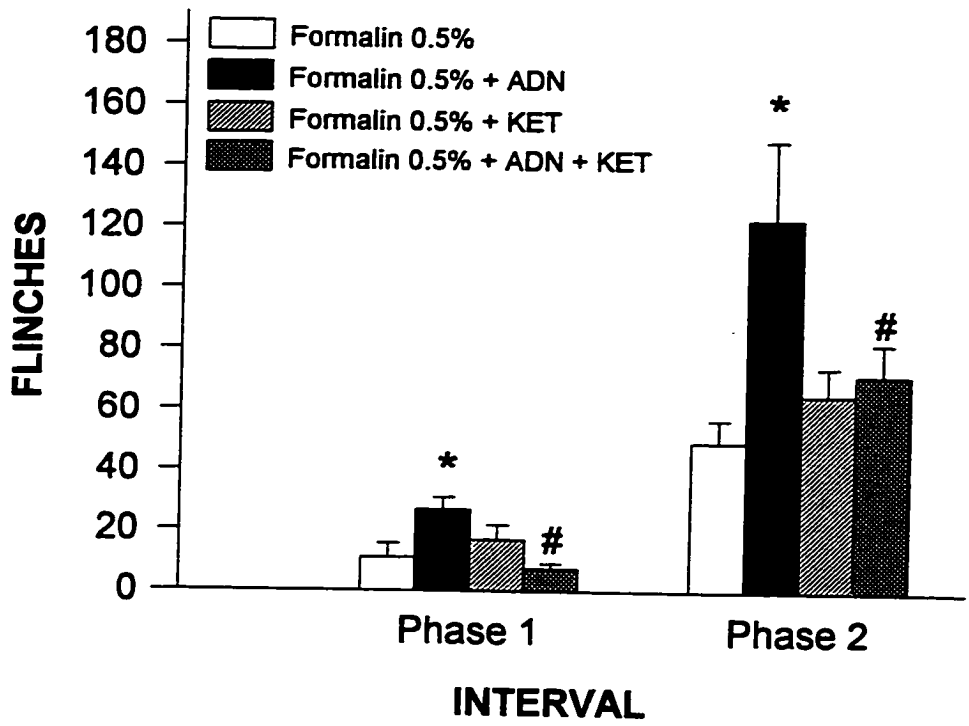


Figure 30. Flinching response in rats following subcutaneous injections of 0.5% formalin alone, formalin plus adenosine (ADN) 50 nmol, formalin plus ketanserin (KET) 500 nmol, or formalin plus adenosine and ketanserin (n=6 rats/group, * $P < 0.05$ vs formalin alone, # $P < 0.05$ vs formalin + ADN). Values are mean ± S.E.M. Phase 1 response (cumulative flinches in alternate 2 min bins between 2 and 12 min). Phase 2 response (cumulative flinches in alternate 2 min bins between 14 and 60 min).

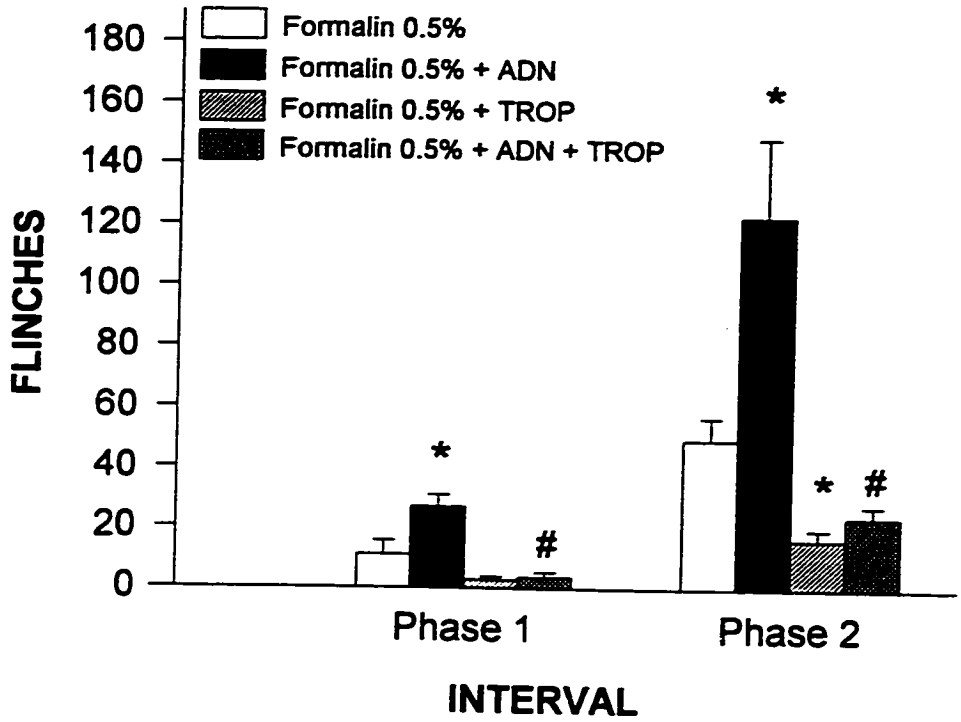


Figure 31. Flinching response in rats following subcutaneous injections of 0.5% formalin alone, formalin plus adenosine (ADN) 50 nmol, formalin plus tropisetron (TROP) 150 nmol, or formalin plus adenosine and tropisetron (n=6 rats/group,

* P <0.05 vs formalin alone, # P < 0.05 vs formalin + ADN). Values are mean ± S.E.M. Phase 1 response (cumulative flinches in alternate 2 min bins between 2 and 12 min). Phase 2 response (cumulative flinches in alternate 2 min bins between 14 and 60 min).

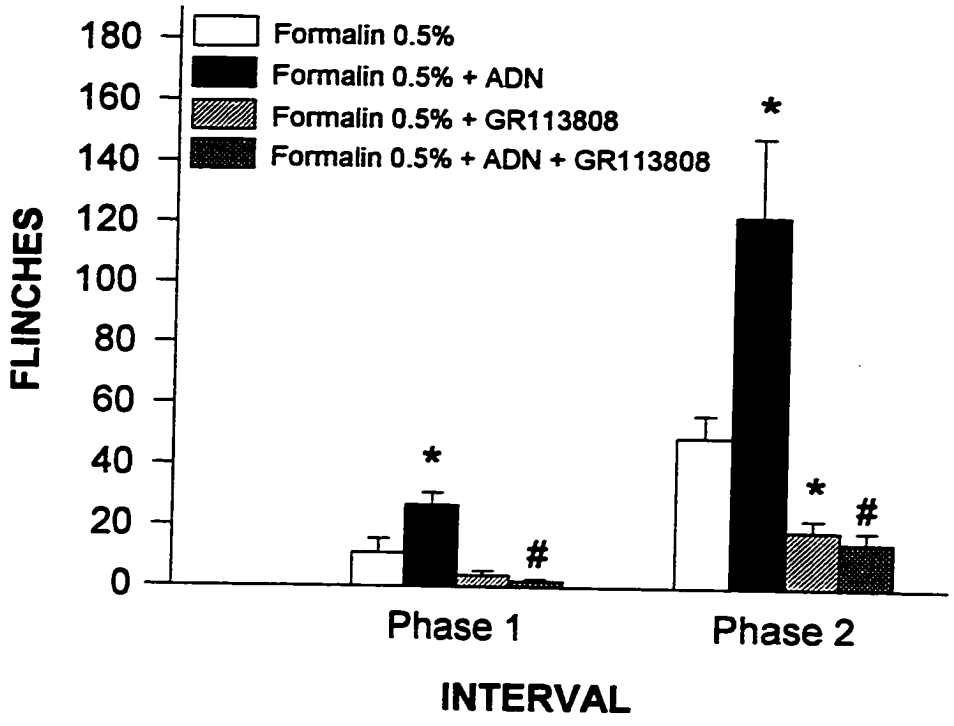


Figure 32. Flinching response in rats following subcutaneous injections of 0.5% formalin alone, formalin plus adenosine (ADN) 50 nmol, formalin plus GR113808A 500 nmol, or formalin plus adenosine and GR113808A (n=6 rats/group, * P <0.05 vs formalin alone, # P < 0.05 vs formalin + ADN). Values are mean ± S.E.M. Phase 1 response (cumulative flinches in alternate 2 min bins between 2 and 12 min). Phase 2 response (cumulative flinches in alternate 2 min bins between 14 and 60 min).

The 5-HT_3 receptor antagonist, tropisetron (150 nmol) reduced phase 2 flinching associated with 0.5% formalin alone ($P < 0.05$) and in both phases eliminated the augmentation of flinching seen with the addition of adenosine ($P < 0.05$, Fig. 31).

The highly selective 5-HT_4 receptor antagonist GR113808A (500 nmol) reduced phase 2 flinching induced by 0.5% formalin ($P < 0.05$) and in both phases eliminated the augmentation of flinching seen with the addition of adenosine ($P < 0.05$, Fig. 32).

Formalin induced paw swelling. As before, 0.5% formalin resulted in significant paw swelling ($P < 0.05$) that was unaffected by the addition of adenosine 50 nmol. None of the 5-HT receptor antagonists, at the doses tested (see above), had any effect on formalin induced paw swelling.

DISCUSSION

I. The formalin test model

The formalin test as a model for acute inflammatory pain

Subcutaneous formalin injection in the hindpaw of rats results in a dose related increase in flinching. Phase 1 of the formalin test has been suggested to result from direct sensory activation, while phase 2 involves peripheral inflammatory mediators (Dubuisson and Dennis 1977; Hunskaar *et al.* 1986) and central sensitization (Coderre *et al.* 1990). The relative importance of peripheral inflammation versus central sensitization to the phase 2 response is controversial (Coderre *et al.* 1990, Haley *et al.* 1990), however the evidence suggests that spinal perception of phase 1 is not necessary for development of the phase 2 response (Haley *et al.* 1990; Dallel *et al.* 1995).

When prepared with saline as the diluent, formalin solutions (0.5 - 5.0%) are acidic, having a pH ranging from 4.15 to 3.33 (Table V). This may account for some of its irritant properties, particularly in the first phase, since protons are known to excite and sensitize nociceptors (Bevan and Geppetti 1994; Steen *et al.* 1995). Formaldehyde is highly reactive and binds to free amino groups, resulting in change or destruction of proteins. This may occur with concentrations as low as 0.5% formaldehyde (*i.e.* 1.35% stock formalin solution) (Harvey 1985). Damage to cell surface proteins, particularly in the presence of formalin concentrations in excess of 1%, may contribute to more prolonged reactions, possibly provoking the inflammatory response. Indeed, Rosland *et al.* (1990) observed marked histological changes in the paws of mice injected with

formalin concentrations greater than 0.2%. The highest concentrations produced a depilated scar (1%) or ulceration (5%) at an interval of 24 hours post-injection.

The phase 2 behavioural response in rats appears to plateau at formalin concentrations of 2.5-5% so there appears to be little to be gained by using higher concentrations. Indeed, it has been suggested that termination of the response, after 60 minutes or so, is due to a toxic effect of formalin on the peripheral nerves, since the inflammatory response is ongoing (Tjølsen *et al.* 1992). Conversely, use of lower concentrations of formalin may produce a submaximal flinching response that may reveal the antinociceptive effects of mild analgesics or may be augmented by pronociceptive substances.

Methodological issues

As with any behavioural paradigm, as the formalin test evolved, a number of methodological issues have surfaced, including methods of scoring, site of injection, and characteristics of the test environment.

The quantification of the nociceptive response as described by Dubuisson and Dennis (1977) was based on time spent by the animal in each of 4 mutually exclusive behaviours (0-3). This resulted in averaged pain scores from 30 second blocks versus time. Originally, the forepaw was injected but in some species normal grooming behaviour could be confused with pain related behaviour, so it is now customary to inject the hindpaw. This method of weighted scores assumes that the rated behaviours are related to nociception, that the different behaviours are not fundamentally different but represent

degrees of a single nociceptive experience, and finally that the numbers assigned to each behaviour are directly related to the perceived intensity of the pain. Despite these assumptions, the weighted scoring technique, with some modifications, has been widely used (reviewed, Tjølsen *et al.* 1992) and appears to be a valid measure of analgesic activity (Coderre *et al.* 1993).

In an effort to simplify and increase the objectivity of the scoring method, scoring of a single parameter such as time spent licking the affected paw (Sugimoto *et al.* 1986) or counting paw flinching (or lifting) within specific intervals of time (Ryan *et al.* 1985; Wheeler-Aceto *et al.* 1990) has been advocated. In rats, the time course and analgesic sensitivity of single parameter scoring such as flinching correlates well with the weighted scoring technique of Dubuisson and Dennis (1977) and the electrophysiological data from Dickenson and Sullivan (1987). Formalin induced flinching behaviour has been shown to be more robust than the paw licking response and less affected by other behavioural influences (Wheeler-Aceto and Cowan, 1991).

