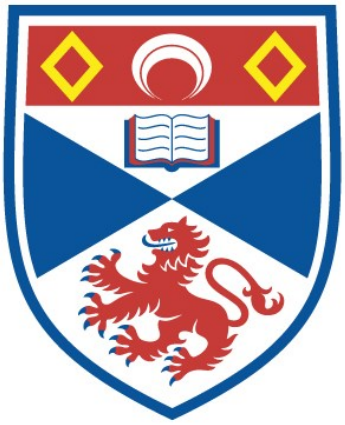
CHARACTERIZATION OF 5-HYDROXYTRYPTAMINE RECEPTORS IN THE SNAIL, 'HELIX ASPERSA'

Anna-Karina Cadogan

A Thesis Submitted for the Degree of PhD
at the
University of St Andrews



1992

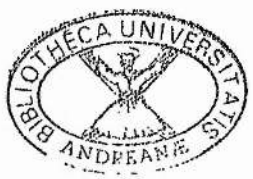
CHARACTERIZATION OF 5-HYDROXYTRYPTAMINE RECEPTORS IN THE SNAIL, *HELIX ASPERSA*

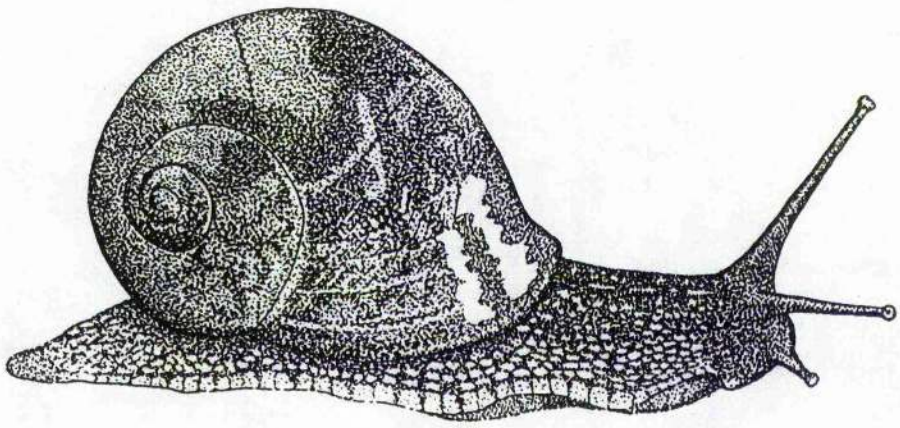
by Anna-Karina Cadogan

A thesis submitted to the University of St. Andrews in candidature for the degree of Doctor of Philosophy.

Department of Biology and
Preclinical Medicine,
St. Andrews University.

July 1991





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I, Anna-Karina Cadogan, hereby certify that this thesis has been composed by myself, that it is a record of my own work, and that it has not been accepted in partial or complete fulfilment of any other degree of professional qualification.

Signed:

Date: 5.4.91

I was admitted to the Faculty of Science of the University of St. Andrews under Ordinance General No 12 in October, 1987 and as a canditate for the degree of Ph.D. in October, 1987.

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Date: 5. 4. 91.

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ACKNOWLEDGEMENTS

Firstly I would like to express my sincere thanks to my academic supervisor, Professor Glen Cottrell, for his help and guidance and to my supervisor at Glaxo, Dr. Pat Humphrey, for his inspiration and support. Had it not been for Pat's encouragement, I would never have been cajoled into giving a talk at the British Pharmacological Society last december and for that opportunity, I am eternally grateful.

At St. Andrews, I am indebted to Dr. Jim Aiton for his patience in dealing with my enquiries about the Macintosh, to Dr. Gordon Cramb for allowing me laboratory space to finish my assays, to the photographic unit for their help and advice with figures and photographs, to Dr. Colin Thomson and his wife, Maureen for their support during this period and to Christina Lamb who typed a draft of this thesis into my computer. I am also extremely grateful to my fellow workers who have helped me out on numerous occasions.

At Glaxo, where I spent nine happy months, I would like to thank Dr. Wasyl Feniuk for his thoughtful discussion and his support at the British Pharmacological Society meeting in december, Mike Sumner for putting up with me in his group for that period and to all the other Glaxo staff who, not only welcomed me in but also helped me in every way possible.

I wish also to acknowledge the financial support I received from the Science and Engineering Research Council and Glaxo who supported me as a CASE student.

Finally, I must express my heartfelt gratitude to Claire and Chris for their constant moral support, friendship and encouragement and to Gary for his considerable patience and understanding.

ABSTRACT

The aim of this investigation was to characterize those 5-HT receptors present in three different tissues of the common garden snail, *Helix aspersa*, into one or more of the categories already described for vertebrate 5-HT receptors. Specific 5-HT receptor agonists and antagonists which had been developed and used to help characterize, and subsequently classify, the various types of 5-HT receptor in vertebrates, were utilized in this study. The three preparations from *Helix* included: i) identified neurones in the visceral ganglion ii) the heart and iii) the pharyngeal retractor muscle (PRM).

The action of 5-HT on identified neurones in the visceral ganglion was studied using the electrophysiological techniques of both voltage- and current-clamp. Under voltage-clamp conditions the response of the identified neurones to iontophoretic application of 5-HT was seen to be an inward current of approximately 3-10 nA. Under current-clamp conditions the response to 5-HT was an excitatory depolarization leading to the firing of action potentials of approximately 3-15 mV. Both responses showed rapid desensitization to repetitive applications of 5-HT and were blocked by tubocurarine. No specific 5-HT receptor antagonist to this 5-HT response in *Helix* neurones was found. The action of 5-HT was mimicked by 5-CT and α-Me-5-HT both of which showed similar-sized responses to 5-HT, whereas sumatriptan gave smaller responses than those of 5-HT.

5-HT had a positive inotropic effect on the heart. The excitatory action of 5-HT on the heart was studied using an organ bath methodology with application of the 5-HT receptor agonists and antagonists at suitable concentrations. No specific 5-HT receptor antagonist was found for the cardioexcitatory effect of 5-HT. The full rank order of potency for the 5-HT receptor agonists tested was 5-HT > methylergometrine = ergotamine = 5-CT > α-Me-5-HT = sumatriptan > methysergide = 2-Me-5-HT = tryptamine ≥ 8-OH-DPAT.

5-HT caused relaxation in the PRM and was found to inhibit, in a dose-dependent manner, acetylcholine (ACh)-induced contraction in the muscle. This inhibition of ACh-induced contraction by 5-HT in the PRM was studied using an organ bath methodology with application of 5-HT receptor agonists

and antagonists at suitable concentrations. No specific 5-HT receptor antagonist for the inhibition of ACh-induced contraction was found. The rank order of 5-HT receptor agonist potency was 5-HT > 5-CT > sumatriptan = ergotamine = methysergide >> α-Me-5-HT = 2-Me-5-HT.

The effect of 5-HT on cyclic adenosine 3',5'-monophosphate (cAMP) levels within *Helix* heart and pharyngeal retractor muscle (PRM) tissue were monitored in this investigation. 5-HT caused a dose-dependent increase in cAMP both in *Helix* heart and PRM tissue.

The 5-HT receptors within *Helix* are not readily characterized into any of the categories of 5-HT receptor that have been already classified in vertebrates: *Helix* 5-HT receptors appear to be unique in the fact that they are unclassifiable in terms of the vertebrate 5-HT receptor classification. The evidence presented in this investigation is discussed in terms of the molecular biology of receptors: this includes the hypothesis that the 5-HT receptors particularly in *Helix* heart and PRM tissue could be related to a family of G-protein-coupled receptors whereas the neuronal 5-HT receptors in Helix are more likely to be integral to an ion channel.

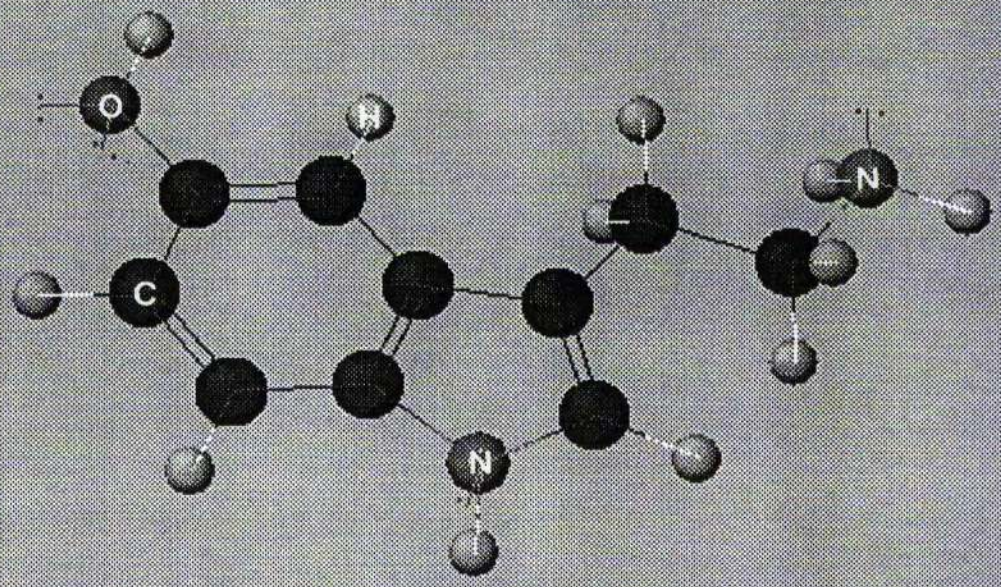
ABBREVIATIONS

ABRM	anterior byssus retractor muscle
AC	alternating current
ACh	acetylcholine
ATP	adenosine triphosphate
AV	atrio-ventricular
BSA	bovine serum albumen
cAMP	cyclic adenosine 3', 5'-monophosphate
cGMP	cyclic guanosine 3', 5'-monophosphate
CNS	central nervous system
D	aspartic acid
DC	direct current
DMSO	dimethyl sulphoxide
EC	excitation-contraction
EDTA	ethylene diamine tetraacetic acid
F	phenylalanine
g	gram
Hepes	N-2-Hydroxyethylpiperazine-N'-2-ethanesulfonic acid
Hz	Hertz
IBMX	3-isobutyl-1-methylxanthine
ICS 205-930	(3α-tropanyl)-1H-indole-3-carboxylic acid ester
KΩ	kilohm
l	litre
L	leucine
LSD	lysergic acid diethylamide
M	molar
M	methionine
MAO	monoamine oxidase
mCPP	1-(3-chlorophenyl)piperazine
mg	milligram
MHz	megahertz
min	minute
ml	millilitre
mM	millimolar
mm	millimetre
MDL 72222	1αH,3α,5αH-tropan-3-yl-3,5-dichlorobenzoate
MK 212	6-chloro-2-(piperazinyl)-pyrazine