Either the dorsal or plantar surface of the hindpaw has been used as the injection site. Qualitatively there appears to be little difference in behavioural outcome, although a recent study has suggested that plantar injections, because there is less diffusion into surrounding tissue, may result in higher formalin concentrations and greater desensitization (Puig and Sorkin 1995).

The test environment has been shown to have a significant influence on the animals' behaviour in response to formalin injection. Stress, in itself, may induce analgesia or a characteristic "freeze" response in rats (Faneslow 1984; Helmstetter 1993).

Stressors may include sounds, odors, bright lights, or improper handling. Creation of a dedicated testing area, in a quiet, low traffic area has been recommended and allowing the animals time to explore the test environment prior to injection results in more consistent nociceptive behaviour, particularly in the first phase (Tjølsen *et al.* 1992). The ambient temperature appears to influence nociceptive behaviour in response to formalin, in particular, lower room temperatures result in reduced responses, at least in mice (Rosland 1991). Whether this is important in larger species (*i.e.* rats) is not clear, but avoidance of extremes and consistency among trials has been recommended.

Priming the nociceptor - utility of low and high concentration formalin

There have been a number of approaches to the investigation of peripheral nerve excitation/sensitization including behavioural and electrophysiological approaches (reviewed, Treede *et al.* 1992). Many of these approaches have involved application of putative mediators, alone or in combination, and looking for some response signalling activation of nociceptors. Recently, Hong and Abbott (1994) described a behavioural paradigm utilizing a behavioural rating scale modelled after the formalin test. They injected various combinations of inflammatory mediators and observed behaviours representative of hyperalgesia (favouring) or pain (lifting / licking). They were able to demonstrate the dependence of the pronociceptive effect of some mediators such as SP, histamine, or norepinephrine on the presence of another, such as 5-HT. This underlines the importance of the appropriate conditions for the manifestation of some mediators' effects. Although multiple combinations of mediators such as employed by Hong and

Abbott (1994) provide valuable information, the appropriate "mix" in the "inflammatory soup" can only be arrived at empirically and the possibility exists of inadvertently excluding a key ingredient.

Higher concentrations of formalin (2.5% and 5%) result in near maximal flinching responses in phase 1 and phase 2 and are associated with a well developed inflammatory response. These are concentrations which have been used by others to detect analgesic effects (reviewed, Tjølsen *et al.* 1992). Lower concentrations of formalin (*e.g.* 0.5%) produce less flinching and minimal edema. Although the inflammatory response resulting from injection of 0.5% formalin is proportionately reduced, it is presumed to include all the necessary components. This submaximal activation of the inflammatory-nociceptive process sets up a milieu into which may be co-injected potential inflammatory or nociceptive mediators, which by themselves may be inactive, but which may become inflammatory or pronociceptive in sensitized tissues.

Formalin induced paw swelling

Subcutaneous formalin injection in the rat hindpaw produces paw swelling that is dose related. Although in this study, paw swelling was measured only once post-formalin injection, at 60 min, previous work indicates that almost 75% of the swelling occurs within the first 60 minutes after formalin injection (Wheeler-Aceto *et al.* 1990). The peak in formalin induced paw swelling is reported to occur at about 4 hours, well after the peak behavioural response which is usually seen at 25-35 minutes (Wheeler-Aceto *et al.* 1990). This apparent divergence between nociceptive behaviour and degree of inflammation as

measured by paw swelling was also seen in the context of the various adenosine and 5-HT analogues tested (see below). Other irritant substances, such as yeast or carrageenan, typically result in significant edema without any spontaneous display of pain behaviour (*i.e.* vocalization or paw flinching), although hyperalgesia to heat or pressure may be seen (Wheeler-Aceto *et al.* 1990). It is possible that some degree of peripheral nerve desensitization may occur at the injection site, particularly with higher formalin concentrations. This appears to be the case with Aβ fibres and may also apply to C- and Aδ fibres (Puig and Sorkin 1995). If this were to occur, the result might be a decreasing nociceptive signal (and behavioural response) in the face of ongoing or even increasing inflammation.

Limitations of the present model

The conclusions that may be derived from the data generated using the formalin model may be limited in their generalizability. A number of issues must be considered when interpreting the data and attempting to apply the conclusions to other species or pain of possibly different origin.

Although the rodent model is valuable because of its low cost, adaptability, and uniformity as compared to a primate model, the possibility of important interspecies differences must be considered. The rodent mast cell is a potential source of 5-HT while human mast cells do not contain 5-HT. Obviously such a difference has to be considered in interpreting the present data concerning the role of 5-HT in inflammatory pain. Differences in receptor morphology and functional distribution (*e.g.* 5-HT_{1B} vs 5-HT_{1D})

among species is another example of the limitations imposed by animal models. Conclusions about the mechanisms of inflammatory pain in other species cannot be inferred from the present data.

Interpretation of the behavioural reponse and quantifying this in terms of a nociceptive score has already been alluded to (see above), but it is also important to recognize the kind of pain that is being modelled and how any conclusions about the mechanism can be applied to other types of pain. It is well established that analgesics may act at multiple levels of the CNS (*e.g.* peripheral nerves, spinal cord, and brain) and it is becoming increasingly evident that there are different types of pain with different underlying mechanisms (Franklin and Abbott 1989). As previously discussed, the formalin model is one of mild tissue injury with an inflammatory component. The presence of tissue injury (and the mediators of inflammation released by this injury) differentiates this from threshold tests such as the hot-plate or tail-flick models. The duration of the inflammatory component is less than that associated with chronic inflammatory models such as adjuvant-induced arthritis, which are associated with development of an autoimmune response (Franklin and Abbott 1989). The formalin model has been reported to demonstrate neuroplastic changes at the spinal cord level, in the form of increased responsiveness to peripheral stimuli (Coderre et al. 1990). The experimental design used in this thesis did not allow any observation of spinal neuroplasticity and focused only on interactions at the peripheral nerve ending. Although there is a significant inflammatory component to the second phase of the formalin model, conclusions about the mediators and

receptor subtypes involved cannot necessarily be applied to models of chronic inflammatory pain.

II. Adenosine and formalin induced inflammatory pain

The adenosine data suggest a dual role for adenosine in the periphery in the development of tonic inflammatory pain associated with the formalin test. The effect of endogenous adenosine under conditions of inflammation may involve coincidental activation of adenosine A_1 and A_2 receptor subtypes resulting in antinociceptive and pronociceptive effects, respectively. At the same time adenosine may exert an antiinflammatory effect on cellular components such as neutrophils and other inflammatory cells (Cronstein 1994). The absence of any independent pronociceptive effect of adenosine suggests the requirement of co-mediators for adenosine to exert its hyperalgesic effects.

Adenosine and formalin induced nociception

Adenosine augmented the pain associated with 0.5% formalin injection while eliciting no intrinsic effect on behaviour. This pronociceptive effect appears to be due to adenosine A_2 receptor activation since the A_2 selective agonist, CGS21680 is also pronociceptive. This is consistent with the response to adenosine analogues seen in the phasic paw-pressure withdrawal test (Taiwo and Levine 1990) and the first phase of the formalin test in mice, as determined by the time spent licking or biting the injected paw (Karlsten *et al.* 1992). In both of these studies, adenosine A_2 receptor analogues were hyperalgesic or pronociceptive. It should be noted that both of these pain paradigms are

considered to be a phasic stimulus while the second phase of the formalin test involves a tonic stimulus with an inflammatory component (Dubuisson and Dennis 1977). When adenosine was administered in conjunction with a concentration of formalin producing a minimal nociceptive response (0.5%), flinching behaviour throughout phase 2 was augmented. As the concentration of formalin was increased, the ability of adenosine to further augment the response became insignificant. This suggests two things. First, while adenosine alone resulted in no significant behavioural response, the minimal inflammatory reaction following formalin injection seems to either sensitize the peripheral nociceptor to the stimulatory effect of adenosine or provides co-mediators which somehow facilitate adenosine's pronociceptive effect. Although application of adenosine to a human blister base preparation produces pain (Bleehen and Keele 1977), one could argue that the blister base model involves some degree of low level tissue injury and sensitization. Secondly, with higher formalin concentrations the effect of endogenous adenosine becomes masked, possibly because endogenous adenosine is already being generated by the developing inflammatory response (*cf.* Cronstein 1994). Alternatively, at higher formalin concentrations there may be some element of damage (and desensitization) to the peripheral nerve terminal (Puig and Sorkin 1995), making adenosine receptor stimulation ineffective. The latter seems less likely since the adenosine antagonists remain active in the presence of higher formalin concentrations, suggesting ongoing adenosine receptor stimulation.

Further evidence for a pronociceptive effect of adenosine A_2 receptor stimulation is derived from observations with antagonists. Thus, the A_2 receptor antagonist DMPX

can inhibit the nociceptive response generated by higher concentrations of formalin. The overall importance of endogenous adenosine to the inflammatory component of formalin pain may be limited, as DMPX reduced flinching in phase 2A by a maximum of only about 40%. On the other hand, DMPX has relatively low potency and limited selectivity for antagonism of adenosine A_2 receptors (Table III). This could be manifested as limited efficacy in the antagonism of adenosine mediated nociceptor stimulation. Other endogenous peripheral mediators of the formalin response are bradykinin (reviewed, Dray and Perkins 1993), norepinephrine (Coderre *et al.* 1984), histamine (Shibata *et al.* 1989), 5-HT (Giordano and Rogers 1989; Rueff and Dray 1992), and eicosanoid products of the lipoxygenase or cyclo-oxygenase pathways of arachidonic acid metabolism (Hunskaar *et al.* 1986; Dray and Bevan 1993). It may be that the real significance of the role of adenosine in inflammatory pain can only be appreciated in the presence of some of these other inflammatory mediators.

The hyperalgesic effect of peripheral adenosine A_2 receptor activation may be partially countered by an apparent analgesic effect mediated via A_1 receptor activation. Thus, when the system is stimulated by 2.5% formalin injection, A_1 receptor antagonism by CPT augments flinching. Interestingly, the augmentation of the nociceptive response was not seen when CPT was given in conjunction with 0.5% formalin. This suggests that only at the higher concentration of formalin is there a significant concentration of endogenous adenosine present to stimulate the inhibitory receptor sensitive to CPT. An analgesic effect resulting from peripheral A_1 receptor stimulation by CHA could not be detected, as the central depressant effect of CHA could not be separated from any

peripheral analgesic effect it might have. The apparent opposing effect of adenosine on the second phase of the formalin test, which we have inferred using the antagonist, was seen directly by Karlsten *et al.* (1992) using an agonist in the first phase in mice, and by Taiwo and Levine (1990) using the paw-withdrawal threshold test.

It should be noted that the analgesic effect attributed to peripheral adenosine A_1 receptor stimulation is in contrast to human data which suggests that peripheral A_1 receptor stimulation induces the pain associated with direct intradermal injection of adenosine (Pappagallo *et al.* 1993). Ischemic pain, including angina-like pain, can be induced by adenosine infusion and is sensitive to theophylline blockade (reviewed, Sylvén 1993). This ischemic pain has been attributed to adenosine A_1 receptor stimulation on the basis of sensitivity to the selective A_1 receptor antagonist bamiphylline (Gaspardone *et al.* 1995). The reasons for this apparent discrepancy are not clear but the doses of bamiphylline used by Pappagallo *et al.* (1993) and Gaspardone *et al.* (1995) may have resulted in adenosine A_2 receptor blockade. Most of the cardiac afferents are sympathetic fibres (Sylvén 1993). It is possible they are fundamentally different from primary afferent fibres in their adenosine receptor population. There may also be interspecies differences. Finally, it is difficult to reconcile stimulation of a peripheral inhibitory receptor (*i.e.* A_1 receptor) resulting in pronociception.