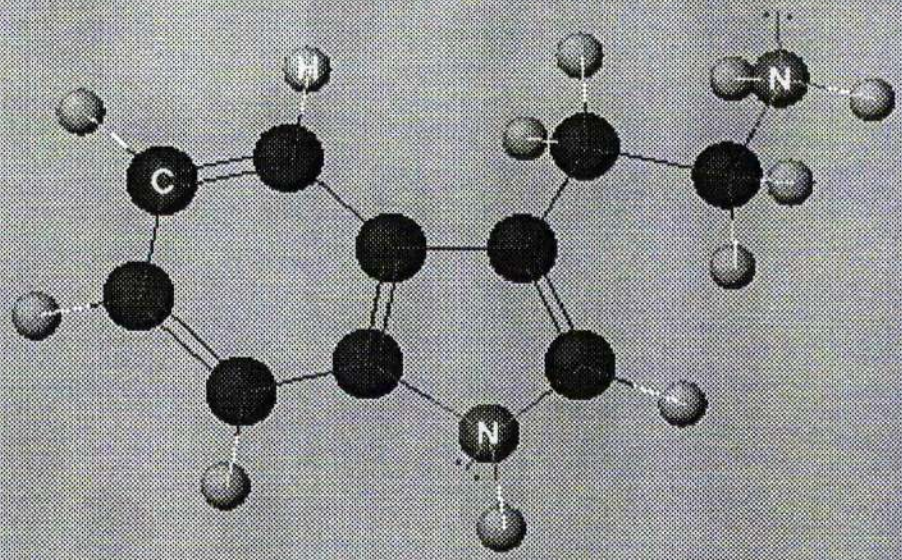
MΩ	megohm
m V	millivolt
µF	microfarad
µg	microgram
µM	micromolar
µm	micron
µl	microlitre
nA	nanoamp
nm	nanometre
nM	nanomolar
P	proline
PAPP	p-amino-phenylTFMPP
pmole	picomole
pQ	pyroglutamate
PRM	pharyngeal retractor muscle
R	arginine
RU 24969	5-methoxy-3(1,2,3,6-tetrahydropyridin-4-yl)1H indole
s	second
TFMPP	m-trifluoromethylphenyl pierazine
TRM	tentacle retractor muscle
UV	ultraviolet
4-HT	4-hydroxytryptamine
5-CT	5-carboxamidotryptamine
5-HT	5-hydroxytryptamine
5-HTP	5-hdroxytryptophan
5-MeOT	5-methoxytryptamine
5-MeODMT	5-methoxy-N,N-dimethyltryptamine
6-HT	6-hydroxytryptamine
7-HT	7-hydroxytryptamine
8-OH-DPAT	8-hydroxy-2-(di-n-propylamino)tetralin

MOLECULAR STRUCTURES OF SOME OF THE RELEVANT 5-HT AGONISTS AND ANTAGONISTS

The following pages give the structures of the most frequently used 5-HT compounds in the present study. The structures were calculated with the CAChe molecular modelling system (Tektronix). The compounds include 5-HT, 5-CT, tryptamine, α-Me-5-HT, 2-Me-5-HT, methysergide, LSD, ketanserin and ondansetron. The atoms in each of the structures are labelled: C for carbon, H for hydrogen, O for oxygen, N for nitrogen, F for fluorine and S for sulphur. The small black dots, attached to some of the atoms, indicate lone pairs of electrons. My sincere thanks to Dr. Colin Thomson who kindly gave up his time to help me optimize the structures of these compounds with this molecular modelling programme.



5—HT



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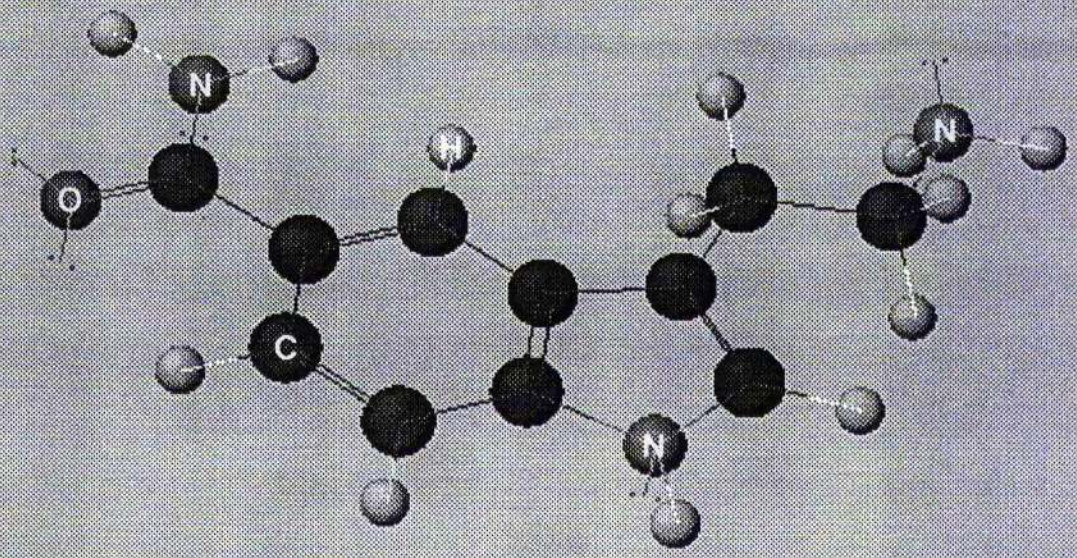
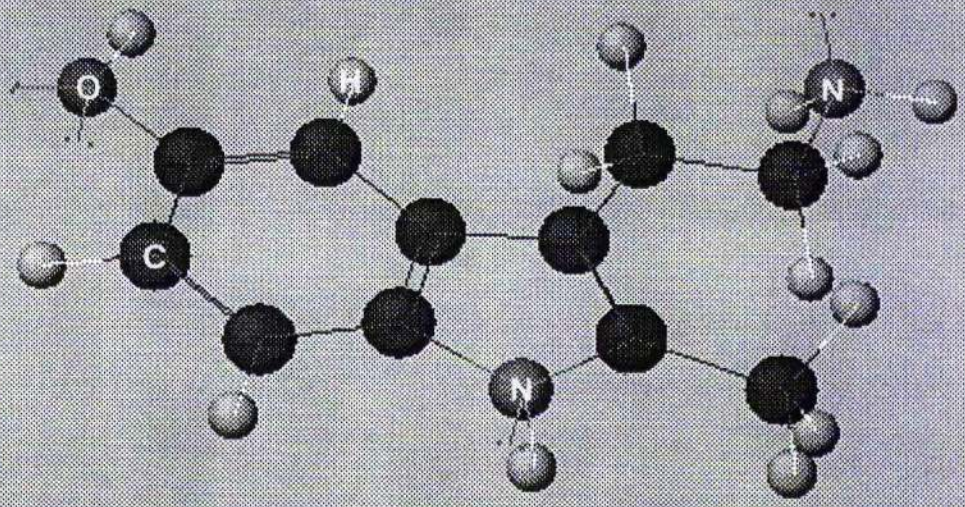
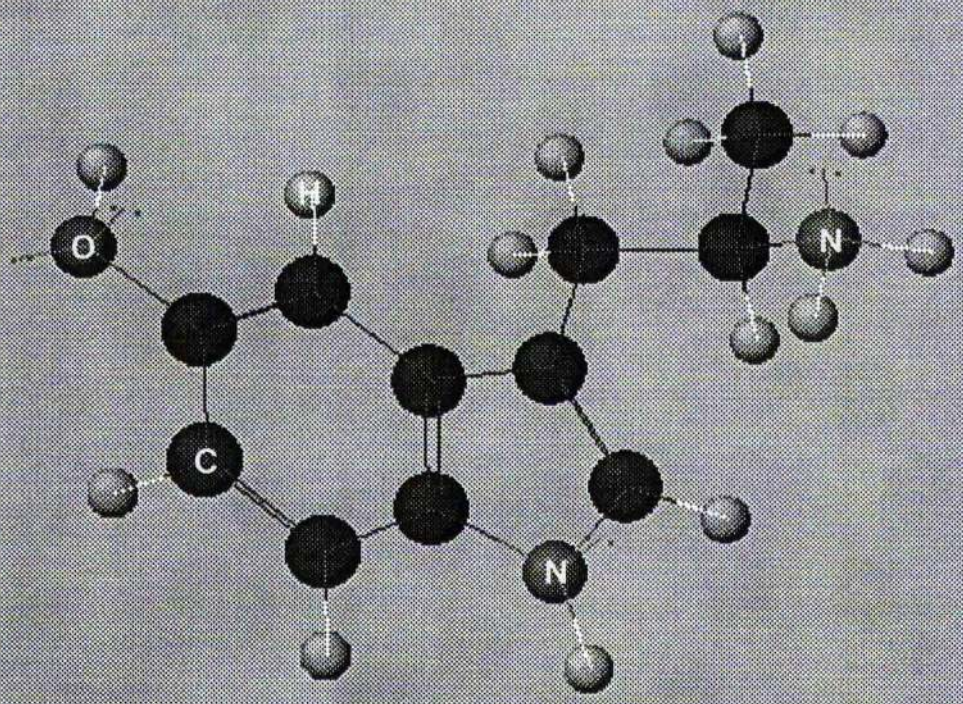