The mechanism by which adenosine exerts simultaneous hyperalgesic and analgesic actions is not clear, but there is some evidence to suggest adenosine A_2 receptor mediated hyperalgesia is mediated via a direct action on the primary afferent nerve terminal (Taiwo and Levine 1990). Thus, the onset of action is rapid and comparable to other direct acting

mediators, and interventions designed to remove indirectly generated responses do not alter the effect of adenosine (Taiwo and Levine 1990). Adenosine receptors are located on dorsal root ganglion cell bodies (Dolphin *et al.* 1986; MacDonald *et al.* 1986; Santicioli *et al.* 1992) and on the soma and central terminals of vagal afferents (Castillo-Meléndez *et al.* 1994), providing some support for the notion of a direct rather than indirect activation mechanism. The increase in adenosine-induced hyperalgesia in the presence of phosphodiesterase inhibition links the hyperalgesic action of adenosine to other directly acting hyperalgesic agents through the cAMP second messenger system (Taiwo and Levine 1991). The mechanism involved in the adenosine A_1 receptor mediated analgesia is less clear. In cultured dorsal root ganglion cell bodies, A_1 receptor mediated inhibition of Ca^{2+} entry has been demonstrated (MacDonald *et al.* 1986; Dolphin *et al.* 1986). It is possible that adenosine A_1 receptor activation on the peripheral end of primary afferent nerve terminals results in inhibition of Ca^{2+} entry or cAMP production or changes in IP_3 generation. There is evidence to suggest that both adenosine A_1 and A_2 receptors are present on afferent neuronal cell bodies in the inferior vagal ganglion, where they exert opposing effects (Castillo-Meléndez *et al.* 1994). The possibility of a similar co-existence on afferent nerve terminals is plausible. The fact that the dose of adenosine which enhanced the formalin response was not itself algesic suggests that in this model adenosine acts to either sensitize the nociceptors to the effects of other algesic substances or requires the nociceptor to be sensitized to exert its effects. This would be consistent with adenosine exerting a low-level of stimulation of adenylate cyclase which is magnified in the presence of other direct acting agents (see above).

The possibility of adenosine exerting pronociceptive effects through stimulation of adenosine A_3 or A_4 receptors cannot be excluded. The adenosine A_3 receptor, for example, is found on mast cells, and stimulation results in mast cell degranulation and subsequent liberation of 5-HT and histamine (Hannon *et al.* 1995; Fozard *et al.* 1996). Recently, the adenosine A_3 receptor agonist N^6-benzyl-5'-N-ethylcarboxamidoadenosine (B-NECA) has been demonstrated to evoke spontaneous pain behaviour upon injection into the rat hindpaw and to augment the response to 0.5% formalin (Sawynok *et al.* 1997). The response appears to involve release of histamine and 5-HT. The importance of the less well characterized adenosine A_4 receptor to the formalin induced nociceptive response remains speculative.

Caffeine produces analgesia in both human and animal nociceptive trials (reviewed, Sawynok and Yaksh 1993). It was postulated that antagonism of peripheral adenosine A_2 receptors might reflect part of the mechanism of caffeine-induced analgesia, in view of the peripheral pronociceptive actions of A_2 agonists (Taiwo and Levine 1990; Karlsten *et al.* 1992). However, despite the analgesic activity of caffeine in the formalin test following systemic administration, no analgesic effect was seen following peripheral administration of caffeine (Sawynok *et al.* 1995) in conjunction with 5% formalin. The inference of adenosine A_1 receptor activation having an analgesic effect which can oppose the pronociceptive effect of A_2 receptor activation may provide an explanation for the apparent lack of effect of caffeine, a nonselective adenosine receptor antagonist, when administered peripherally. The analgesic efficacy of caffeine appears to result primarily from interactions at supraspinal sites (Sawynok *et al.* 1995).

It was difficult to separate sedative and locomotor effects of adenosine analogues from local effects on nociception. Higher doses of both CGS21680 and CHA produced definite systemic effects (sedation, decreased locomotor activity and flattened posture) that were apparent within 2-4 minutes of injection. This rapid appearance of systemic behavioural phenomena following local injection of adenosine analogues has been reported by others (Karlsten *et al.* 1992). It is possible that combining the drug injection with formalin, and the presence of DMSO, may have enhanced systemic absorption of the drugs, contributing to the early onset action of these agents.

Adenosine and formalin induced paw swelling

There appears to be a separation between nociceptive and inflammatory effects of adenosine in this model. In contrast to its pronociceptive effect, adenosine appears to contribute little to the development of inflammatory edema as the addition of exogenous adenosine to 0.5% formalin resulted in no further augmentation of paw swelling. Furthermore, DMPX had no effect on the paw swelling resulting from injection of 2.5% formalin. This was somewhat unexpected since adenosine, via A_2 receptor stimulation, is known to mediate vasodilation which could contribute to paw swelling (Collis and Hourani 1993; Costa and Biaggioni 1993). On the other hand, this supports the concept of a direct action of adenosine on primary afferents, without the requirements for intermediates such as prostaglandins, as has been proposed by Taiwo and Levine (1990). Although no reduction in paw swelling was seen in the presence of adenosine, the possibility for this exists since adenosine may inhibit free radical production and

phagocytosis by neutrophils through A_2 receptor activation (Cronstein 1994) and may inhibit platelet aggregation (Cooper 1995), both of which may contribute to inflammation. Adenosine has been shown to contribute to bradykinin-induced plasma extravasation in the rat knee joint (Green *et al.* 1991). In this model, adenosine A_2 receptor stimulation enhanced, while A_1 receptor stimulation inhibited, plasma extravasation, but the site of action was not identified. The failure to observe any changes in paw swelling in the formalin model may reflect different mechanisms inherent to the model or it may be that this particular model is not sensitive enough to detect adenosine mediated changes in vascular permeability or it is possible that these changes may be subtle and may be masked by the more vigorous effects attributable to histamine or 5-HT (see below).

III. The role of 5-HT in formalin induced inflammatory pain

The data, using the 0.5% formalin model to detect pronociceptive actions of inflammatory mediators, confirms the involvement of 5-HT in the development of inflammation following injection of subcutaneous formalin in the rat hindpaw. As well as confirming the role of peripheral 5-HT_1 and 5-HT_3 receptors in nociception, this is the first study to suggest involvement of peripheral 5-HT_4 receptors in augmentation of the pain signal. Activation of peripheral 5-HT_2 receptors does not seem to play a part in nociception in this model of tonic inflammatory pain. Although subcutaneous injection of even small doses of 5-HT alone, or co-injected with 0.5% formalin, produced significant edema, the edema formation associated with 2.5% formalin could not be attenuated by any single 5-HT receptor antagonist tested. This suggests a dissociation between pain and

edema formation where 5-HT is concerned. Edema formation may involve activation of multiple 5-HT receptor subtypes, or activation of other receptors such as for histamine, bradykinin, SP, and CGRP (Di Rosa *et al.* 1971; Maling *et al.* 1974; Amann *et al.* 1995).

5-HT and formalin induced nociception

Endogenously released 5-HT has been shown to be a significant mediator of formalin induced nociceptive behaviour in the rat, based on antagonism by 5-HT receptor subtype-selective antagonists. Activation of peripheral receptors by exogenous administration of 5-HT, at a dose which by itself is not algesic (15 nmol) but which is profoundly hyperalgesic in the presence of low dose formalin, supports this conclusion. Considering the magnitude of the edema formation seen with subcutaneous doses of 5-HT of 15 nmol and lower compared to that seen with high dose formalin alone, it would seem unlikely that amounts of 5-HT released from endogenous sources, as a result of high dose formalin injection, would exceed 15 nmol. This would suggest that under these conditions, 5-HT is not by itself algesic but rather acts (directly or indirectly) on previously sensitized nociceptors, or sensitizes them to the effects of other substances (reviewed, Dray 1995). This is somewhat at odds with other studies which have suggested 5-HT to be algesic. Richardson *et al.* (1985) found application of 5-HT to the human blister base to evoke pain, however it may be argued that the blister base may represent a "sensitized" model. Sufka *et al.* (1991) observed lifting and licking behaviour in rats following plantar injection of 5-HT (250 - 1000 nmol). The difference between observation of hyperalgesia and algesia may be a reflection of dose since 5-HT has been

shown to produce sensitization at low concentrations and direct excitation of nociceptors at higher concentrations (Dray 1995).

Observations from 5-HT receptor subtype-selective antagonists co-injected with 2.5% formalin and from co-injection of 5-HT receptor subtype-selective agonist with 0.5% formalin suggest the involvement of 5-HT_1 , 5-HT_3 ,and 5-HT_4 but not 5-HT_2 receptor subtypes in nociceptive responses. Previous studies have suggested involvement of "5-HT_1 like" receptors in peripheral nociception. Sufka *et al.* (1991) found methysergide to be the most potent in antagonizing the edema and algesic responses of intraplantar 5-HT injections and implicated 5-HT_1 receptors, although methysergide has antagonist properties at 5-HT_2 receptors as well (reviewed, Zifa and Fillion 1992). A direct nociceptive action on primary afferents via 5-HT_{1A} receptor activation in the rat has been proposed by Taiwo and Levine (1992); an action proposed to be mediated via positive coupling to cAMP (Taiwo *et al.* 1992). Interestingly, in this model of noninflammatory nociception, they found no involvement of 5-HT_2 or 5-HT_3 receptors.

The data from the present study suggest 5-HT_3 receptors to be important mediators of inflammatory pain. Considerable evidence supports this view. In the human blister base, tropisetron blocks 5-HT evoked pain (Richardson *et al.* 1985). Giordano and Rogers (1989) examined the effects of two 5-HT receptor antagonists, tropisetron and MDL 72222, against formalin induced acute (phase 1 only) and Freunds adjuvant induced chronic inflammatory pain in rats. They found both agents to be effective but the effects were greater against the inflammatory pain. Tropisetron was similarly effective in attenuating carrageenan induced hyperalgesia in rats (Eschalier *et al.* 1989).

The 5-HT_4 receptor has not been previously linked to peripheral nociceptor activation. It is only recently that the potent, highly selective 5-HT_4 receptor antagonist GR113808A has become available (Gale *et al.* 1994). G113808A was as effective as tropisetron in suppressing both phases of flinching induced by high dose formalin. When co-injected with low dose formalin, 5-MeOT resulted in significant hyperalgesia. 5-MeOT stimulates 5-HT_4 but not 5-HT_3 receptors (Zifia and Fillion 1992). Although 5-MeOT may also activate 5-HT_1 and 5-HT_2 receptors, it seems unlikely that 5-HT_2 receptors are involved since neither the 5-HT_2 receptor antagonist ketanserin or the agonist DOI demonstrated any activity. Clearly 5-HT_1 receptors are involved, and the agonist and antagonist data support this. The effects of 5-HT_1 receptor stimulation appear to include a vascular component since the 5-HT_1 agonist 5-CT produced marked erythema of the injected paw at doses which seemed to parallel hyperalgesia. This vascular phenomenon was not seen with 5-MeOT, even at the highest dose, suggesting that 5-MeOT was not activating these 5-HT_1 receptors. This would suggest that activation of peripheral 5-HT_4 receptors is an important part of the nociceptive response to acute inflammation.

Although tropisetron is highly selective for 5-HT_3 receptors, it does antagonize 5-HT_4 receptors at high enough concentrations (Table IV). The efficacy of tropisetron in the present study and in the study by Giordano and Rogers (1989) may reflect antagonism of both 5-HT_3 and 5-HT_4 receptors simultaneously. The effects of 5-HT_4 receptor activation appear to be mediated via an increase in adenylate cyclase activity and cAMP production (Zifa and Fillion 1992). This would be consistent with the observations of Taiwo *et al.* (1992) on the role of increased cAMP in 5-HT mediated hyperalgesia. Whereas 5-HT_3

receptor stimulation activates a cation channel leading to neuronal depolarization and direct stimulation, 5-HT_4 mediated increases in cAMP would most likely result in neuronal sensitization.

The lack of nociceptive response attributable to 5-HT_2 receptor stimulation is interesting. Platelet aggregation (Drummond and Gordon 1975) and increased capillary permeability (Ortmann *et al.* 1982) are 5-HT_2 receptor mediated effects that might be expected to contribute to inflammation and nociception in this model. Although some additional paw swelling was seen in the presence of DOI (see below) no hyperalgesic effect was seen. Others have been able to demonstrate 5-HT_2 receptor-mediated effects, but under different test conditions.

Sufka *et al.* (1991) found methysergide and ketanserin to inhibit 5-HT induced inflammatory and nociceptive responses, suggesting a 5-HT_2 receptor mediated mechanism. They used plantar injections of 5-HT at a dose of 250 nmol to elicit a nociceptive response that was antagonized by methysergide or ketanserin. This is a dose of 5-HT which resulted in direct activation of nociceptors and is significantly higher than used in the present study. Plantar injections may result in less dispersion of the drug (and higher concentrations) than dorsal paw injections (Puig and Sorkin 1995). It may be that in order to activate 5-HT_2 receptors, higher local concentrations of 5-HT are required than are seen in the formalin test.