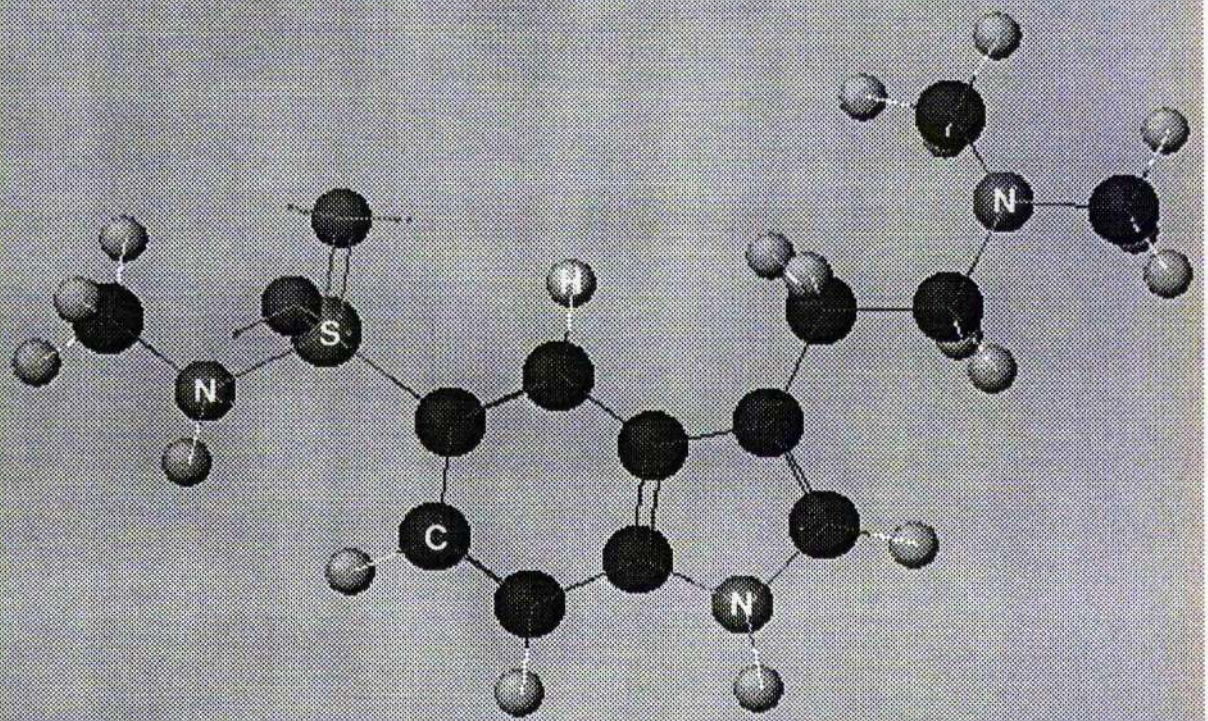
5-CT



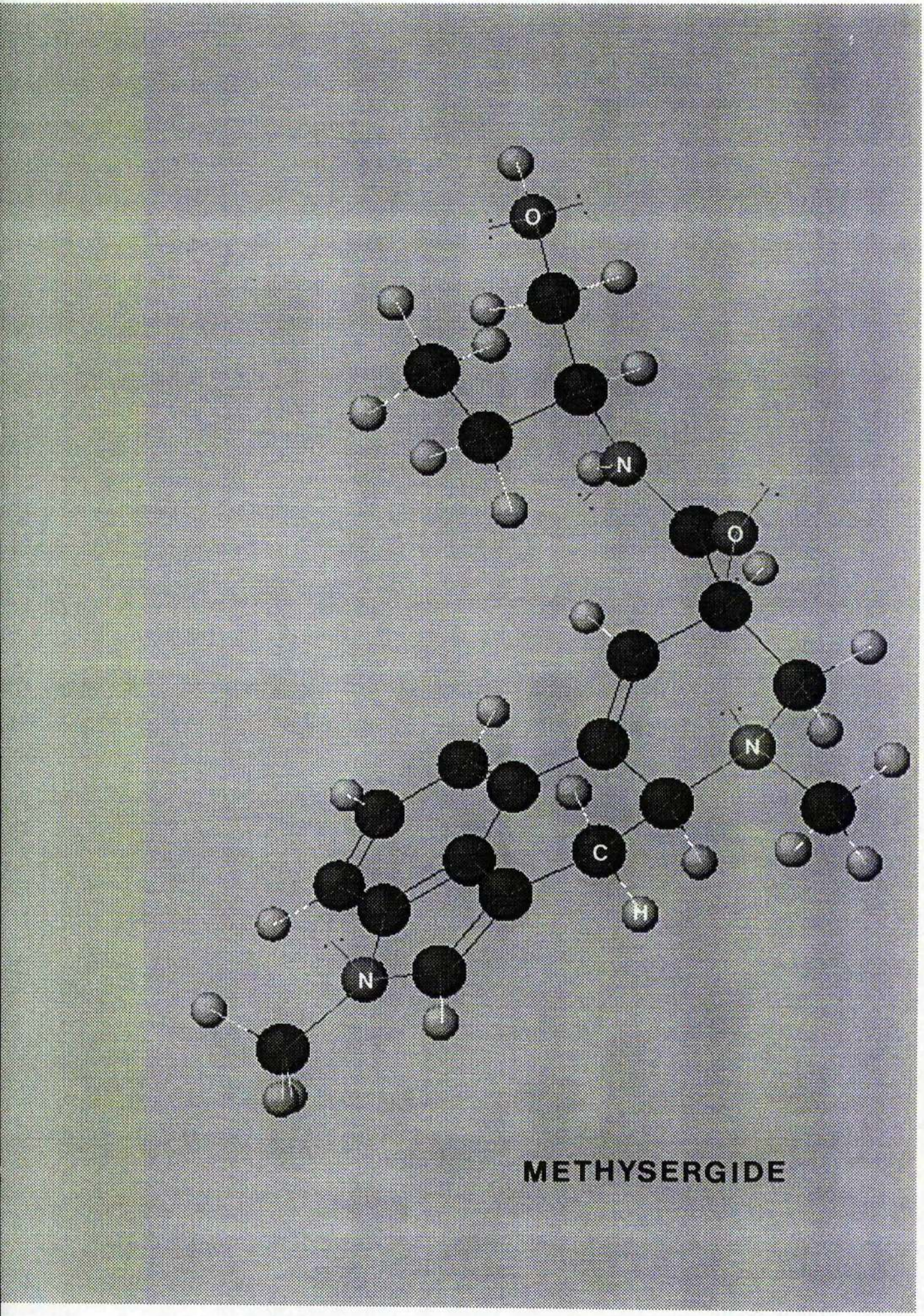
2-ME-5-HT



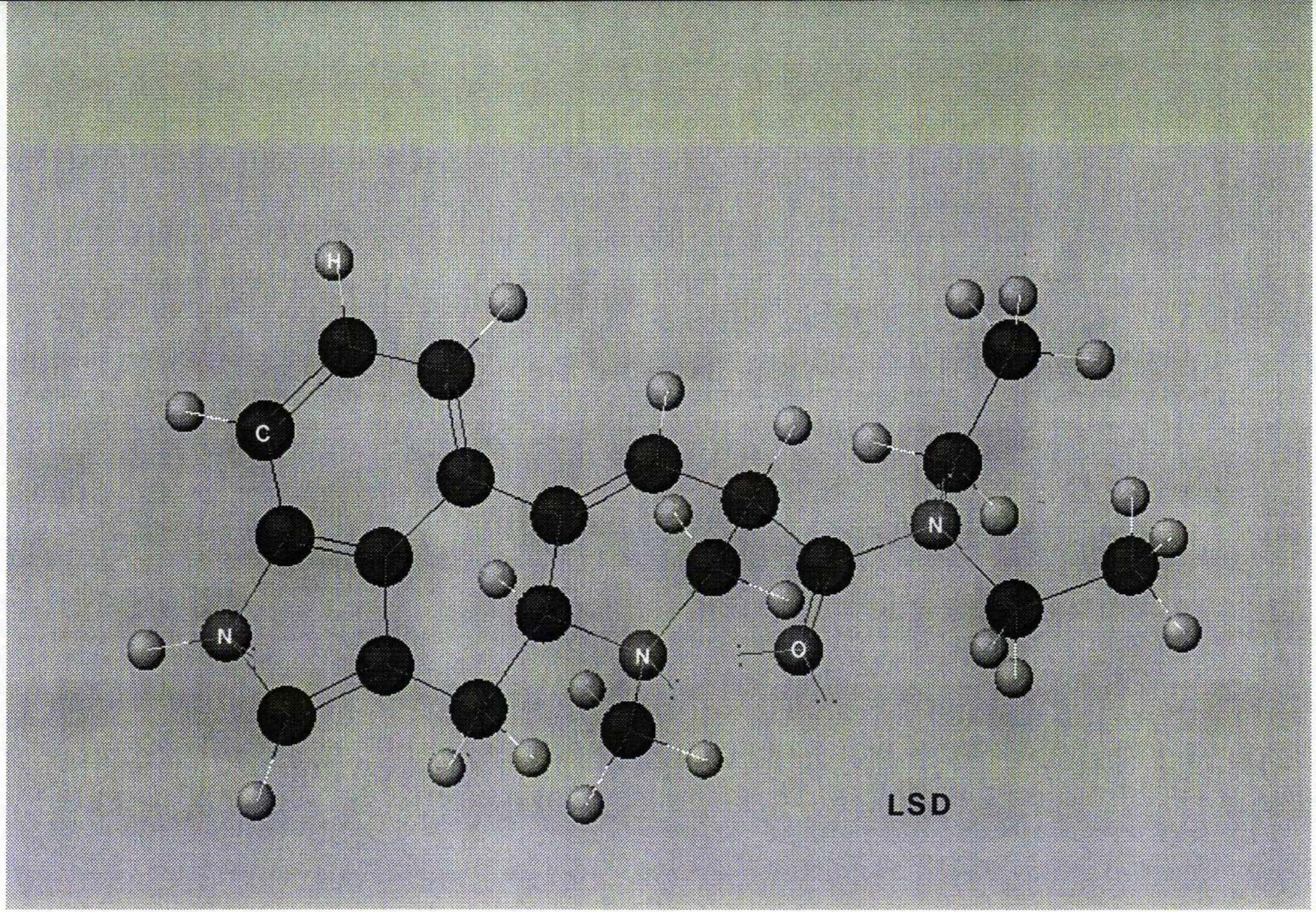
∝– ME–5–HT



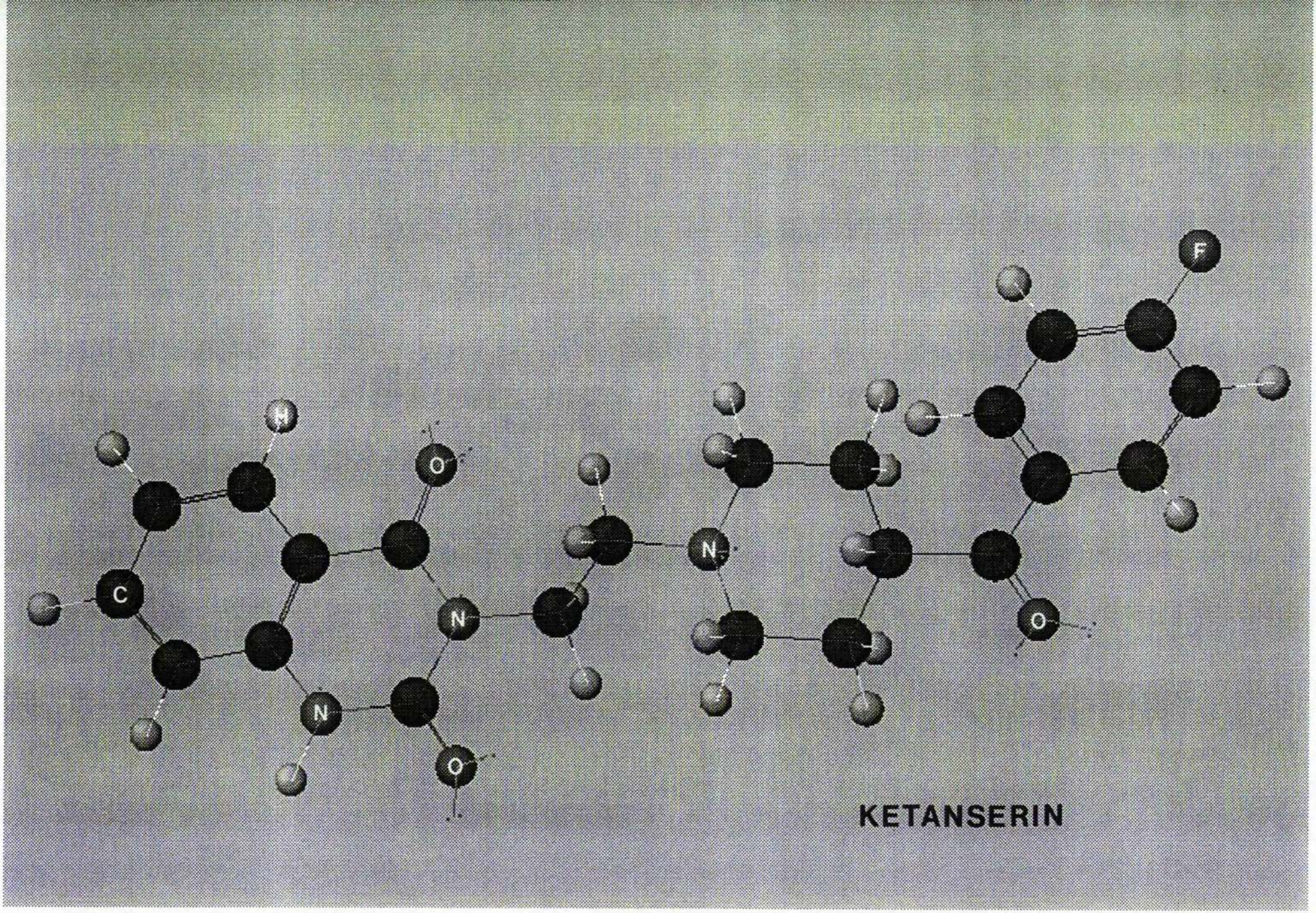
SUMATRIPTAN



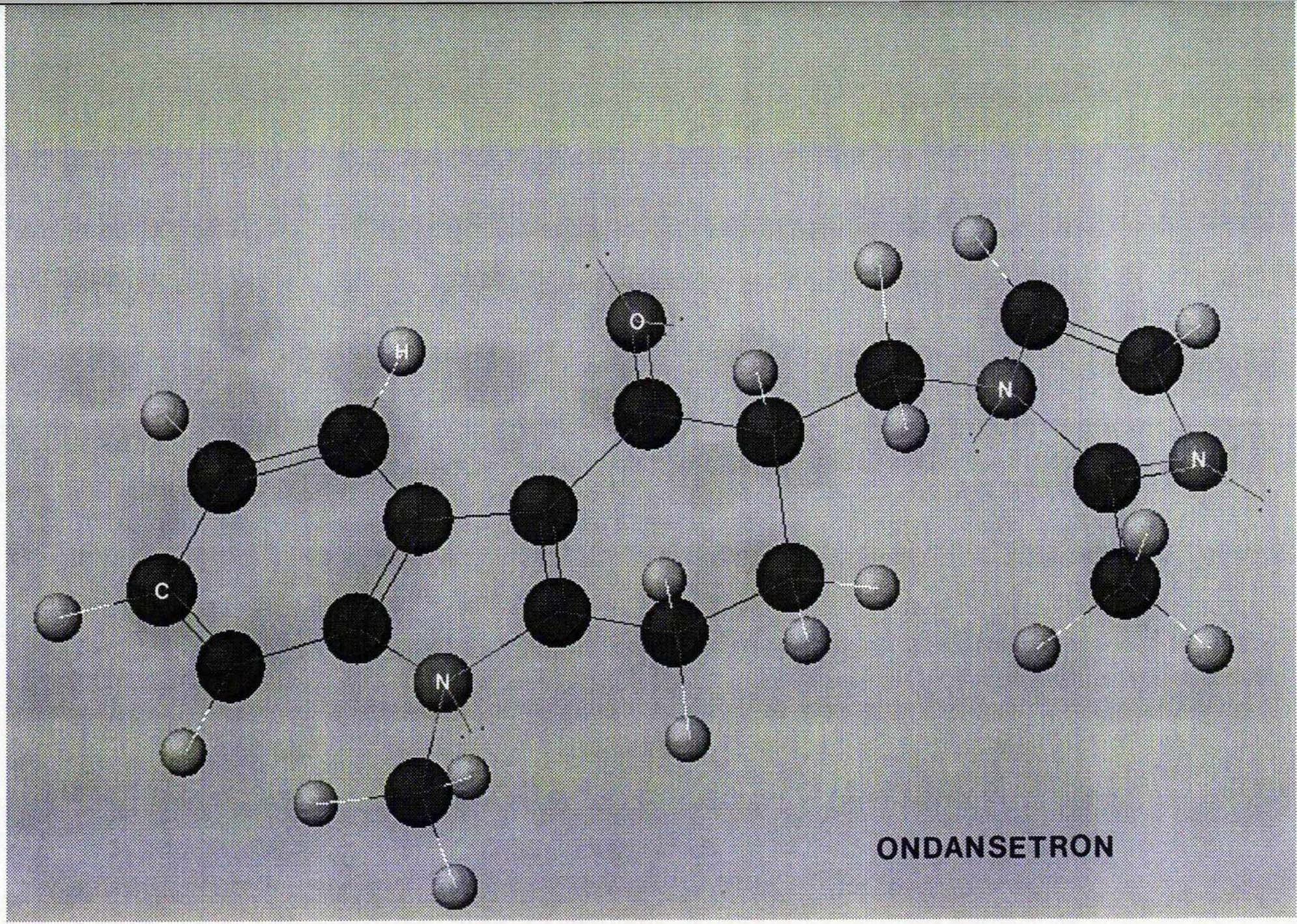
METHYSERGIDE



LSD



KETANSERIN



ONDANSETRON

CONTENTS

CHAPTER ONE

GENERAL INTRODUCTION

Historical perspective

5-hydroxytryptamine (5-HT) or serotonin, as it is also known, is one of the biogenic amines. However, unlike dopamine and noradrenaline which are catecholamines, 5-HT is derived from an indole nucleus and is therefore classified as an indoleamine.

Physiologists of the 19th century were aware of a substance in the blood serum that caused constriction of blood vessels and increased vascular tone. In the late 1940s, investigators were successful in isolating this serum-born substance, and named it serotonin (Rapport *et al.*, 1948). They later deduced that the active component of their crystalline complex was 5-hydroxytryptamine (Rapport, 1949). About the same time, a group of Italian workers had been investigating a substance found in high concentrations in enterochromaffin cells of the intestinal mucosa in the gut which they had called enteramine (Erspamer, 1940). Enteramine also occurred in the skin and salivary glands of lower animals such as toads, salamanders and octopuses. Erspamer and Asero (1952) went on to identify enteramine as being 5-hydroxytryptamine, identical to serotonin.

The distribution and possible roles of 5-HT

5-HT has a unusual distribution in nature and is not confined to vertebrates. Jacques and Schacter (1954) discovered 5-HT in the venom of the wasp, *Vespa vulgaris,* and it has been found in a number of fruits including bananas (Udenfriend *et al.*, 1959) and in the stinging apparatus of the nettle *Urtica dioica* (Brittain and Collier, 1957). It was found in the enterochromaffin cells of the intestine in all classes of vertebrates and in blood platelets and mast cells in mammals. Extensive listings of such occurrences have been given by Erspamer (1954).

The presence of 5-HT in the vertebrate central nervous system (CNS) was demonstrated first by Twarog and Page (1953). At that time, interest in 5-HT was also greatly increased by its discovery in the nervous system of the dog (Amin *et al.*, 1954). In the early 1960s, a group of Swedish scientists made

an influential and important discovery in the history of 5-HT research. Using the Falck-Hillarp technique (Falck *et al.*, 1962) to visualise 5-HT within cells, with formaldehyde-induced fluorescence, they found that virtually all the 5-HT-containing neurones of the CNS of the rat were located in discrete clusters within the midbrain, pons and medulla (Dahlström and Fuxe, 1964). Over the years improvements in techniques have helped to define more clearly the locations of serotonergic cell bodies, fibres and terminals.

5-HT has been found to have multiple actions in the vertebrate nervous system. It affects sensory, motor, autonomic and enteric neuronal activity as well as influencing vascular and other smooth muscle responses. Within the CNS, 5-HT seemed to function in both an inhibitory and excitatory manner. In contrast, in the periphery, 5-HT has mainly excitatory postsynaptic actions on autonomic and enteric nervous system neurones, as well as producing contraction of the uterus, ileum and vasculature smooth muscle (see Vandermaelen, 1985).

The first systematic survey of the occurrence of 5-HT in invertebrates was undertaken by Welsh and Moorhead (1960). Using the fluorescence method to estimate the 5-HT content of nervous and non-nervous tissue from a variety of invertebrates, large amounts of 5-HT were detected both in annelids and molluscs. Although Welsh and Moorhead (1960) showed that 5-HT is widely distributed in both nervous and non-nervous tissues, they were not able to locate it in specific cell types. With the introduction of the Falck-Hillarp fluorescence microscopy technique (Falck *et al.*, 1962) for visualizing indoleamines and catecholamines in single cells and nerve tracts, it was possible to identify specific amine-containing cells. A further refinement was the introduction of biochemical techniques whereby the amine content of single neurones could be established quantitatively. Since then there have been many studies of the localization and possible function of 5-HT in a wide range of invertebrates, particularly in the molluscs.

5-HT has potent actions on many invertebrate tissues but it was the molluscan heart, particularly that of the quahog clam, *Mercenaria mercenaria*, which was the first tissue to be examined in detail with respect to the identity of the 5-HT receptor (Greenberg, 1960a). In most cases the major action of 5-HT was one of excitation but there were clear examples where 5-HT was