Rueff and Dray (1992) found 5-HT to sensitize peripheral nerve fibres of neonatal rats to the effects of exogenously applied capsaicin or bradykinin. This effect could be inhibited by ketanserin but not methiothepin or tropisetron. Conversely, in the same

preparation, pretreatment with low-dose capsaicin or bradykinin sensitized peripheral nociceptors to the effect of exogenous 5-HT, an effect that was blocked by methiothepin but not ketanserin. The authors suggested that 5-HT_2 receptor activation sensitized peripheral fibres to the effects of chemical and thermal stimuli. Since this was an *in vitro* preparation, indirect effects on microvasculature and platelets could be excluded. Caution was advised in extrapolation of the data to the mature nervous system.

Abbott's group (Hong and Abbott 1994) have developed a behavioural model for assessing the effects of intraplantar injection of inflammatory mediators and have identified 5-HT_2, but not 5-HT_1 or 5-HT_3 receptors, as important mediators of hyperalgesia (Abbott *et al.* 1996). They also found the 5-HT_2 antagonists ketanserin, ritanserin and spiperone to attenuate the phase 2 response to 1% formalin injection. Interestingly, they observed no decrease in phase 2 with tropisetron. Intraplantar injection (Abbott *et al.* 1996) vs dorsal subcutaneous injection of the hindpaw has been proposed to result in differences in concentrations of the injected agents, and possible differences in neuronal activation-desensitization (Puig and Sorkin 1995). Differences between Abbott's data and the present data are difficult to reconcile, however the present data, using a 5-HT_2 *agonist and antagonist in combination with formalin*, are consistent.

5-HT and formalin induced paw swelling

Subcutaneous 5-HT was potent in producing edema by itself. Co-injection of 15 nmol 5-HT with 0.5% formalin resulted in significantly greater paw swelling than was seen with formalin alone but this was comparable to that seen with 5-HT alone. Thus, there was no additional effect attributable to the formalin. The data from the co-administration of 0.5% formalin and various 5-HT receptor subtype-selective agonists suggest that activation of 5-HT_3 receptors is not involved in edema formation, in contrast to their role in nociception, but 5-HT_1, 5-HT_2, and 5-HT_4 receptors may be involved in edema formation. 5-HT_1, 5-HT_2, 5-HT_3 and 5-HT_4 receptors have all been implicated in direct neuronal activation (see above) and mRNAs for all but the 5-HT_4 receptor have been isolated from dorsal root ganglia (Pierce *et al.* 1996). Stimulation of peripheral nerve terminals may release vasoactive neuropeptides such as SP (Levine *et al.* 1993) and CGRP (Amann *et al.* 1995), important mediators of inflammatory edema (Dray and Perkins 1993). This may be one mechanism of 5-HT induced paw swelling but 5-HT causes edema that is unaffected by capsaicin-denervation or pretreatment with a CGRP antagonist (Amann *et al.* 1995).

The vasodilatory effect of 5-CT is evident from the erythema of the injected paw at lower doses and subsequent involvement of the ears, tail, and contralateral paws with higher doses. This is consistent with its known pre-synaptic sympathetic inhibitory effect mediated through a 5-HT_1-like receptor (Mylecharane and Phillips 1989; Martin 1994). This vasodilatory effect would certainly contribute to paw swelling, and may contribute

to hyperalgesia indirectly by increasing the availability of other inflammatory mediators through increased blood flow.

Although 5-HT_2 receptor activation did not appear to be involved in the nociceptive response, the 5-HT_2 receptor agonist DOI augmented edema associated with 0.5% formalin. This dissociation suggests that a direct neuronal activation via 5-HT_2 receptors, and subsequent neurogenic edema, is not involved. Rather, activation of platelets via a 5-HT_{2A} receptor (Drummond and Gordon 1975), or endothelium-mediated vascular relaxation via an atypical 5-HT receptor (Martin 1994), may be responsible.

Of the subtype-selective 5-HT agonists, 5-MeOT was most effective in augmenting edema formation associated with 0.5% formalin. 5-MeOT is useful to differentiate 5-HT_3 and 5-HT_4 receptors since it is a potent agonist at the 5-HT_4 receptor while being almost inactive at the 5-HT_3 receptor (Hoyer *et al.* 1994). However, 5-MeOT also has significant activity at some 5-HT_1 and 5-HT_2 sites. It is possible that some of the edema formation may result from stimulation of a combination of these receptors. A significant contribution from the same receptors that are stimulated by 5-CT seems unlikely since none of the paw erythema seen with 5-CT occurred with 5-MeOT. It has been suggested that there are different subpopulations of afferent C-fibres containing predominately CGRP (Donnerer *et al.* 1992). If such fibres selectively exhibited, for example, 5-HT_2 and 5-HT_4 but not 5-HT_3 receptors, it could explain the apparent differences between the agonist profiles for nociception and paw swelling (*cf.* Amann *et al.* 1995).

In view of the activity of the 5-HT receptor agonists, it is important to consider why the 5-HT receptor antagonists are not effective against 2.5% formalin-induced edema.

There is evidence that in carrageenan-induced edema histamine and 5-HT are released simultaneously and either is capable of producing near maximal edema. Inhibition of one or the other alone is insufficient to significantly reduce edema but combinations of mepyramine and cyproheptadine are effective (Di Rosa *et al.* 1971). Methysergide has been more effective than more selective 5-HT receptor antagonists in blocking 5-HT-induced edema (Sufka *et al.* 1991). Methysergide has significant antagonist activity at both 5-HT_1 and 5-HT_2 receptors (Zifa and Fillion 1992). Thus, the inability of any single 5-HT receptor antagonist to significantly alter paw swelling may reflect the effect of multiple, possibly redundant mechanisms. This suggests that simultaneous blockade of multiple 5-HT receptor subtypes may be necessary to inhibit formalin induced edema.

IV. Adenosine and 5-HT interactions in formalin induced inflammatory pain

Adenosine, 5-HT and formalin induced nociception

It was hypothesized that the opportunity exists for adenosine and 5-HT to interact on the peripheral nerve terminal, resulting in a nociceptive signal. 5-HT could release adenosine. Behavioural evidence suggests that 5-HT mediated analgesia at the level of the spinal cord is at least partially dependent on adenosine release (Delander and Hopkins 1987; Sawynok and Reid 1991). This is directly supported by biochemical evidence of 5-HT stimulated release of adenosine from spinal cord synaptosomes (Sweeney *et al.* 1988) and *in vivo* superfused spinal cord (Sweeney *et al.* 1990). This released adenosine is derived from capsaicin sensitive afferent terminals. The possibility of peripheral 5-HT receptor stimulation leading to adenosine release, from peripheral nerve terminals or other

cell types, was examined by determining if nociceptive behaviour resulting from co-injection of 5-HT and 0.5% formalin could be blocked by adenosine receptor antagonists.

Adenosine could release 5-HT. It may be that adenosine receptor stimulation resulted in release of 5-HT from sources such as mast cells, platelets, or other inflammatory cells. Adenosine has been shown to promote rat mast cell degranulation in the presence of inflammatory mediators, likely via adenosine A_3 receptor stimulation (Church *et al.* 1986). There is considerable evidence to support adenosine mediated release of 5-HT as a mode of action in adenosine induced bronchoconstriction (Nordström and Delbro 1986) and capsaicin-sensitive afferent nerves are involved (Manzini and Ballati 1990). The possibility of peripheral adenosine receptor stimulation leading to 5-HT release was examined by determining if nociceptive behaviour resulting from co-injection of adenosine and 0.5% formalin could be blocked by 5-HT receptor antagonists. Finally, the potential for additive or synergistic activity of adenosine and 5-HT as co-mediators in the "inflammatory soup" was considered.

The pronociceptive effect of adenosine in the rodent formalin test appears to be mediated through adenosine A_2 receptors, since the A_2 receptor antagonist DMPX significantly reduces 2.5% formalin induced flinching. If the pronociceptive effect of 5-HT, in the presence of formalin, was related to adenosine release and subsequent activation of A_2 receptors, some attenuation of this pronociceptive effect by DMPX should have been seen. Such was not the case (Fig. 27). It seems unlikely then, that 5-HT exerts its hyperalgesic effects in this model through release of adenosine. Although it is possible that the effects of any adenosine released is overshadowed by the direct

effects of 5-HT in producing pain, this seems less likely since adenosine clearly plays a significant role in the behavioural response to 2.5% formalin. If additional adenosine is being released, an effect should be visible.

5-HT is clearly a powerful player in the peripheral nociceptive milieu. Likely sources of 5-HT are platelets, mast cells, and basophils and there is good evidence for adenosine mediated release of 5-HT from mast cells (Church *et al.* 1986) and basophils (Abbracchio *et al.* 1992). The present data are consistent with adenosine mediated release of 5-HT with subsequent activation of peripheral nociceptors since the adenosine mediated augmentation of flinching induced by 0.5% formalin was completely blocked in the presence of multiple 5-HT antagonists. Adenosine mediated release of 5-HT from basophils results from adenosine A_2 receptor stimulation (Abbracchio *et al.* 1992) and is entirely consistent with this scenario. Interestingly, adenosine A_1 receptor stimulation inhibits 5-HT release from basophils, a possible explanation for the pronociceptive effect of the A_1 antagonist CPT in the presence of 2.5% formalin.

In some cases, another explanation exists. Thus, the 5-HT antagonists S(-) propranolol, tropisetron, and GR113808A each were effective in reducing the flinching response to 2.5% formalin and 0.5% formalin, indicating significant levels of 5-HT present even with minimal inflammation (*e.g.* 0.5% formalin). This contrasts with the adenosine data which suggests significant levels only with inflammation associated with 2.5% formalin (see above). It is also clear that for adenosine to exert its hyperalgesic effect the system needs to be "primed", in the sense that some low level of tissue injury appears necessary, since adenosine in the absence of formalin elicits no behavioural

response. It is possible that 5-HT serves to sensitize the peripheral system to the pronociceptive effects of adenosine, and blockade of 5-HT receptors blunts this "priming" effect and the effects of adenosine receptor activation are no longer manifest.

In the case of 5-HT_2 receptor involvement, this explanation is lacking. Thus, ketanserin, a selective 5-HT_2 receptor antagonist, had no effect on the flinching response to either 0.5% or 2.5% formalin. It was surprising then, to see that the additional pronociceptive effect of adenosine, when coinjected with 0.5% formalin, was blocked completely by ketanserin. For this to occur we must postulate that the *administration of exogenous adenosine releases 5-HT* which in turn stimulates 5-HT_2 receptors and results in a nociceptive signal, while *adenosine released from endogenous stores*, in response to 2.5% formalin injection, does not result in 5-HT_2 activation. It also requires that these 5-HT_2 receptors are somehow not responsive to exogenous 5-HT or 5-HT_2 agonists. The effects of ketanserin in the presence of adenosine and 0.5% formalin more closely resemble the data from Abbott *et al.* (1996), who found 5-HT_{2A} receptor stimulation had a significant pronociceptive effect. How can this be reconciled with the rest of the data?

One possible explanation is that 5-HT_1, 5-HT_3, and 5-HT_4 receptors are part of an essential pathway in the nociceptive process, integral to the sensitization of the peripheral nerve terminal to the effects of other inflammatory mediators, including adenosine. Location of these receptors directly on the nerve terminal, as has been suggested by others (Eschalier *et al.* 1989; Taiwo and Levine 1992), could account for this (Fig. 33). The 5-HT_2 receptors may be involved in an indirect and perhaps ancillary way, where stimulation of 5-HT_2 receptors may augment the nociceptive signal under certain circumstances (*e.g.*

in the presence of excess adenosine) but is not critical to the process. Thus, 5-HT_2 receptor activation may be a redundant mechanism which may augment nociceptive behaviour in some situations, but its absence may be inconsequential in a full-blown inflammatory response. The platelet 5-HT_2 receptor may be one such example, where stimulation of the receptor promotes platelet aggregation, but this is by no means the only way for platelet aggregation to occur (Page 1989; Hourani and Cusack 1991). Lower concentrations of formalin (0.5%) result in a minimal inflammatory response. The addition of adenosine under these conditions may stimulate the local release of 5-HT (from mast cells?) that interacts with 5-HT_2 receptors on platelets to promote aggregation and further inflammation (Fig. 33). Higher concentrations of formalin or more significant levels of tissue injury may bypass this, causing release of multiple mediators (Fig. 1) and masking the 5-HT_2 mediated effects.