basically inhibitory, e.g. on the heart of *Modiolus* (Wilkens and Greenberg, 1973). At molluscan peripheral sites, 5-HT was not always found to be excitatory. Twarog and Cole (1972) found that 5-HT acted as a relaxing agent at the anterior byssus retractor muscle (ABRM) of *Mytilus* and was released at this site following nerve stimulation (Satchell and Twarog, 1978). 5-HT has also been shown to have a relaxing action of the penis retractor muscle (Wabnitz and von Watchendonk, 1976) and of the pharyngeal retractor muscle (PRM) (Lloyd, 1980a) of *Helix*.

Evidence for 5-HT as a transmitter in molluscan ganglia

The idea that 5-HT is a neurotransmitter in invertebrates had been proposed by Welsh (1954). His study with Moorhead (1960) had yielded important data on 5-HT levels in the nervous tissue of invertebrates with particularly high 5-HT levels in both annelids and molluscs. 5-HT has been demonstrated histochemically in the nervous systems of *Helix*, *Anodonta*, *Buccinium* and *Limax* (Dahl *et al.*, 1966, Sedden *et al.*, 1968, Osborne and Cottrell, 1971). The levels of 5-HT in identified neurones of *Tritonia* and *Aplysia* ganglia were found to be between 4 and 6 pmol/cell body (Weinreich *et al.*, 1973). The synthesis of 5-HT from its precursor tryptophan has been demonstrated using identified neurones in *Aplysia* (Eisenstadt *et al.*, 1973). In their study [^{3}H]tryptophan was pressure-injected into nerve cell bodies. 5-HT synthesis was found to be specific for the 5-HT-containing neurones since the pressure-injection of labelled tryptophan into cholinergic neurones produced only a negligible amount of 5-HT.

Endogenous 5-HT was shown to be released from the cerebro-buccal ganglionic ring of *Aplysia* (Gerschenfeld *et al.*, 1978). They demonstrated that the rate of spontaneous release of 5-HT varied between 0.4 and 1.2 pmol/hour. Direct stimulation of the gaint 5-HT-containing cerebral neurones resulted in an 80-100% increase in the release of 5-HT but only in the presence of chlorimipramine, a 5-HT uptake blocker.

The best evidence for 5-HT as a central transmitter comes from work on gastropods such as *Helix* (Cottrell and Macon, 1974) and *Aplysia* (Gerschenfeld and Paupardin-Tritsch, 1944a,b). It is the giant 5-HT-containing cerebral neurones in these gastropods which have been the subjects of extensive

studies. These cells were first noted in *Helix pomatia* by Kunze (1921) and were then characterized electrophysiologically and according to their input properties by Kandel and Tauc (1966a,b). More general interest in these neurones was developed by Cottrell and Osborne (1970) who demonstrated that, in both *Helix* and *Limax*, these neurones contained significant amounts of 5-HT. Cottrell (1970) went on to show that these giant 5-HT-containing neurones in *Helix* provided excitatory inputs to three identified neurones in the ipsilateral buccal ganglion, which were subsequently labelled anterior, middle and posterior. It was this pathway in *Helix pomatia* which was studied with electrophysiological methods by Cottrell and Macon (1974) to provide further evidence of a transmitter role for 5-HT. In their study, stimulation of the giant 5-HT-containing cerebral neurones evoked excitatory post synaptic potentials in the middle buccal cell. Repetitive stimulation of the cerebral neurones caused summation of these excitatory post synaptic potentials, giving rise to an action potential. Iontophoretic application of 5-HT onto the middle buccal neurone produced a depolarizing response which was shown to be sodium-dependent and could be antagonized by morphine. Reserpine which depletes 5-HT in the cerebral 5-HT-containing neurone, reduced but not completely abolished the efficacy of transmission between this neurone and the middle buccal neurone.

Evidence for 5-HT as a transmitter in molluscan hearts

Evidence for a role for 5-HT as the excitatory transmitter in molluscan hearts came from a study by Loveland (1963), on *Mercenaria* heart. Methysergide antagonized the response of the heart not only to applied 5-HT but also to stimulation of the accelerating nerve to the heart. Loveland also used the Falck and Hillarp fluorescence histochemical technique for visualisation of monoamine-containing neurones. He made stretched preparations of the atria with attached portions of ventricle. After drying at room temperature, with subsequent exposure to formaldehyde vapour, microscopic observation with ultra-violet (UV) light revealed a yellow fluorescing cardiac nerve passing through each atrium and terminating in the adjacent lateral regions of the ventricle. This yellow fluorescence was thought to be due mainly to the presence of 5-HT although the presence of other amines, particularly the catecholamines which also gave off a yellow fluorescence, could not be eliminated.

That 5-HT served as a mediator for cardio-excitation in molluscs was probable. However confirmation of the release of 5-HT from the cardioregulatory nerves had to be achieved. A two *Helix* heart/Loewi-type experiment was set up by S-Rózsa and Perényi (1966) in order that the release of 5-HT from the cardioregulatory nerves might be demonstrated. On stimulation of the nerve going to one heart, the perfusate from this first heart was found to contain material which activated the second heart. 5-HT was subsequently detected in the perfusate both by spectrophotometric and chromatographic techniques.

Evidence for 5-HT as a transmitter in molluscan visceral and smooth muscle.

The other molluscan tissues where the action of 5-HT has been well investigated are the somatic and visceral muscles. These have included ABRM of *Mytilus*, the penis retractor muscle of *Helix* and *Strophocheilos* and the PRM of *Helix*. The visceral muscles which have been investigated include the rectum of *Mercenaria* and *Tapes* and the crop of *Helix*.

There is substantial evidence for 5-HT as a neurotransmitter mediating relaxation at the ABRM in *Mytilus*. 5-HT was found to mimic the effects of stimulating the relaxing nerves. When the muscle was exposed to 5-HT at concentrations >1nM, relaxation of catch contraction in the muscle was proportional to the $\log_{10}$ of 5-HT concentration (Twarog ,1954). Von Watchendonk and Käppler (1977), using liquid scintillation counting and microchromatography, found 5-HT levels up to $4.3\mu g.g^{-1}$wet weight for the ABRM. 5-HT was also released from the ABRM during stimulation of the pedal ganglion (Satchell and Twarog, 1978). Relaxation from either ACh- or electrically-induced contraction was faster following prolonged stimulation of the muscle (York and Twarog ,1973); another indication of the release of 5-HT from the nerves supplying the muscle. The pharmacological evidence for 5-HT as a transmitter in the ABRM was less extensive. LSD and ergometrine relaxed the ABRM while other ergot derivatives showed no relaxant action (Twarog, 1959). Northrop (1964), reported that methysergide blocked neural relaxation in the ABRM, while 2-Bromo-LSD was also observed to delay neural relaxation in the ABRM presumably by acting as a competitive inhibitor of 5-HT (Bullard, 1967). The organic mercurial, mersalyl, effectively

blocked both the response to 5-HT in the ABRM and the relaxant effect induced by inhibiting nerve stimulation. The blocking action of mersalyl was thought to be attributable to its combination with a sulphydryl group at or near the receptor site to which the indole nitrogen of 5-HT attached (Twarog *et al.*, 1977).

Evidence was accumulating also for the involvement of 5-HT in neuromuscular transmission in the *Helix* penis retractor muscle. The presence of 5-HT was confirmed by von Watchendonk and Käppler (1977), who found 5-HT levels of $3.2\mu g.g^{-1}$ wet weight. Wabnitz and von Watchendonk (1976) showed that 5-HT relaxed the muscle and reversed tonic contractions induced by ACh. In contrast, a study of the penis retractor of another snail, *Strophocheilos oblongus*, showed that the muscle was excited by 5-HT. At lower concentrations 5-HT relaxed the muscle (Jaeger, 1963). LSD activated the muscle while 2-Bromo-LSD caused relaxation and antagonised the action of 5-HT. The pharyngeal retractor muscle (PRM) of *Helix* was also sensitive to 5-HT. Kerkut and Leake (1966) found that 5-HT increased the rate of relaxation of the muscle following electrical stimulation of the brain. In an earlier study, Kerkut and Cottrell (1962) had found that 5-HT increased the rate of relaxation following contractions with ACh.

Studies on the actions of 5-HT have not been limited solely to the molluscan somatic muscles but have also focused on the molluscan visceral muscles. Greenberg and Jegla (1963) investigated the actions of 5-HT on the rectum of *Mercenaria*. 5-HT excited the rectum, inducing rhythmical activity at lower concentrations, while at high concentrations 5-HT increased the tone of the rectum.

Inactivation of 5-HT within molluscs

5-HT was known to be inactivated by the enzyme, monoamine oxidase (MAO) in tissues such as the mammalian CNS (see Werman, 1966). This inactivation mechanism might also extend to invertebrates because MAO has been shown in molluscan muscle (Blaschko and Hope, 1957). It was likely that also 5-HT was partly inactivated by presynaptic uptake because an imipramine-sensitive uptake mechanism was found to exist in an excitatory 5-HT-mediated synapse within *Helix* ganglia (Cottrell, 1971). Further studies on

5-HT uptake were carried out on the *Helix* brain by Stahl *et al.* (1977). They found that 5-HT was taken up by an active transport process which required extracellular sodium and was inhibited by 1-10mM ouabain. These studies indicated that several different mechanisms existed within the molluscs for the inactivation of 5-HT, the particular pathway depending upon the tissue in which the inactivation of 5-HT was taking place.

Multiple 5-HT receptors

The concept of multiple receptors for 5-HT was advanced first by Gaddum and Picarelli (1957) for the guinea pig ileum. They classified the receptors for 5-HT as M and D, based upon pharmacological studies in isolated segments of ileum. The D receptor, sited in the smooth muscle, was blocked by dibenzyline whereas the M receptor found in neuronal tissue was blocked by morphine. The existence also of multiple 5-HT receptors in the vertebrate brain had been suggested by electrophysiological studies in cat cerebral cortex, where the microiontophoresis of 5-HT produced either excitation or inhibition in the population of neurones tested (Roberts and Straughan, 1967). In their study, methysergide, LSD and cianserin blocked the excitatory but not the inhibitory effects. On the basis of these data, Roberts and Straughan (1967) proposed that there may be both excitatory and inhibitory types of 5-HT receptor in the brain. A similar multiple excitatory and inhibitory receptors concept was proposed for the invertebrate nervous system based on the diverse electrophysiological actions of 5-HT at several synaptic junctions in *Aplysia* and *Helix* by Gerschenfeld and Paupardin-Tritsch (1974a).

Peroutka and Snyder (1979) presented evidence for two distinct 5-HT binding sites for which LSD had a high affinity. At one site, termed 5-HT_1, 5-HT had a high affinity while at the other, termed 5-HT_2, spiperone had a high affinity. Subsequent investigations have, however, revealed a far greater complexity, with at least five subtypes of 5-HT_1 recognition sites (termed 5-HT_{1A}, 5-HT_{1B}, 5-HT_{1C}, 5-HT_{1D} and 5-HT_{1E}) being demonstrated to date (see Peroutka, 1988; Leonhardt *et al.*, 1989). Furthermore there was also a suggestion of possible heterogeneity, or multiple affinity states, within the 5-HT_2 category (McKenna and Peroutka, 1989). In an attempt to reconcile these differing terminologies introduced by Gaddum and Picarelli (1957) and Peroutka and Snyder (1979), Bradley *et al.* (1986) proposed a new classification

and nomenclature for functional 5-HT receptors, based upon data obtained from isolated peripheral tissues. A comparison of the pharmacological profiles of the 5-HT_2 recognition site and the D receptor led to the conclusion that the two were essentially identical. The so-called D receptor therefore became redundant in favour of the more prevalent term 5-HT_2. The category "5-HT_1-like" was introduced to include those functional receptors which demonstrated pharmacological similarities to the heterogeneous 5-HT_1 binding sites described for the CNS, and the M receptor was incorporated into a newly defined 5-HT_3 class. The central binding site equivalent of the 5-HT_3 receptor (Kilpatrick *et al.*, 1987) emerged subsequent to the recommendations of Bradley *et al.* (1986), along with the discovery of further subtypes of 5-HT receptor such as 5-HT_4 (Clarke *et al.*, 1989). The scheme developed by Bradley *et al.* (1986) stressed the role of functional responses and the use of potent selective and competitively acting receptor antagonists, and, to a lesser extent, agonists in the classification of 5-HT receptors.

Specific agonists and antagonists for the different 5-HT receptor types

The 5-HT_1 like group of functional receptors is the least well-defined because there are, as yet, no selective antagonists available. Some 5-HT_2 receptor antagonists, such as methysergide and methiothepin, have been found to have appreciable affinity for the 5-HT_1 binding sites and were found not to discriminate between the various 5-HT_1 subtypes or 5-HT_1-like receptor-mediated responses (Peroutka and Snyder, 1979). 5-carboxamidotryptamine (5-CT) was found to be a selective agonist and, at most 5-HT_1-like receptors, 5-CT was either equipotent or more potent than 5-HT (Bradley *et al.*, 1986). Selective agents have been defined for the four 5-HT_1 subtypes; 8-hydroxy-2-(di-n-propylamine) tetralin (8-OH-DPAT) (5-HT_{1A}), propranolol (5-HT_{1B}), cyanopinodolol (5-HT_{1A} and 5-HT_{1B}), mesulergine (5-HT_{1C}), metergoline (5-HT_{1C} and 5-HT_{1D}), rauwolscine (5-HT_{1D}) and yohimbine (5-HT_{1A} and 5-HT_{1D}) (see Peroutka, 1988). Another selective agonist at some 5-HT_1-like receptors, particularly those in the vasculature, is sumatriptan (Humphrey *et al.*, 1988).

Functional 5-HT_2 receptors were identified by the potent blocking action of ketanserin (Bradley *et al.*, 1986). Humphrey and Feniuk (1987), have

suggested that α-Methyl-5-HT was a useful selective agonist for identifying 5-HT_2 receptors.

Until seven years ago no satisfactory antagonist of the 5-HT_3 receptors was available. Early investigations demonstrated blockade of 5-HT_3 receptor-mediated responses by metoclopramide, quipazine and cocaine. MDL 72222 and ICS 205-930, the first potent and selective 5-HT_3 receptor antagonists, were recognised in the mid-1980s (Fozard, 1984c; Richardson *et al.*, 1985). These were followed by other selective 5-HT_3 antagonists, which included ondansetron (Butler *et al.*, 1988), and 2-Methyl-5-HT proved to be a selective 5-HT_3 receptor agonist (Richardson *et al.*, 1985).

5-HT actions at the molecular level in vertebrates

Recently three 5-HT receptor subtypes, 5-HT_{1C}, 5-HT_{1A} and 5-HT_2 have been cloned by three different laboratories. All three receptors are single subunit proteins and members of the G-protein receptor superfamily. This receptor family was characterized by the presence of seven transmembrane domains for each receptor and the ability to activate G-protein-dependent processes, including activation or inhibition of adenylate cyclase activity and activation of phosphoinositide turnover (Hartig, 1989). Activation of 5-HT_2 and 5-HT_{1C} receptors increased the activity of phopholipase C in hydrolysing phosphatidylinositol. The resulting diacylglycerol and inositol triphosphate products caused subsequent cellular responses. Agonists at 5-HT_{1A} receptors led to the activation of a different G-protein which inhibited adenylate cyclase activity (see Pierce and Peroutka, 1989). 5-HT_{1B} and 5-HT_{1D} were proposed to be equivalent receptors in different species, with the 5-HT_{1D} receptor being possibly a recent evolutionary derivation of the 5-HT_{1B} receptor (Hoyer and Middlemiss, 1989). Both these receptors have been shown to be linked to the inhibition of adenylate cyclase at the cellular level (see Pierce and Peroutka, 1989). In contrast to the 5-HT_1 receptor subtypes, the novel 5-HT_4 receptor has been shown to be positively linked to cyclic adenosine 3',5'-monophosphate (cAMP) production, not only in guinea pig hippocampus but also for human atrial tissue and mouse colliculus neurones (Clarke *et al.*, 1989; Kaumann *et al.*, 1989). It has also been shown recently that 5-HT_3 receptors are ligand-gated cation channels. 5-HT caused the opening of discrete channels in outside-out

patches of membrane excised from guinea-pig submucous plexus neurones (Derkach *et al.*, 1989; Lambert *et al.*, 1989).