The dual role of 5-HT as a pain mediator, as a direct acting agent and as a mediator which sensitizes the nociceptor to the effects of other chemicals, is evident when 5-HT is combined with adenosine. The combination of doses of adenosine (15 nmol) and 5-HT (1.5 nmol), which by themselves had no effect on 0.5% formalin induced flinching, resulted in a marked augmentation of flinching to near maximal levels (Figs. 25-26). When we consider the sensitivity of adenosine mediated hyperalgesia to 5-HT receptor blockade and the resistance of 5-HT mediated hyperalgesia to adenosine receptor blockade, it seems reasonable to conclude that the 5-HT is the key mediator in the relationship. It is likely that 5-HT is responsible, at least in part, for sensitizing the peripheral nociceptor

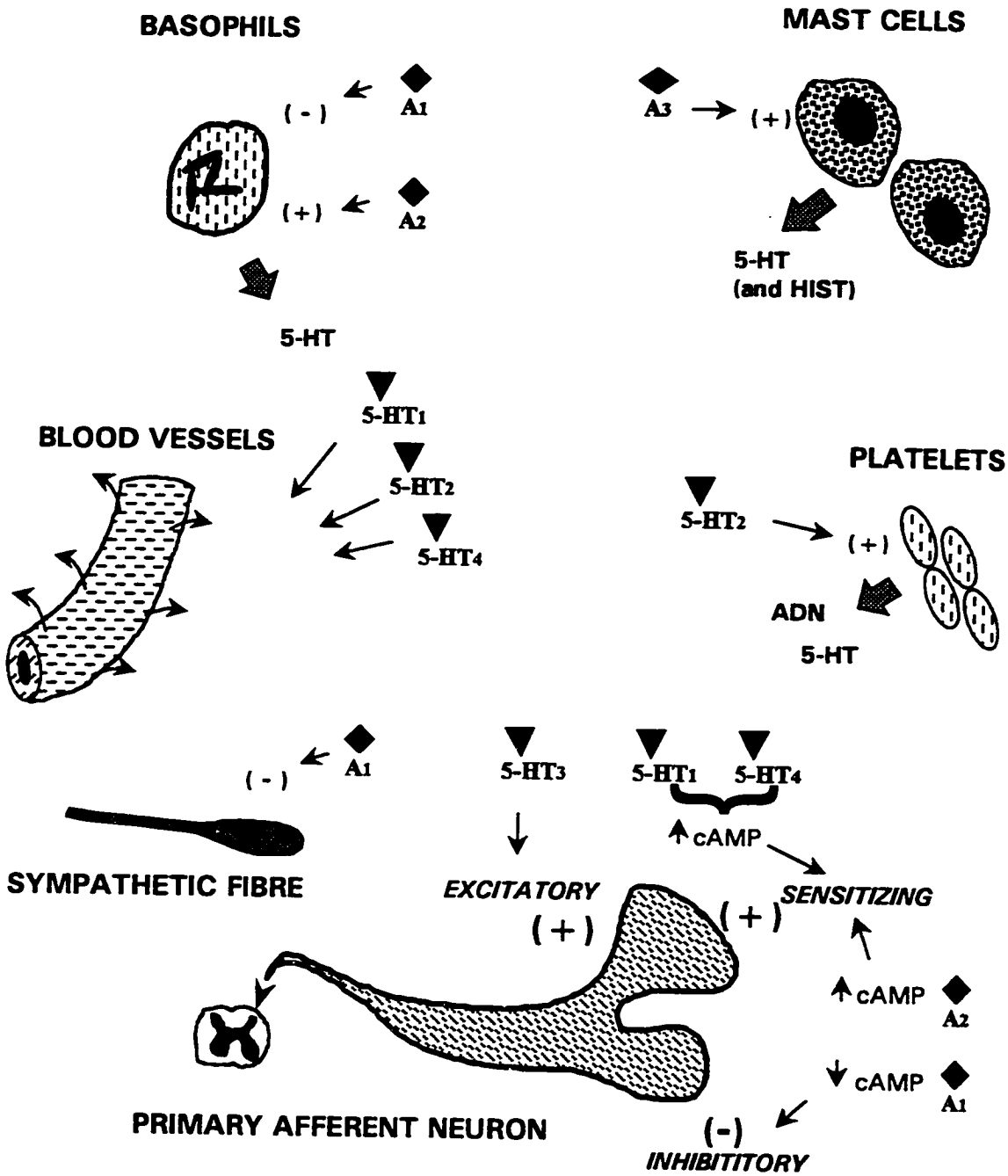


Figure 33. Summary of 5-HT and adenosine interactions in the production of inflammatory pain.

to the effects of adenosine, as it does for other mediators such as SP, norepinephrine, and histamine (Hong and Abbott 1994).

Adenosine, 5-HT and formalin induced paw swelling

As previously discussed, a significant contribution by adenosine, to the paw swelling associated with formalin injection, could not be detected. The data derived from combinations of adenosine and 5-HT and their respective antagonists are consistent with this. Recently, Sawynok *et al.* (1997) determined that adenosine A_3 receptor stimulation by B-NECA significantly augmented the paw swelling associated with 0.5% formalin injection and this could be antagonized by ketanserin. The present data did not specifically address the role of adenosine A_3 receptors but the lack of significant paw swelling in the presence of exogenous adenosine suggests that concentrations of adenosine that are higher than generated by 2.5% formalin injection are required to activate the A_3 receptor mechanism. Alternatively, the present model may not be sensitive enough to detect adenosine mediated changes in paw swelling. While 5-HT clearly plays an important role in the paw swelling in response to formalin injection, adenosine, at least, would appear to be a lesser contributor. Again there appears to be a divergence between interactions and effects on nociception and the inflammatory response as measured by paw swelling.

CONCLUSIONS

In summary, it has been demonstrated that endogenous adenosine plays a significant role in tonic, inflammatory pain as revealed by the formalin test. Adenosine may exert simultaneous pro- and antinociceptive effects via activation of adenosine A_2 and A_1 receptors respectively (Fig. 33). The dissociation between adenosine's effects on nociception and paw swelling supports the concept of a direct effect on primary afferents, circumventing indirect effects on other cell types. This does not exclude a role for adenosine receptors on inflammatory cells such as basophils (A_1 and A_2) or mast cells (A_3). It may be that peripheral administration of a selective adenosine A_2 receptor antagonist and an A_1 agonist in combination might produce effective analgesia, while a peripherally acting nonselective adenosine antagonist could be of limited benefit. Given the opposing nociceptive effects of adenosine at the A_1 and A_2 receptors, the effect of altering endogenous adenosine levels during inflammation using, for example, adenosine kinase inhibitors, would be difficult to predict. The analgesic potential of adenosine A_3 receptor antagonism is yet to be explored.

5-HT has been shown to be a key component of the inflammatory response to subcutaneous formalin injection in the rat. Involvement of 5-HT_1 and 5-HT_3 receptor subtypes has again been implicated but a role for the more recently characterized 5-HT_4 receptor has been identified. Although the location of the receptors that were involved was speculative, it is likely that the 5-HT_1, 5-HT_3, and 5-HT_4 receptors were located on the primary afferent neuron. The importance of the 5-HT_2 receptor to the nociceptive process would appear to be minimal, although it may play a secondary role through augmentation

of platelet aggregation (Fig. 33). Although 5-HT was clearly an important contributor to the inflammatory response, as measured by paw swelling, no single receptor type appeared to be crucial. It is possible that activation of any one of several 5-HT receptor types may be capable of initiating a maximal inflammatory response. Furthermore, as with adenosine, there appears to be a dissociation between the inflammatory and nociceptive effects mediated through 5-HT receptors. Peripherally active 5-HT_4 receptor antagonists may represent a novel approach to analgesia in conditions of acute inflammation.

The data do not support the hypothesis of a peripheral nociceptive effect of 5-HT being mediated through adenosine release. It is also unlikely that the pronociceptive effect of peripheral A_2 adenosine receptor stimulation is the result of subsequent release of 5-HT. A more likely explanation of their relationship is that other factors result in their co-release under conditions of injury or inflammation and that the pronociceptive effects of adenosine are at least partially dependent upon a sensitizing effect of 5-HT on the peripheral nerve terminal.

REFERENCES

Abbott, F.V., Hong, Y., and Blier, P.: Activation of 5-HT_{2A} receptors potentiates pain produced by inflammatory mediators. Neuropharmacology 35: 99-110, 1996.

Abbracchio, M.P., Paoletti, A.M., Luini, A, Cattabeni, F., and De Matteis, M.A.: Adenosine receptors in rat basophilic leukaemia cells: transductional mechanisms and effects on 5-hydroxytryptamine release. Br. J. Pharmacol. 105: 405-411, 1992.

Alhaider, A.A., Hamon, M., and Wilcox, G.L.: Intrathecal 5-methoxy-N,N-dimethyltryptamine in mice modulates 5-HT_1 and 5-HT_3 receptors. Eur. J. Pharmacol. 249: 151-160, 1993.

Alhaider, A.A., and Wilcox, G.L.: Differential roles of 5-hydroxytryptamine$_{1A}$ and 5-hydroxytryptamine$_{1B}$ receptor subtypes in modulating spinal nociceptive transmission in mice. J. Pharmacol. Exp. Ther. 265: 378-385, 1993.

Amann, R., Schuligoi, R., Lanz, I., and Donnerer, J.: Histamine-induced edema in the rat paw - effect of capsaicin denervation and a CGRP receptor antagonist. Eur. J. Pharmacol. 279: 227-231, 1995.

Archer, T., Arweström, E., Minor, B.G., Persson, M.L., Post, C., Sundstrom, E., and Jonsson, G.: (+)-8-OH-DPAT and 5-MeODMT induced analgesia is antagonized by noradrenaline depletion. Physiol. Behav. 39: 95-102, 1987.

Beck, P.W., and Handwerker, H.O.: Bradykinin and serotonin effects on various types of cutaneous nerve fibres. Pflügers Arch. 347: 209-222, 1974.

Bevan, S., and Geppetti, P.: Protons: small stimulants of capsaicin-sensitive sensory nerves. Trends Neurosci. 17: 509-512, 1994.

Birrell, G.J., McQueen, D.S., Iggo, A., Coleman, R.A., and Grubb, B.D.: PGI_2-induced activation and sensitization of articular mechanonociceptors. Neurosci. Lett. 124: 5-8, 1991.

Bleehen, T., and Keele, C.A.: Observations on the algogenic actions of adenosine compounds on the human blister base preparation. Pain 3: 367-377, 1977.

Bowker, R.M., and Abbott, L.C.: Quantitative re-evaluation of descending serotonergic and non-serotonergic projections from the medulla of the rodent: evidence for extensive co-existence of serotonin and peptides in the same spinally projecting neurons, but not from the nucleus raphe magnus. Brain Res. 512: 15-25, 1990.

Braas, K.M., Newby, A.C., Wilson, V.S., and Snyder, S.H.: Adenosine-containing neurons in the brain localized by immunocytochemistry. J. Neurosci. 6: 1952-1961, 1986.

Brown, L.M., Smith, D.L., Williams, G.M., and Smith, D.J.: Alterations in serotonin binding after 5,7-dihydroxytryptamine treatment in the rat spinal cord. Neurosci. Lett. 102: 103-107, 1989.

Bruns, R.F., Lu, G.H., and Pugsley, G.H.: Characterization of the A_2 adenosine receptor labelled by [^{3}H]-NECA in rat striatal membranes. Mol. Pharmacol. 29: 331-346, 1986.

Burnstock, G.: A basis for distinguishing two types of purinergic receptor. *In:* Cell Membrane Receptors for Drugs and Hormones: A Multidisciplinary Approach, ed. by R.W. Straub and L. Bolis, pp. 107-118, Raven Press, New York, 1978.

Buzzi, M.G., Moskowitz, M.A., Peroutka, S.J., and Byun, B.: Further characterization of the putative 5-HT receptor which mediates blockade of neurogenic plasma extravasation in rat dura mater. Br. J. Pharmacol. 103: 1421-1428, 1991.

Cashman, J., and McAnulty, G.: Nonsteroidal anti-inflammatory drugs in perisurgical pain management: Mechanisms of action and rationale for optimum use. Drugs 49: 51-70, 1995.

Castillo-Meléndez, M., Krstew, E., Lawrence, A.J., and Jarrott, B.: Presynaptic adenosine A_{2a} receptors on soma and central terminals of rat vagal afferent neurons. Brain Res. 652: 137-144, 1994.

Cazzola, M., Matera, M.G., Santangelo, G., Assogna, G., D'Amato, G., Rossi, F., and Girbino, G.: Effects of the selective 5-HT_2 antagonist ketanserin on adenosine-induced bronchoconstriction in asthmatic subjects. Immunopharmacol. 23: 21-28, 1992.

Cesselin, F., Laporte, A.-M., Miquel, M.C., Bourgoin, S., and Hamon, M.: Serotonergic mechanisms of pain control. In: Proceedings of the 7th World Congress on Pain, Progress in Pain Research and Management, Vol 2., ed. by G.F. Gebhart, D.L. Hammond, and T.S. Jensen, pp. 669-695, IASP Press, Seattle, 1994.