5-HT actions at the molecular level in molluscs

The second messenger concept of hormone or transmitter action, involving adenylate cyclase, which had been developed by Sutherland and Robison (1966), had been well established in the control of certain biochemical processes in vertebrates. Furthermore Mansour *et al.* (1960) had shown that adenylate cyclase of the liver fluke, *Fasciola*, could be activated by 5-HT. The existence of various transmitter-sensitive adenylate cyclases implied that transmitters might act through the alteration of cAMP, which was itself formed from adenosine triphosphate (ATP) by the membrane-bound adenylate cyclases. Cyclic AMP-dependent protein kinase activity had been shown in a wide range of invertebrates (Kuo and Greengard, 1969). The latter authors suggested that a variety of actions attributable to cAMP was mediated by the single reaction mechanism of phosphorylation of protein kinases, which itself increased their activity.

5-HT had been implicated in the activation of cyclic nucleotides in many invertebrate tissues: for example in neurones of *Helix*, 5-HT could induce an inward current which was associated with a decrease in potassium conductance (Deterre *et al.*, 1982). This response could also be induced by cAMP and dopamine. When the response to one compound was maximal, in terms of inward current, the response to the other two was blocked. This indicated that 5-HT and dopamine activated adenylate cyclase via two distinct receptors, although it was not possible to find specific antagonists for 5-HT and dopamine.

The distribution of receptors for 5-HT and dopamine in *Aplysia* and *Helix* tissues was studied using [^{3}H]LSD binding and adenylate cyclase stimulation techniques (Drummond *et al.*, 1980a,b). Using [^{3}H]LSD they identified dopamine and serotonin receptors in a particulate fraction derived from *Helix* CNS. These authors concluded that, in gastropod tissues, 5-HT-sensitive [^{3}H]LSD binding was related to a 5-HT receptor coupled to adenylate cyclase. In a subsequent paper, Drummond *et al.* (1980b) found that there was a high level of specific [^{3}H]LSD binding in all the examined ganglia and

nerves. The ability of 5-HT and dopamine to inhibit [H^3]LSD binding varied depending on the tissue; in muscle most of the binding was sensitive to 5-HT whereas in the nervous system the ganglia contained up to 50% dopamine sensitive binding. The IC_{50} values (concentration required to inhibit 50% of the binding) for *Aplysia* CNS and gill muscle and *Helix* CNS have been compared with data from the rat CNS (Bennett and Snyder, 1976), and found to be remarkably similar.

Adenylate cyclase was found in *Spisula solida* heart and 5-HT stimulated this enzyme (Cottrell and Osborne, 1969). In heart muscle microsomes of a molluscan bivalve, *Macrocallistra nimbosa*, 5-HT and cAMP both stimulated phosphorylation of microsomal calcium uptake (Higgins and Greenberg, 1974). 5-HT and cAMP induced a similar increase directly. Higgins and Greenberg suggested that 5-HT increased intracellular cAMP and altered the amount of calcium available for contraction, whereas cAMP mediated the action of 5-HT, either by phosphorylation and subsequent activation of the protein kinase or by direct activation of calcium uptake.

cAMP was also implicated in having a second messenger function in molluscan smooth muscle. Cole and Twarog (1972) suggested that cAMP was involved in the 5-HT-induced catch relaxation of the ABRM. An investigation by Achazi *et al.* (1974), of the cAMP system in the molluscan ABRM, showed an increase of cAMP levels following application of 5-HT. The possible involvement of cAMP in the effect of 5-HT within the different molluscan tissues will be discussed at greater length in the relevant chapters.

Aims of the present study

From the evidence presented here, 5-HT has potent actions on molluscan tissues and it is within this phylum that a transmitter role for 5-HT has been established most clearly. Very few studies of the action of 5-HT on nervous and non-nervous tissue have focused on the nature of the receptor or receptors responsible for the 5-HT effect seen in these tissues. The molluscan 5-HT receptor(s) has not been well characterized in contrast to those of vertebrates. This lack of characterization of molluscan receptors can, in part, be attributed to the fact that the demands of modern medicine have led to the development of specific 5-HT agonists and antagonists in vertebrates, with a

commercial view to drug development. As a consequence, investigations into the invertebrate, and in particular the molluscan, 5-HT receptor characterization have been overlooked in favour of the more financially rewarding vertebrate 5-HT receptor studies. Invertebrate research by physiologists has, therefore, concentrated on the mechanism of action of 5-HT rather than the 5-HT receptor itself. In the present study an attempt was made to characterize the 5-HT receptor for three preparations from the common garden snail, *Helix aspersa*. This was achieved using previously developed specific 5-HT agonists and antagonists: these have helped, not only in the characterization of the different 5-HT receptors in vertebrates, but also in their classification. The three preparations that were utilised in this study were (i)identified cells in the visceral ganglion, which showed a fast depolarising response to 5-HT, (ii)the isolated heart, which showed a positive inotropic and chronotropic response to 5-HT and (iii)the isolated pharyngeal retractor muscle (PRM), which showed a relaxant response to 5-HT. The 5-HT receptor of *Helix* heart was studied using a standard organ bath technique with the specific 5-HT agonists and antagonists being bath-applied at suitable concentrations. The same method was used to study the 5-HT receptor mediating relaxation, or, in this case, inhibition of acetylcholine (ACh) induced contraction in *Helix* PRM. In the latter two cases the effect of 5-HT on levels of cAMP was also investigated in order to give a clearer insight into the mechanism of action of 5-HT at the receptor. The 5-HT receptor in identified cells of *Helix* visceral ganglion was studied using the electrophysiological techniques of current- and voltage-clamp. Each preparation is described separately with each chapter including a separate introduction, methods and materials, results and discussion sections. The final chapter includes a general discussion in order to compare and contrast the conclusions drawn from each previous chapter.

CHAPTER TWO

THE 5-HT RECEPTOR IN IDENTIFIED *HELIX* NEURONES

INTRODUCTION

The 5-HT receptor in molluscan ganglia

Electrophysiological evidence: The molluscs, and in particular gastropods such as *Aplysia* and *Helix*, offer ideal preparations for neurobiological studies since they possess nervous systems with large neurones which can be easily identified from preparation to preparation. Many of these identified neurones have also been shown to contain 5-HT as discussed earlier. With the development of electrophysiological methods, such as the single electrode circuit which allows for both current and voltage recording with a single electrode (Wilson and Goldner, 1975), 5-HT has been shown to have potent actions on many central gastropod neurones. Electrophysiological studies of neurones in the abdomino-visceral ganglionic mass of a gastropod, *Cryptophallus aspersa*, showed that 5-HT depolarized the neurones through a selective increase of their membrane permeability to sodium ions (Gerschenfeld and Stefani, 1966, 1968). They postulated that the 5-HT receptors were located at or near the axon hillock. These 5-HT receptors could be blocked by LSD and its derivative Bromo-LSD, dibenamine, morphine and tryptamine. In a later study of the central ganglia of *Helix aspersa*, Gerschenfeld (1971) distinguished 5-HT inhibition mediated via two different mechanisms. 5-HT B receptors inhibited neurones by causing a selective increase in potassium permeability whereas 5-HT C receptors caused inhibition by selectively increasing the membrane permeability to chloride ions.

There is now evidence to suggest six different types of response, within molluscan neurones, to the iontophoretic application of 5-HT (Gerschenfeld and Paupardin-Tritsch, 1974a). These workers found that the iontophoretic application of 5-HT onto *Helix* and *Aplysia* neurones caused three different types of excitation. 5-HT could also evoke three kinds of inhibition. Table 2.1 overpage, taken from their paper, summarizes these 5-HT responses.

TABLE 2.1 Summary of different types of 5-HT response of ganglionic neurones of *Aplysia* and *Helix*

Response	Effect	Reversal Potential(mV)	Ionic mechanism
A	Fast depolarisation	O	Increase in sodium conductance
A'	Slow depolarisation	O	Increase in sodium conductance
B	Slow hyper-polarisation	-75	Increase in potassium conductance
C	Fast hyper-polarisation	-56	Increase in chloride conductance
α	Depolarisation	-75	Decrease in potassium conductance
β	Hyper-polarisation	-30	Decrease in sodium and potassium conductance

The reversal potential values for A and A' were estimated by extrapolation (Taken from Gerschenfeld and Paupardin-Tritsch, 1974a).

Four of the responses which they labelled A, A', B and C were consequent upon an increase in membrane conductance while the other two, labelled α and β, were caused by a decrease in membrane conductance. Four distinct receptors involved in the 5-HT responses associated with conductance increases were characterized by the action of specific antagonists. These data are summarized in Table 2.2 overpage.

TABLE 2.2 Pharmacological profile of four 5-HT responses of *Helix* and *Aplysia* neurones.

Antagonist	A	A'	B	C
Tubocurarine	block	none	none	block
LSD	block	none	block	block
Tryptamine	block	none	block	block
7-Methyl-tryptamine	block	none	none	none
Bufotenine	block	block	block	none
5-Methoxy-gramine	none	none	block	none
Neostigmine	none	none	none	block

The block was obtained with 10μM antagonist (Taken from Gerschenfeld and Paupardin-Tritsch, 1974a).

7-Methyltryptamine blocked only the A-receptors, 5-methoxygramine only the B-receptors and neostigmine only the C-receptors. Curare was found to block the A- and C-receptors while bufotenine the A-, A'- abd B-receptors. No specific antagonists were found for the 5-HT responses caused by conductance decreases.

In a subsequent paper Gerschenfeld and Paupardin-Tritsch (1974b) studied the connexions made by a pair of gaint neurones, in *Aplysia* cerebral ganglia, to neurones situated in the ipsilateral buccal ganglion in order to investigate the possible physiological roles for the 5-HT responses they had previously described in the preceding paper. They found that of the six 5-HT responses, at least four intervened in the generation of synaptic potentials i.e. 5-HT released from a single neurone was then able to mediate opposite synaptic actions on different buccal cells : excitation associated with sodium conductance increases and inhibition resulting from an increase in potassium conductance.

An alternative approach to attempt to analyze the gastropod central 5-HT receptors in terms of the mammalian central classification into 5-HT_1 and 5-HT_2 as proposed by Peroutka and Snyder (1979) was undertaken by Bokisch *et al.* (1983). The 5-HT excitatory type A response was used as the standard 5-HT response. RU 24969, a potent 5-HT_1 receptor agonist was tested together with MK 212, a preferential 5-HT_2 agonist. MK 212 had clear agonist activity but it was 60-70 times less potent than 5-HT. In contrast RU 24969 had no clear agonist action. In this study MK 212 was also observed to be a specific antagonist of 5-HT. RU 24969 was also found to possess 5-HT antagonist activity though it was much less potent than MK 212. These data implied that the *Helix* excitatory type A receptor appeared to be similar to the mammalian 5-HT_2 receptor.

The structural requirements for the molluscan 5-HT receptors has not been fully determined but there are indications that in binding the 5-HT molecule the 5-hydroxy group, the indole nucleus, the length of the side chain and its charged nitrogen group have vital importance (Walker and Woodruff, 1972, Walker, 1985).