Choca, J.I., Green, R.D., and Proudfit, H.K.: Adenosine A_1 and A_2 receptors of the substantia gelatinosa are located predominantly on intrinsic neurons: an autoradiography study. J. Pharmacol. Exp. Ther. 247: 757-764, 1988.

Choca, J.I., Proudfit, H.K., and Green, R.D.: Identification of A_1 and A_2 adenosine receptors in the rat spinal cord. J. Pharmacol. Exp. Ther. 242: 905-910, 1987.

Church, M.K., Hughes, P.J., and Vardey, C.J.: Studies on the receptor mediating cyclic AMP-independent enhancement by adenosine of IgE-dependent mediator release from rat mast cells. Br. J. Pharmacol. 87: 233-242, 1986.

Coderre, T.J., Abbott, F.V., and Melzack, R.: Effects of peripheral antisympathetic treatments in the tail-flick, formalin and autonomy tests. Pain 18: 13-23, 1984.

Coderre, T.J., Vaccarino, A.L., and Melzack, R.: Central nervous system plasticity in the tonic pain response to subcutaneous formalin injection. Brain Res. 535: 155-158, 1990.

Coderre, T.J., Fundytus, M.E., McKenna, J.E., Dalal, S., and Melzack, R.: The formalin test: a validation of the weighted-scores method of behavioural pain rating. Pain 54: 43-50, 1993.

Collis, M.G., and Hourani, S.M.: Adenosine receptor subtypes. Trends Pharmacol. Sci. 14: 360-366, 1993.

Cooper, J.A., Hill, S.J., Alexander, S.P., Rubin P.C., and Horn, E.H.: Adenosine receptor-induced cyclic AMP generation and inhibition of 5-hydroxytryptamine release in human platelets. Br. J. Clin. Pharmacol. 40: 43-50, 1995.

Cornfield, L.J., Hu, S., Hurt, S.D., and Sills, M.A.: [^{3}H]-2-Phenylaminoadenosine ([^{3}H]-CV 1808) labels a novel adenosine receptor in rat brain. J. Pharmacol. Exp. Ther. 263: 552-561, 1992.

Costa, F., and Biaggioni, I.: Adenosine activates afferent fibres in the forearm, producing sympathetic stimulation in humans. J. Pharmacol. Exp. Ther. 267: 1369-1374, 1993.

Cronstein, B.N.: Adenosine, an endogenous anti-inflammatory agent. J. Appl. Physiol. 76: 5-13, 1994.

Dallel, R., Raboisson, P., Clavelou, P., Saade, M., and Woda, A.: Evidence for a peripheral origin of the tonic nociceptive response to subcutaneous formalin. Pain 61: 11-16, 1995.

Dalziel, H.H., and Westfall, D.P.: Receptors for adenine nucleotides and nucleosides: subclassification, distribution, and molecular characterization. Pharmacol. Rev. 46: 449-466, 1994.

DeLander, G.E., and Hopkins, C.J.: Interdependence of spinal adenosinergic, serotonergic and noradrenergic systems mediating antinociception. Neuropharmacology 26: 1791-1794, 1987.

DeLander, G.E., and Wahl, J.J.: Behavior induced by putative nociceptive neurotransmitters is inhibited by adenosine or adenosine analogs coadministered intrathecally. J. Pharmacol. Exp. Ther. 246: 565-570, 1988.

Dickenson, A.H., and Sullivan, A.F.: Peripheral origins and central modulation of subcutaneous formalin-induced activity of rat dorsal horn neurones. Neurosci. Lett. 83: 207-211, 1987.

Di Rosa, M., Giroud, J.P., and Willoughby, D.A.: Studies of the mediators of the acute inflammatory response induced in rats in different sites by carrageenan and turpentine. J. Path. 104: 15-29, 1971.

Doi, T., Kuzuna, S., and Maki, Y.: Spinal antinociceptive effects of adenosine compounds in mice. Eur. J. Pharmacol. 137: 227-231, 1987.

Dolphin, A.C., Forda, S.R., and Scott, R.H.: Calcium-dependent currents in cultured rat dorsal root ganglion neurones are inhibited by an adenosine analogue. J. Physiol. (London) 373: 47-61, 1986.

Donnerer, J., Schuligoi, R., and Stein, C.: Increased content and transport of substance P and calcitonin gene-related peptide in sensory nerves innervating inflamed tissue: evidence for a regulatory function of nerve growth factor *in vivo*. Neuroscience 49: 693-698, 1992.

Dray, A.: Inflammatory mediators of pain. Br. J. Anaesth. 75: 125-131, 1995.

Dray, A., and Bevan, S.: Inflammation and hyperalgesia: highlighting the team effort. Trends Pharmacol. Sci. 14: 287-290, 1993.

Dray, A., and Perkins, M.: Bradykinin and inflammatory pain. Trends Neurosci. 16: 99-104, 1993.

Dray, A., Urban, L., and Dickenson, A.: Pharmacology of chronic pain. Trends Pharmacol. Sci. 15: 190-197, 1994.

Drummond, A.H., and Gordon, J.L.: Specific binding sites for 5-hydroxytryptamine on rat blood platelets. Biochem. J. 150: 129-132, 1975.

Dubuisson, D., and Dennis, S.G.: The formalin test: a quantitative study of the analgesic effects of morphine, meperidine, and brain stem stimulation in rats and cats. Pain 4: 161-174, 1977.

Eide, P.K., and Hole, K.: Different role of 5-HT_{1A} and 5-HT_2 receptors in spinal cord in the control of nociceptive responsiveness. Neuropharmacology 30: 727-731, 1991.

Eide, P.K., and Tjolsen, A.: Effects of serotonin receptor antagonists and agonists on the tail-flick response in mice involve altered tail-skin temperature. Neuropharmacology 27: 889-893, 1988.

Eschalier, A., Kayser, V., and Guilbaud, G.: Influence of a specific 5-HT_3 antagonist on carrageenan-induced hyperalgesia in rats. Pain 36: 249-255, 1989.

Faneslow, M.S.: Shock-induced analgesia on the rat formalin test: Effects of shock severity, naloxone, hypophysectomy, and associative variables. Behav. Neurosci. 98: 79-95, 1984.

Fasmer, O.B., Berge, O.G., Post, C., and Hole, K.: Effects of the putative 5-HT_{1A} receptor agonist 8-OH-2-(di-n-propylamino)tetralin on nociceptive sensitivity in mice. Pharmacol. Biochem. Behav. 25: 883-888, 1986.

Ford, A.P., and Clarke, D.E.: The 5-HT_4 receptor. Med. Res. Rev. 13: 633-662, 1993.

Fox, A.J., Urban, L., Barnes, P.J., and Dray, A.: Effects of capsazepine against capsaicin- and proton-evoked excitation of single airway C-fibres and vagus nerve from the guinea-pig. Neuroscience 67: 741-752, 1995.

Fozard, J.R.: Neuronal 5-HT receptors in the periphery. Neuropharmacology 23: 1473-1486, 1984.

Fozard, J.R., Pfannkuche, H.-J., and Schuurman, H.-J.: Mast cell degranulation following adenosine A_3 receptor activation in rats. Eur. J. Pharmacol. 298: 293-297, 1996.

Franklin, K.B.J., and Abbott, F.V.: Techniques for assessing the effects of drugs on nociceptive responses. Neuromethods (Pharmacology) 13: 145-216, 1989.

Fredholm, B.B., Abbracchio, M.P., Burnstock, G., Daly, J.W., Harden, T.K., Jacobson, K.A., Leff, P., and Williams, M.: Nomenclature and classification of purinoceptors. Pharmacol. Rev. 46: 143-156, 1994.

Freissmuth, M., Schütz, W., and Linder, M.E.: Interactions of the bovine brain A1-adenosine receptor with recombinant G protein α-subunits. Selectivity for $rG_{i\alpha\text{-}3}$. J. Biol. Chem. 266: 17778-17783, 1991.

Gale, J.D., Grossman, C.J., Whitehead, J.W., Oxford, A.W., Bunce, K.T., and Humphrey, P.P.: GR113808: a novel, selective antagonist with high affinity at the 5-HT_4 receptor. Br. J. Pharmacol. 111: 332-338, 1994.

Gaspardone, A., Crea, F., Tomai, F., Versaci, F., Iamele, M., Gioffrè, G., Chiariello, L., and Gioffrè, P.A.: Muscular and cardiac adenosine-induced pain is mediated by A_1 receptors. J. Am. Coll. Cardiol. 25: 251-257, 1995.

Geiger, J.D., LaBella, F.S., and Nagy, J.I.: Characterization and localization of adenosine receptors in rat spinal cord. J. Neurosci. 4: 2303-2310, 1984.

Geiger, J.D., and Nagy, J.I.: Heterogeneous distribution of adenosine transport sites labelled by [^{3}H]nitrobenzylthioinosine in rat brain: an autoradiographic and membrane binding study. Brain Res. Bull. 13: 657-666, 1984.

Geiger, J.D., and Nagy, J.I.: Localization of [^{3}H]nitrobenzylthioinosine binding sites in rat spinal cord and primary afferent neurons. Brain Res. 347: 321-327, 1985.

Gerwins, P., and Fredholm, B.B.: ATP and its metabolite adenosine act synergistically to mobilize intracellular calcium via the formation of inositol 1,4,5-trisphosphate in a smooth muscle cell line. J. Biol. Chem. 267: 16081-16087, 1992.

Giordano, J., and Rogers, L.V.: Peripherally administered serotonin 5-HT_3 receptor antagonists reduce inflammatory pain in rats. Eur. J. Pharmacol. 170: 83-86, 1989.

Glaum, S.R., Proudfit, H.K., and Anderson, E.G.: 5-HT_3 receptors modulate spinal nociceptive reflexes. Brain Res. 510: 12-16, 1990.

Göthert, M., Schlicker, E., and Kollecker, P.: Receptor-mediated effects of serotonin and 5-methoxytryptamine on noradrenaline release in the rat vena cava and in the heart of the pithed rat. Naunyn-Schmiedeberg's Arch. Pharmacol. 332: 124-130, 1986.

Green, P.G., Basbaum, A.I., Helms, C., and Levine, J.D.: Purinergic regulation of bradykinin-induced plasma extravasation and adjuvant-induced arthritis in the rat. Proc. Natl. Acad. Sci. USA 88: 4162-4165, 1991.

Grossman, C.J., Kilpatrick, G.J., and Bunce, K.T.: Development of a radioligand binding assay for 5-HT_4 receptors in guinea-pig and rat brain. Br. J. Pharmacol. 109: 618-624, 1993.

Grubb, B.D., McQueen, D.S., Iggo, A., Birrell, G.J., and Dutia, M.B.: A study of 5-HT-receptors associated with afferent nerves located in normal and inflamed rat ankle joints. Agents Actions 25: 216-218, 1988.

Guilbaud, G., Benoist, J.M., Eschalier, A., Gautron, M., and Kayser, V.: Evidence for peripheral serotonergic mechanisms in the early sensitization after carrageenin-induced inflammation: electrophysiological studies in the ventrobasal complex of the rat thalamus using a potent specific antagonist of peripheral 5-HT receptors. Brain Res. 502: 187-197, 1989.

Gustafsson, L.E., Wiklund, C.U., Wiklund, N.P., and Stelius, L.: Subclassification of neuronal adenosine receptors. *In:* Purines in Cellular Signalling: Targets for New Drugs. ed. by K.A. Jacobson, J.W. Daly, and V. Manganiello, pp. 200-205, Springer-Verlag, New York, 1990.

Haley, J.E., Sullivan, A.F., and Dickenson, A.H.: Evidence for spinal N-methyl-D-aspartate receptor involvement in prolonged chemical nociception in the rat. Brain Res. 518: 218-226, 1990.

Haley, J.E., Dickenson, A.H., and Schachter, M.: Electrophysiological evidence for a role of nitric oxide in prolonged chemical nociception in the rat. Neuropharmacology 31: 251-258, 1992.

Handwerker, H.O., and Reeh, P.W.: Pain and inflammation. *In:* Proceedings of the VIth World Congress on Pain, ed. by M.R. Bond, J.E. Charlton, and C.J. Woolf, pp. 59-70, Elsevier Science Publishers, Amsterdam, 1991.

Hannon, J.P., Pfannkuche, H.J., and Fozard, J.R.: A role for mast cells in adenosine A_3 receptor-mediated hypotension in the rat. Br. J. Pharmacol. 115: 945-952, 1995.

Harvey, S.C.: Antiseptics and disinfectants; fungicides; ectoparasiticides. *In:* The Pharmacological Basis of Therapeutics, ed. by A.G. Gilman, L.S. Goodman, T.W. Rall, and F. Murad, pp. 959-979, Macmillan, New York, 1985.

Helmstetter, F.J.: Stress-induced hypoanalgesia and defensive freezing are attenuated by application of diazepam to the amygdala. Pharmacol. Biochem. Behav. 44: 433-438, 1993.

Herbert, M.K., and Schmidt, R.F.: Activation of normal and inflamed fine articular afferent units by serotonin. Pain 50: 79-88, 1992.

Herrick-Davis, K., Chippari, S., Luttinger, D., Ward, S.J.: Evaluation of adenosine agonists as potential analgesics. Eur. J. Pharmacol. 162: 365-369, 1989.

Holmgren, M., Hedner, J., Mellstrand, T., Nordberg, G., and Hedner, T.: Characterization of the antinociceptive effects of some adenosine analogues in the rat. Naunyn-Schmiedeberg's Arch. Pharmacol. 334: 290-293, 1986.

Holthusen, H., and Arndt, J.O.: Nitric oxide evokes pain in humans on intracutaneous injection. Neurosci. Lett. 165: 71-74, 1994.

Hong, Y., and Abbott, F.V.: Behavioural effects of intraplantar injection of inflammatory mediators in the rat. Neuroscience 63: 827-836, 1994.

Hourani, S.M., and Cusack, N.J.: Pharmacological receptors on blood platelets. Pharmacol. Rev. 43: 243-298, 1991.

Hoyer, D., Clarke, D.E., Fozard, J.R., Hartig, P.R., Martin, G.R., Mylecharane, E.J., Saxena, P.R., and Humphrey, P.P.: International Union of Pharmacology classification of receptors for 5-hydroxytryptamine (serotonin). Pharmacol. Rev. 46: 157-203, 1994.

Huang, J.C., and Peroutka, S.J.: Identification of 5-hydroxytryptamine$_1$ binding site subtypes in rat spinal cord. Brain Res. 436: 173-176, 1987.

Humphrey, P.P.A., and Feniuk, W.: Mode of action of the anti-migraine drug sumatripan. Trends Pharmacol. Sci. 12: 444-446, 1991.

Hunskaar, S., Berge, O.-G., and Hole, K.: Dissociation between antinociceptive and anti-inflammatory effects of acetylsalicylic acid and indomethacin in the formalin test. Pain 25: 125-132, 1986.

Hunskaar, S., and Hole, K.: The formalin test: dissociation between inflammatory and non-inflammatory pain. Pain 30: 103-114, 1987.

Ialenti, A., Ianaro, A., Moncada, S., and Di Rosa, M.: Modulation of acute inflammation by endogenous nitric oxide. Eur. J. Pharmacol. 211: 177-182, 1992.

Jensen, K., Tuxen, C., Pedersen-Bjergaard, U., Jansen, I., Edvinsson, L., and Olesen, J.: Pain, wheal and flare in human forearm skin induced by bradykinin and 5-hydroxytryptamine. Peptides 11: 1133-1138, 1990.

Karlsten, R., Gordh, T., and Post, C.: Local antinociceptive and hyperalgesic effects in the formalin test after peripheral administration of adenosine analogues in mice. Pharmacol. Toxicol. 70: 434-438, 1992.

Kazakov, V.N., Kravtsov, P.Y., Krakhotnika, E.D., and Maisky, V.A.: Sources of cortical, hypothalamic and spinal serotonergic projections: topical organization within the nucleus raphe dorsalis. Neuroscience 56: 157-164, 1993.

Kessler, W., Kirchhoff, C., Reeh, P.W., and Handwerker, H.O.: Excitation of cutaneous afferent nerve endings *in vitro* by a combination of inflammatory mediators and conditioning effect of substance P. Exp. Brain Res. 91: 467-476, 1992.

Kwiat, G.C., and Basbaum, A.I.: The origin of brainstem noradrenergic and serotonergic projections to the spinal cord dorsal horn of the rat. Somatosens. Mot. Res. 9: 157-173, 1992.

Lagerqvist, B., Sylvén, C., Beermann, B., Helmius, G., and Waldenströom, A.: Intracoronary adenosine causes angina pectoris like pain - an inquiry into the nature of visceral pain. Cardiovasc. Res. 24: 609-613, 1990.

Lang, E., Novak, A., Reeh, P.W., and Handwerker, H.O.: Chemosensitivity of fine afferents from rat skin *in vitro*. J. Neurophysiol. 63: 887-901, 1990.

Levine, J.D., Taiwo, Y.O., Collins, S.D., and Tam, J.K.: Noradrenaline hyperalgesia is mediated through interaction with sympathetic postganglionic neurone terminals rather than activation of primary afferent nociceptors. Nature 323: 158-160, 1986.

Levine, J.D., Fields, H.L., and Basbaum, A.I.: Peptides and the primary afferent nociceptor. J. Neurosci. 13: 2273-2286, 1993.

MacDonald, R.L., Skerritt, J.H., and Werz, M.A.: Adenosine agonists reduce voltage-dependent calcium conductance of mouse sensory neurones in cell culture. J. Physiol. (London) 370: 75-90, 1986.

Maling, H.M., Webster, M.E., Williams, M.A., Saul, W., and Anderson, W. Jr.: Inflammation induced by histamine, serotonin, bradykinin and compound 48/80 in the rat: antagonists and mechanisms of action. J. Pharmacol. Exp. Ther. 191: 300-310, 1974.

Mantegazza, P., Tammiso, R., Zambotti, F., Zecca, L., Zonta, N.: Purine involvement in morphine antinociception. Br. J. Pharmacol. 83: 883-888, 1984.

Manzini, S., and Ballati, L.: 2-Chloroadenosine induction of vagally-mediated and atropine-resistant bronchomotor responses in anaesthetized guinea-pigs. Br. J. Pharmacol. 100: 251-256, 1990.

Martin, G.R.: Vascular receptors for 5-hydroxytryptamine: distribution, function and classification. Pharmac. Ther. 62: 283-324, 1994.

Matherne, G.P., Headrick, J.P., Coleman, S.D., and Berne, R.M.: Interstitial transudate purines in normoxic and hypoxic immature and mature rabbit hearts. Pediatr. Res. 28: 348-353, 1990.

Matsuzaki, S., Takada, M., Li, Y.Q., Tokuno, H., and Mizuno, N.: Serotonergic projections from the dorsal raphe nucleus to the nucleus submedius in the rat and cat. Neuroscience 55: 403-416, 1993.

Munshi, R., Pang, I.-H., Sternweis, P.C., and Linden, J.: A1 adenosine receptors of bovine brain couple to guanine nucleotide-binding proteins G_{i1}, G_{i2} and G_o. J. Biol. Chem. 266: 22285-22289, 1991.

Mylecharane, E.J., and Phillips, C.A.: Pre-synaptic sympathetic inhibition and 5-hydroxytryptamine-induced vasodilation. *In:* Serotonin: Actions, receptors, pathophysiology, Satellite Symposia of the IUPHAR 10th International Congress of Pharmacology, ed. by E.J. Mylecharane, J.A. Angus, I.S. de la Lande and P.P.A. Humphrey, pp. 137-145, Macmillan Press, Hampshire, 1989.

Nordström, O., and Delbro, D.: Adenosine-induced contractions of the isolated rat trachea are mediated by 5-hydroxytryptamine. Acta Physiol. Scand. 127: 557-558, 1986.

Ortmann, R., Bischoff, S., Radeke, E., Buech, O., and Deline-Stula, A.: Correlations between different measures of antiserotonin activity of drugs: study with neuroleptics and serotonin receptor blockers. Naunyn-Schmiedeberg's Arch. Pharmacol. 321: 265-270, 1982.

Page, C.P.: Platelets as inflammatory cells. Immunopharmacology 17: 51-59, 1989.

Pappagallo, M., Gaspardone, A., Tomai, F., Iamele, M., Crea, F., and Gioffré, P.A.: Analgesic effect of bamiphylline on pain induced by intradermal injection of adenosine. Pain 53: 199-204, 1993.

Pierce, P.A., Xie, G.-X., Levine, J.D., and Peroutka, S.J.: 5-hydroxytryptamine receptor subtype messenger RNAs in rat peripheral sensory and sympathetic ganglia: a polymerase chain reaction study. Neuroscience 70: 553-559, 1996.

Puig, S., and Sorkin, L.S.: Formalin-evoked activity in identified primary afferent fibres: systemic lidocaine suppresses phase-2 activity. Pain 64: 345-355, 1995.

Raja, S.N.: Role of the sympathetic nervous system in acute pain and inflammation. Ann. Med. 27: 241-246, 1995.

Ramkumar, V., Stiles, G.L., Bevan, M.A., and Ali, H.: The A3 adenosine receptor is the unique adenosine receptor which facilitates release of allergic mediators in mast cells. J. Biol. Chem. 268: 16887-16890, 1993.

Rhodes, K.F., Coleman, J., and Lattimer, N.: A component of 5-HT-evoked depolarization of the rat isolated vagus nerve is mediated by a putative 5-HT_4 receptor. Naunyn-Schmiedeberg's Arch. Pharmacol. 346: 496-503, 1992.

Richardson, B.P., Engel, G., Donatsch, P., and Stadler, P.A.: Identification of serotonin M-receptor subtypes and their specific blockade by a new class of drugs. Nature 316: 126-131, 1985.

Ringkamp, M., Schmelz, M., Kress, M., Allwang, A., Ogilvie, A., and Reeh, P.W.: Activated human platelets in plasma excite nociceptors in rat skin, *in vitro*, Neurosci. Lett. 170: 103-106, 1994.

Rivot, J.P., Weil-Fugazza, J., Godefroy, F., Bineau-Thurotte, M., Ory-Lavollee, L., and Besson, J.M.: Involvement of serotonin in both morphine and stimulation produced analgesia: Electrochemical and biochemical approaches. Adv. Pain Res. Ther. 6: 135-150, 1984.

Rosland, J.H.: The formalin test in mice: the influence of ambient temperature. Pain 45: 211-216, 1991.

Rosland, J.H., Tjølsen, A., Mæhle, B., and Hole, K.: The formalin test in mice: effect of formalin concentration. Pain 42: 235-242, 1990.

Ruda, M.A., Bennet, G.J., and Dubner, R.: Neurochemistry and neural circuitry in the dorsal horn. *In:* Progress in Brain Research, Vol. 66, ed. by P.C. Emson, M. Rossor and M. Tohyamma, pp. 219-268, Elsevier Science Publishers, Amsterdam, 1986.

Rueff, A., and Dray, A.: 5-Hydroxytryptamine-induced sensitization and activation of peripheral fibres in the neonatal rat are mediated via different 5-hydroxytryptamine-receptors. Neuroscience 50: 899-905, 1992.

Ryan, S.M., Watkins, L.R., Mayer, D.J., and Maier, S.F.: Spinal pain suppression mechanisms may differ for phasic and tonic pain. Brain Res. 334: 172-175, 1985.

Salter, M.W., and Henry, J.L.: Effects of adenosine 5'-monophosphate and adenosine 5'-triphosphate on functionally identified units in the cat spinal dorsal horn. Evidence for a differential effect of adenosine 5'-triphosphate on nociceptive vs non-nociceptive units. Neuroscience 15: 815-825, 1985.

Santicioli, P., Del Bianco, E., and Maggi, C.A.: Adenosine A_1 receptors mediate the presynaptic inhibition of calcitonin gene-related peptide release by adenosine in the rat spinal cord. Eur. J. Pharmacol. 231: 139-142, 1993.

Santicioli, P., Del Bianco, E., Tramontana, M., and Maggi, C.A.: Adenosine inhibits action potential-dependent release of calcitonin gene-related peptide- and substance P-like immunoreactivities from primary afferents in rat spinal cord. Neurosci. Lett. 144: 211-214, 1992.

Sato, J., Suzuki, S., Iseki, T., and Kumazawa, T.: Adrenergic excitation of cutaneous nociceptors in chronically inflamed rats. Neurosci. Lett. 164: 225-228, 1993.

Sawynok, J.: Opioid and nonopioid antinociception as revealed by lesion studies. Meth. Neurosci. 7: 200-216, 1991.

Sawynok, J.: Purines and nociception. *In:* Purinergic Approaches in Experimental Therapeutics, ed. by K. Jacobsen and M.F. Jarvis, (in press), Wiley & Sons, New York, 1997.

Sawynok, J., and Reid, A.: Noradrenergic and purinergic involvement in spinal antinociception by 5-hydroxytryptamine and 2-methyl-5-hydroxytryptamine. Eur. J. Pharmacol. 204: 301-309, 1991.