Electrophysiology of 5-HT_3 receptors in neuronal cell lines

Cells of the murine neuroblastoma cell line, NIE-115, responded to locally applied 5-HT with a rapid membrane depolarisation associated with a conductance increase to monovalent cations (Peters and Usherwood, 1983). Both the 5-HT-induced depolarisation and the transient inward current which underlay it, seen under voltage-clamp conditions, were blocked by selective 5-HT_3 antagonists but were unaffected by antagonists acting at 5-HT_1-like or 5-HT_2 receptors. The responses to iontophoretically applied 5-HT were rapidly desensitized (Nejit *et al.*, 1988). The reversal potential obtained from the current-voltage curve of the peak amplitude of response in the NIE-115 cell line was 20mV (Nejit *et al.*, 1989), while the reversal potential of the neuroblastoma x Chinese hamster brain cell line, NCB-20, was -2mV (Lambert *et al.*, 1989). This rapid depolarizing response to 5-HT, which was due to a transient inward current in these neuronal cell lines, was mediated by 5-HT_3 receptors. However, the response to 5-HT observed in these cell lines was similar to the depolarizing response of 5-HT as noted, and labelled the A

response, by Gerschenfeld and Paupardin-Tritsch (1974a). The responses to 5-HT in both the neuronal cell lines and *Helix* neurones showed rapid desensitization. In the case of the A-depolarizing responses to 5-HT, which had previously been shown to be sensitive to changes in extracellular sodium concentration, voltage-clamp experiments have shown that they reverse near the zero level: this suggested that they involved an increase in both sodium and potassium conductances (Gerschenfeld *et al.*, 1981). More recently Bokisch and Walker (1986) have investigated the ionic mechanisms associated with the actions of putative transmitters on identified neurones of the snail, *Helix aspersa*. The ionic mechanism associated with 5-HT excitation was examined using two identified cells (E1 and E2) in the visceral ganglion. Iontophoretic application of 5-HT induced an inward current which had an extrapolated reversal potential of -3mV. In sodium-free saline the 5-HT-induced inward current was reduced by 30-50% of the control value. When sodium- and calcium-free solution was used the 5-HT-induced current was totally abolished. Bokisch and Walker concluded that this 5-HT excitatory response involved an increase both in sodium and calcium conductance. This was similar to the conductance increases in monovalent cations induced by 5-HT seen in the NIE-115 cell line by Peters and Usherwood (1983). Could it be a 5-HT_3 receptor which mediates the A-depolarizing response in molluscan neurones as described by Gerschenfeld and Paupardin-Tritsch (1974a)?

Aims

The aim of the present study was to investigate the 5-HT receptor present in *Helix aspersa* neurones, in order to attempt characterization of the 5-HT receptor into one of the vertebrate 5-HT receptor subtypes. This was to be achieved using the electrophysiological techniques of voltage-clamp and current-clamp. The neurones in the suboesophageal ganglia of *Helix aspersa* had been well mapped by Kerkut *et al.* (1975) who had also noted which cells were depolarized by the iontophoretic application of 5-HT onto the identified cell soma. A more detailed electrophysiological, pharmacological and fluorescent study on twelve neurones from the suboesophageal ganglia by Loker *et al.* (1975) identified five large 5-HT-containing neurones in the visceral ganglion. The six neurones under investigation in the visceral ganglion were E1, E2, E3, E4, E5 and E6. Five of these neurones contained 5-HT, were depolarized by 5-HT and ACh and were hyperpolarised by dopamine.

In contrast, neurone E4 was found not to contain 5-HT and was hyperpolarized by both ACh and dopamine. It was five of these six large easily identifiable neurones that were utilised in this study. They were E1, E2, E3, E4 and E5. Because of the similarity between the depolarizing response of 5-HT in *Helix* neurones to the 5-HT response observed in neuroblastoma cells which was mediated by a well-described 5-HT_3 receptor, 5-HT_3 receptor agonists and antagonists were tested first on the preparation. This was followed later by other specific 5-HT vertebrate receptor subtype agonists and antagonists.

METHODS AND MATERIALS

Preparation of animals

Common garden snails, *Helix aspersa*, were collected locally and kept in a enclosed bucket at room temperature until they were required. Prior to dissection the snails were enticed out of their shells by being placed in warm water (20-30°C). Once out of its shell, the head of the snail was rapidly cut off using a sharp pair of scissors and the detached head pinned through the mouth to a wax bottomed dissecting dish. The dorsal midline at the back of the head was cut from top to bottom and the skin pulled back exposing the buccal mass. The cerebral ganglia, which comprise part of the circumoesophageal ring, were visible as a collar over the oesophagus which is itself attached to the buccal mass. The oesophagus was pulled from beneath the cerebral ganglia and the buccal mass cut away. A pair of forceps was then placed underneath the cerebral ganglia and these lifted to expose the suboesophageal ganglia beneath. The nerve connections were cut away and the isolated circumoesophageal ring placed in a beaker containing physiological saline (80mM NaCl, 5mM KCl, 5mM $MgCl_2$, 7mM $CaCl_2$, 20mM Hepes pH 7.5) (Meech and Thomas, 1977). The ganglia were then transferred to the recording chamber for further dissection before the experiments were started. Once the suboesophageal ganglia had been pinned to a Sylgard base of the bath, the layers of connective tissue covering the ganglion cells could then be removed. The connective tissue had to be removed before the neurones could be impaled with microelectrodes. This careful microdissection was achieved with fine forceps and a pair of fine scissors. The outer connective sheath is spongy in texture and was relatively easy to remove. However, the inner sheath is thin and transparent, and had to be carefully torn to expose the cells without causing them further damage.

Experimental set up in the recording chamber

The recording chamber was made of a rectangular block of Perspex supported by four small Perspex legs. A 5mm identation in the perspex block formed the small bath. Sylgard was used to coat the bath and this made a suitable base into which dissecting pins could be inserted. The bath was illuminated by a conventional Watson light source. This was achieved by

using an tapered glass rod, the thick end of which was inserted by means of a rubber bung into the light source, while the thin tapering end was positioned in the bath below the level of the liquid. Adequate illumination was provided by the tip of the glass rod when it was positioned close to the preparation. The bath was observed through a Nikon stereoscan microscope.

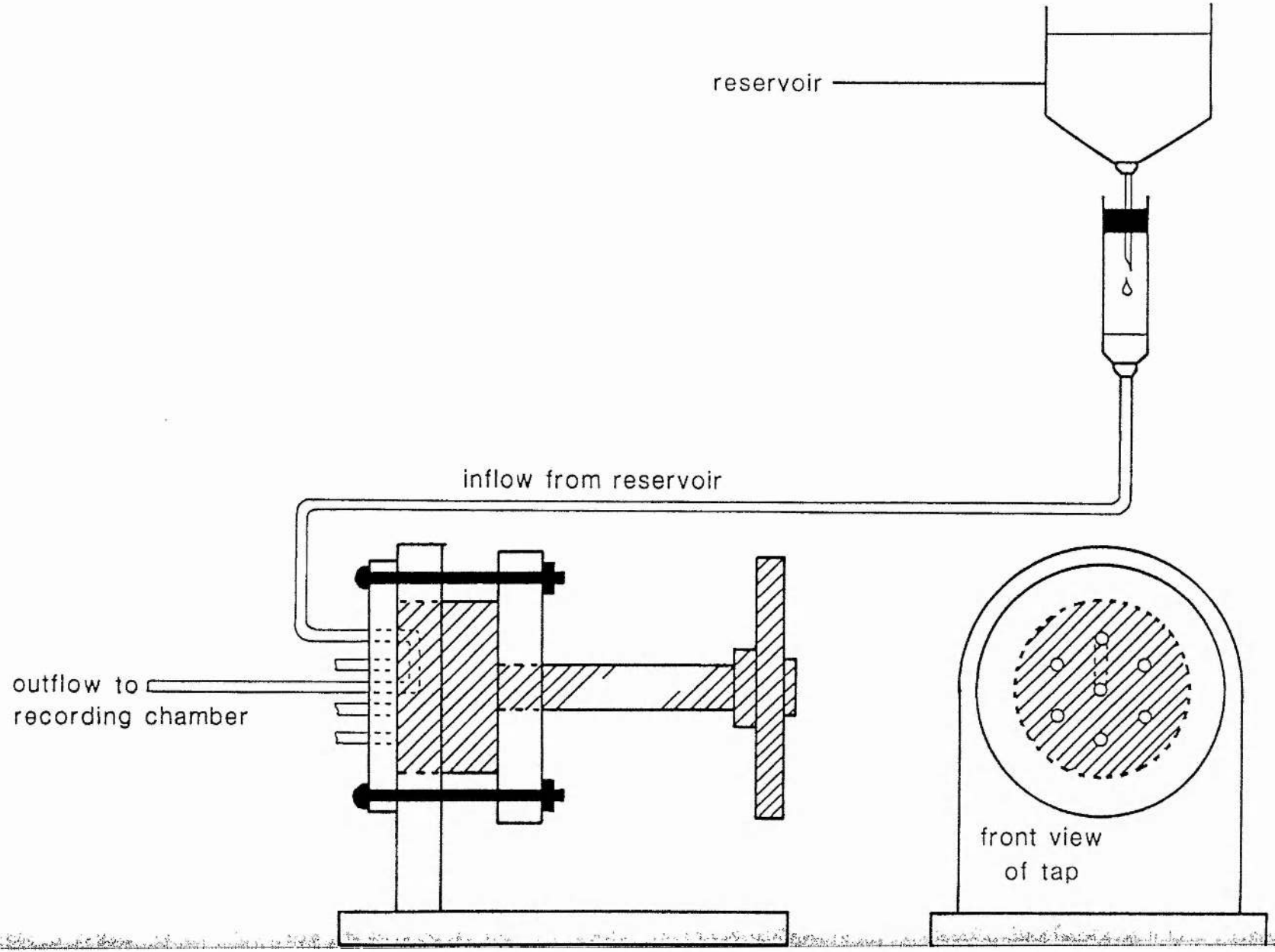
The perfusion system

The preparation could be exposed to various solutions by means of a multi-way tap to which a maximum of six reservoirs could be connected. A diagram of the tap system is shown in Fig. 2.1. Large 100ml syringes were used as reservoirs and these were attached to smaller 5ml syringes. The syringes were held in place on the side of the Faraday cage surrounding the experimental apparatus by means of adjustable clips. Thin plastic tubing attached to the small syringes formed the inflow into the six-way tap. The single outflow of the tap led directly into the bath. The reservoirs were positioned higher than the recording chamber, thus providing the small hydraulic head necessary to create a flow of solution. The different solutions from the different reservoirs could be used to perfuse the bath by turning the tap outflow to the selected reservoir inflow. A clamp on the tubing from the outflow of the tap to the bath made it possible to control the flow of solution. The rate of flow per minute could then be measured by monitoring the amount of solution passing from the large to the small syringe. The flow rate was an important experimental parameter so it was measured periodically by the addition of a small amount of dye to the bath. The dye gave an indication of the movement of solution in the bath. For example, if the flow was too fast the dye would be swept around, but not over, the preparation whereas, if the flow was too slow small pockets of dye would remain in the bath. The flow had to be adjusted carefully in order to allow any drugs in the perfusate to have an effect, but also to allow them to be washed out of the bath once the effect had been observed.

A suction system disposed of the waste perfusate from the bath. The suction tube was positioned carefully so that the flow of solution was constant and the liquid in the bath was maintained at the desired level. The vacuum was produced by connecting the suction tube to an aspirator via a conical flask into which the waste collected.