Sawynok, J., Reid, A.R., and Doak, G.J.: Caffeine analgesia in the rat hot plate and formalin tests. Pain 61: 203-213, 1995.

Sawynok, J., and Yaksh, T.L.: Caffeine as an analgesic adjuvant: a review of pharmacology and mechanisms of action. Pharmacol. Rev. 45: 43-85, 1993.

Sawynok, J., Zarrindast, M.R., Reid, A.R., and Doak, G.J.: Adenosine A_3 receptor activation produces nociceptive behaviour and edema by release of histamine and 5-hydroxytryptamine. Eur. J. Pharmacol. (in press), 1997.

Saxena, P.R.: Serotonin receptors: subtypes, functional responses and therapeutic relevance. Pharmac. Ther. 66: 339-368, 1995.

Scholz, K.P., and Miller, R.J.: Analysis of adenosine actions on Ca2+ currents and synaptic transmission in cultured rat hippocampal pyramidal neurones. J. Physiol. (Lond.) 435: 373-393, 1991.

Seale, T.W., Abla, K.A., Shamin, M.T., Carney, J.M., and Daley, J.W.: 3,7-dimethyl-1-propargylxanthine: a potent and selective *in vivo* antagonist of adenosine analogs. Life Sci. 43: 1671-1684, 1988.

Shibata, M., Ohkubo, T., Takahashi, H., and Inoki, R.: Modified formalin test: characteristic biphasic pain response. Pain 38: 347-352, 1989.

Simone, D.A., Alreja, M., and LaMotte, R.H.: Psychological studies of the itch sensation and itchy skin ("alloknesis") produced by intracutaneous injection of histamine. Somatosens. Mot. Res. 8: 271-279, 1991.

Solomon, R.E., and Gebhart, G.F.: Mechanisms of effects of intrathecal serotonin on nociception and blood pressure in rats. J. Pharmacol. Exp. Ther. 245: 905-911, 1988.

Stamford, J.A.: Descending control of pain. Br. J. Anaesth. 75: 217-227, 1995.

Steen, K.H., Reeh, P.W., Anton, F., and Handwerker, H.O.: Protons selectively induce lasting excitation and sensitization to mechanical stimulation of nociceptors in rat skin, *in vitro*. J. Neurosci. 12: 86-95, 1992.

Steen, K.H., Steen, A.E., and Reeh, P.W.: A dominant role of acid pH in inflammatory excitation and sensitization of nociceptors in rat skin, *in vitro*. J. Neurosci. 15: 3982-3989, 1995.

Sufka, K.J., Schomburg, F.M., and Giordano, J.: Receptor mediation of 5-HT-induced inflammation and nociception in rats. Pharmacol. Biochem. Behav. 41: 53-56, 1991.

Sugimoto, M., Kuraishi, Y., Satoh, M., and Takagi, H.: Involvement of medullary opioid-peptidergic and spinal noradrenergic systems in the regulation of formalin-induced persistent pain. Neuropharmacology 25: 481-485, 1986.

Sweeney, M.I., White, T.D., and Sawynok, J.: Involvement of adenosine in the spinal antinociceptive effects of morphine and noradrenaline. J. Pharmacol. Exp. Ther. 243: 657-665, 1987.

Sweeney, M.I., White, T.D., and Sawynok, J.: 5-Hydroxytryptamine releases adenosine from primary afferent nerve terminals in the spinal cord. Brain Res. 462: 346-349, 1988.

Sweeney, M.I., White, T.D., and Sawynok, J.: 5-Hydroxytryptamine releases adenosine and cyclic AMP from primary afferent nerve terminals in the spinal cord *in vivo*. Brain Res. 528: 55-61, 1990.

Sylvén, C.: Mechanisms of pain in angina pectoris - a critical review of the adenosine hypothesis. Cardiovasc. Drugs Ther. 7: 745-759, 1993.

Sylvén, C., Beermann, B., Edlund, A.,Lewander, R., Jonzon, B., and Mogensen, L.: Provocation of chest pain in patients with coronary insufficiency using the vasodilator adenosine. Eur. Heart J. 9 (Suppl N): 6-10, 1988a.

Sylvén, C., Beermann, B., Jonzon, B., and Brandt, R.: Angina pectoris-like pain provoked by intravenous adenosine in healthy volunteers. Br. Med. J. 293: 227-230, 1986.

Sylvén, C., Jonzon, B., Fredholm, B.B., and Kaijser, L.: Adenosine injected into the brachial artery produces ischaemia like pain or discomfort in the forearm. Cardiovasc. Res. 22: 674-678, 1988b.

Taiwo, Y.O, Heller, P.H., and Levine, J.D.: Mediation of serotonin hyperalgesia by the cAMP second messenger system. Neuroscience 48: 479-483, 1992.

Taiwo, Y.O., and Levine, J.D.: Direct cutaneous hyperalgesia induced by adenosine. Neuroscience 38: 757-762, 1990.

Taiwo, Y.O., and Levine, J.D.: Role of adenyl cyclase and cAMP-dependent protein kinase in primary afferent hyperalgesia. Neuroscience 44: 131-136, 1991.

Taiwo, Y.O., and Levine, J.D.: Serotonin is a directly-acting hyperalgesic agent in the rat. Neuroscience 48: 485-490, 1992.

Tjølsen, A., Berge, O.-G., Hunskaar, S., Rosland, J.H., and Hole, K.: The formalin test: an evaluation of the method. Pain 51: 5-17, 1992.

Treede, R.-D., Meyer, R.A., Raja, S.N., and Campbell, J.N.: Peripheral and central mechanisms of cutaneous hyperalgesia. Prog. Neurobiol. 38: 397-421, 1992.

Trussell, L.O., and Jackson, M.B.: Adenosine-activated potassium conductance in cultured striatal neurons. Proc. Natl. Acad. Sci. USA 82: 4857-4861, 1985.

Van Calker, D., Muller, M., and Hamprecht, B.: Adenosine regulates via two different types of receptors, the accumulation of cyclic AMP in cultured brain cells. J. Neurochem. 33: 999-1005, 1979.

Van Schaick, E.A., Jacobson, K.A., Kim, H.O., Ijzerman, A.P., and Danhof, M.: Hemodynamic effects and histamine release elicited by the selective adenosine A_3 receptor agonist 2-Cl-IB-MECA in conscious rats. Eur. J. Pharmacol. 308: 311-314, 1996.

Vinegar, R., Truax, J.F., Selph, J.L., and Johnston, P.R.: Pharmacological characterization of the algesic response to the subplantar injection of serotonin in the rat. Eur. J. Pharmacol. 164: 497-505, 1989.

Wang, Q.-P., and Nakai, Y.: The dorsal raphe: an important nucleus in pain modulation. Brain Res. Bull. 34: 575-585, 1994.

Wheeler-Aceto, H., and Cowan, A.: Standardization of the rat paw formalin test for evaluation of analgesics. Psychopharmacology 104: 35-44, 1991.

Wheeler-Aceto, H., Porreca, F., and Cowan, A.: The rat paw formalin test: comparison of noxious agents. Pain 40: 229-238, 1990.

Yakel, J.L., and Jackson, M.B.: 5-HT_3 receptors mediate rapid responses in cultured hippocampus and clonal cell line. Neuron 1: 615-621, 1988.

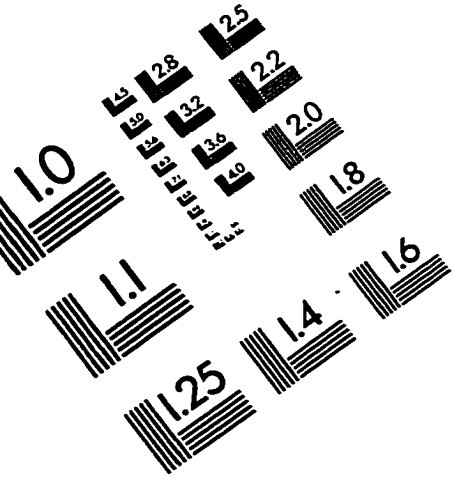
Yarbrough, G.G., and McGuffin-Clineschmidt, J.C.: *In vivo* behavioural assessment of central nervous system purinergic receptors. Eur. J. Pharmacol. 76: 137-144, 1981.

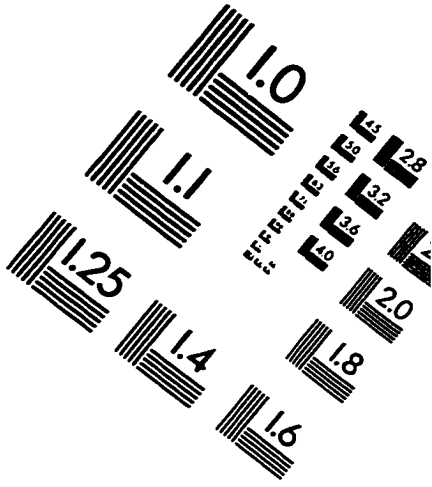
Zemlan, F.P., Schwab, E.F., Murphy, R.M., and Behbehani, M.M.: Identification of a novel 5-HT_1 binding site in the rat spinal cord. Neurochem. Int. 16: 507-513, 1990.

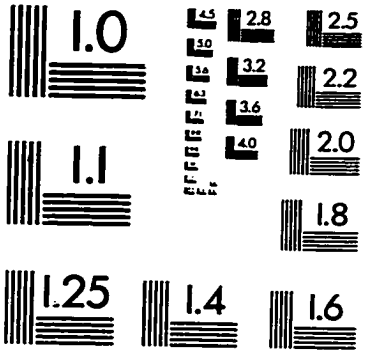
Zhou, Q.-Y., Li, C., Olah, M.E., Johnson, R.A., Stiles, G.L., and Civelli, O.: Molecular cloning and characterization of an adenosine receptor: the A_3 adenosine receptor. Proc. Natl. Acad. Sci. U.S.A. 89: 7432-7436, 1992.

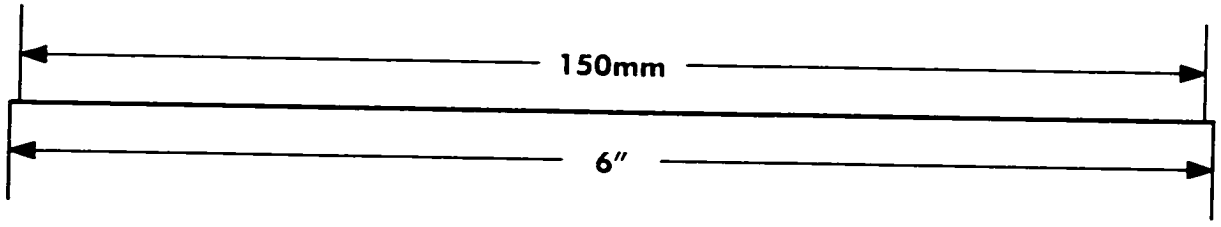
Zifa, E., and Fillion, G.: 5-hydroxytryptamine receptors. Pharmacol. Rev. 44: 401-458, 1992.

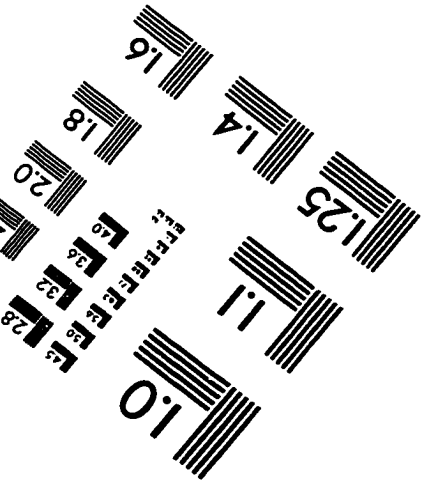
IMAGE EVALUATION TEST TARGET (QA-3)

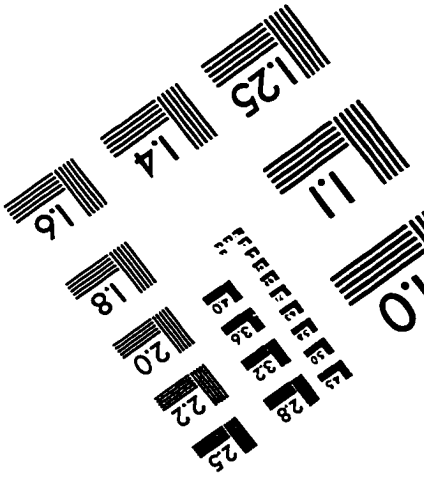












APPLIED IMAGE, Inc
1653 East Main Street
Rochester, NY 14609 USA
Phone: 716/482-0300
Fax: 716/288-5989