FIGURE 2.1

Diagram to show a close up view of the 6-way tap system used in the experiments. Only one inflow is shown in this figure. Under normal conditions all six inflows would be in use, for example, with different concentrations of antagonist that required perfusion through the bath. The reservoir was at least 2 feet above the height of the tap. This drawing was taken from the collection of equipment drawings on the Macintosh which was the work of Grant Gourlay.


reservoir
inflow from reservoir
outflow to
recording chamber
front view
of tap

Intracellular recording

Intracellular recordings were made using the Dagan 8100 single electrode clamp system. This single electrode circuit had been developed by Wilson and Goldner (1975) to provide a means of voltage clamping with a single microelectrode. This technique is described in further detail in a following section. The electrical recordings were monitored with a Gould 20MHz oscilloscope, and permanent records were made on a Gould 200 brush chart recorder. Both current and voltage records were filtered before being displayed on the chart recorder, in order to eliminate high frequency oscillations and noise. This was accomplished by using a low pass filter consisting of a resistor and capacitor in series. A 160kΩ resistor was used with a 0.1μF capacitor. This low pass filter effectively removed noise above 9-10Hz. The experimental responses to 5-HT were sufficently slow to not be distorted by this filter. The bath was grounded with an Ag/AgCl wire connected to the virtual ground of the Dagan 8100 probe. The Ag/AgCl wire was made by coating a piece of silver wire with molten AgCl. A Grass S44 stimulator was connected to the Dagan, via a Grass SIU5 stimulus isolation unit so that pulses could be given to impaled cells. The stimulation rate, the delay between stimuli and the duration of stimuli could all be modified with this system.

Intracellular electrodes

Microelectrodes were made using filamented glass capillary tubes (Clark Electromedical) of 1.5mm diameter. They were pulled on a Narishige PP83 electrode puller and kept on plasticine in petri dishes until required. Recording electrodes were filled with 200mM KCl solution using a syringe and fine needle. The solution was filtered with a Millipore Swinnex 25 filter unit containing 0.22μm filter paper before use. The microelectrodes had a resistance of between 1 and 5MΩ .

Voltage- and current-clamp techniques

The single electrode circuit in the Dagan 8100 requires that a single electrode both records potential and passes current. This is accomplished through a switching circuit that alternates between a current-injecting mode and a voltage recording mode. The percentage of time spent injecting current and recording voltage is called the duty cycle. Current is injected for 50% of the time while membrane potential is recorded only during intervals between applications of current. The rate at which the clamp alternates between the two modes is called the switching frequency. The clamp relies on the time constant of the cell (membrane resistance x membrane capacitance) to smooth out the injected square waves of current and to provide an essentially steady DC current across the cell membrane. It was therefore important that the switching frequency be less than the time constant so that the membrane potential did not have time to wane appreciably. In all experiments the switching frequency was set at 3Hz. The voltage-clamp system used a negative feedback circuit to compare the actual potential of the cell to a commanded holding potential. Current is injected to compensate for any offset, including spontaneous events, that would otherwise shift the membrane potential. The injected current, equal and opposite to that flowing across the cell membrane, is monitored. From the holding potential, voltage commands to other membrane potentials can be applied. The currents required to produce and sustain the voltage step are recorded; they reflect the various currents present in the cell.

The Dagan could also be used in switched current-clamp mode. So that a neurone could be voltage-clamped adequately the electrode had to be able to pass current reliably during the course of the experiment. Electrodes with a resistance of 1-5MΩ were found to be reliable. Before impaling a neurone and with the electrode in solution, the resistance of the recording electrode was tested using a bridge current circuit with the z test facility of the Dagan. Electrodes with resistances higher than 5MΩ were discarded. The recording electrode was held in a micromanipulator. The gain of the negative feedback amplifier was set close to the maximum value because the higher the gain of the clamp, the more accurately the membrane voltage will follow the voltage command. The negative capacity compensation was set to a minimum.

Electrodes were then inserted into the soma of identified neurones once the fine microdissection had exposed the neurones.

Application of agonists by iontophoresis

Drugs such as 5-HT and the other 5-HT agonists were applied onto the soma of the impaled neurones by iontophoresis. Iontophoresis was performed using a microiontophoresis WP1 Model 160 programmer. Iontophoretic electrodes were made from filamented glass capillary tubes (Clark Electromedical) of 1.5mm diameter, pulled on a Narishige PN3 8903 electrode puller. They were kept in petri dishes, held in place with plasticine until required. They were filled with appropriate drug solutions which had been filtered using Costar centrifuge filter units with 0.22μm cellulose acetate filters. The drugs were made up in distilled water at a concentration of 40mM. A small retain current was applied to the iontophoretic electrode to prevent leakage of the drugs into the bath. The retain current of 20nA was used routinely. Ejection currents were in the range of 200nA with an ejection time of two seconds. This could all be set up by connecting the iontophoresis programmer to a custom-made small pulse generator. With this arrangement, the time between pulses could also be set to whatever was required. Blockage of electrodes could be detected easily by either a decrease in retain current or by the appearance of a large artefact during the eject current. Blocked electrodes were replaced immediately.

Before each experiment the eject current was increased to ensure selection of a submaximal ejection pulse. A sigmoid curve was obtained if eject current was plotted against the size of response. It was important that the eject current was on the rising phase and not on the plateau phase of the curve. The iontophoretic electrodes, once in the bath solution, were positioned with their tips close to the impaled neurones. However, care had to be taken not to place the tip too close because the eject current might cause the iontophoretic electrode to impale the cell. With continuous flow through the bath, if the iontophoretic electrode was too far away from the impaled neurone, the drug was automatically washed away without effect. The agonists applied in this way were 5-CT and sumatriptan (both 5-HT_1 receptor agonists), 2-Me-5-HT (a 5-HT_3 receptor agonist) and α-Me-5-HT (a 5-HT_2 receptor agonist). The full names of the drugs and from where they were

obtained are as follows: 5-HT creatinine sulphate (Sigma), 5-CT maleate, α-Me-5-HT maleate, 2-Me-5-HT hydrochloride and sumatriptan (all synthesised by the Chemistry Research Department at Glaxo Group Research).

Antagonists by bath application

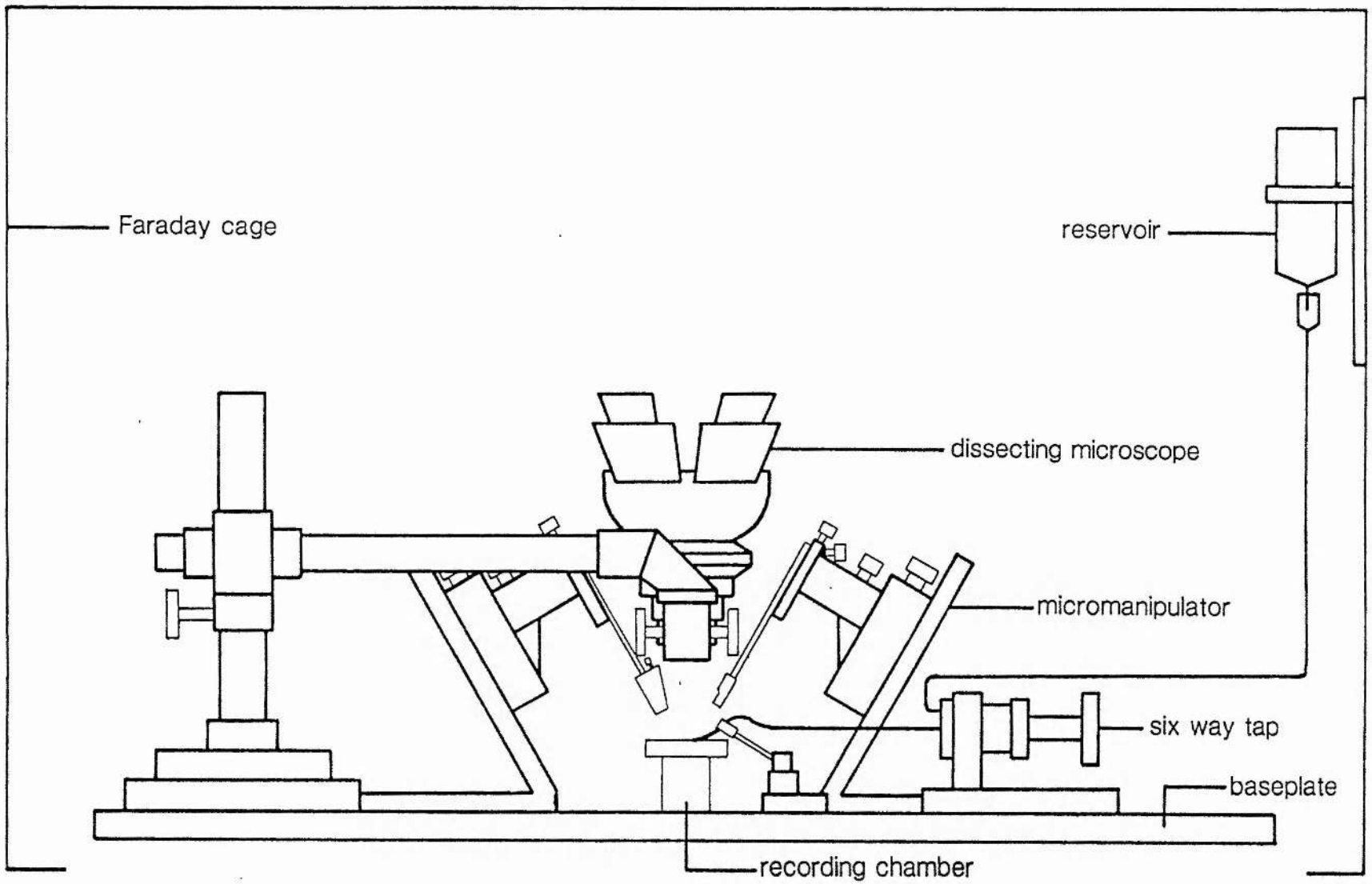
Application of antagonists required that they be perfused through the bath at the relevant concentrations. The antagonists were made up in physiological saline at appropriate concentrations with the exception of ketanserin and ondansetron which were dissolved in 0.1M tartaric acid to give a 1mM stock solution. The subsequent dilutions were then made in physiological saline. All antagonists were made up fresh on the day of use and kept in the fridge (4°C) until required. The antagonists tested were methiothepin (a 5-HT_1 receptor antagonist), ketanserin (a 5-HT_2 receptor antagonist), ondansetron, MDL 72222 and ICS 205-930 (all 5-HT_3 receptor antagonists). Methysergide was tested as an antagonist. The full names of the drugs tested and from where they were obtained are listed: methiothepin maleate (Hoffman La Roche), ketanserin tartrate (Janssen), MDL 72222 (Merrel Dow), ICS 205-930 (Sandoz), ondansetron (Glaxo) and methysergide maleate (Sandoz).

Layout of equipment

The recording chamber, micromanipulators, lighting system, microscope and perfusion system were all placed on a steel baseplate which was supported by four specially reinforced legs. This reduced vibration and aided stable recordings. A diagram of the layout is shown in Fig. 2.2. The baseplate with all the equipment on it was enclosed in a Faraday cage which was connected to earth to reduce electrical interference. The microscope baseplate and micromanipulators were also earthed. All the electronic apparatus was stacked in a freely moveable rack to the left of the cage.

FIGURE 2.2

Diagram to show the layout of the experimental apparatus within the Faraday cage. The light source, which is not visible, was placed behind the recording chamber along with the aspirator flask which was used to collect the waste perfusate from the bath. All the equipment was positioned on a baseplate in order to give good stability. This drawing was taken from the collection of equipment drawings on the Macintosh computer which was the work of Grant Gourlay.


Faraday cage
reservoir
dissecting microscope
micromanipulator
six way tap
baseplate
recording chamber

RESULTS

Position of identified neurones E1, E2, E3, E4 and E5 in the visceral ganglion.

All the identified neurones (E1, E2, E3, E4 and E5) in the visceral ganglion gave a depolarizing response to 5-HT. Their position within the visceral ganglion is shown in Fig. 2.3. The photograph (Fig. 2.4) gives a more detailed picture of the cells, although here they are still enclosed within the inner connective sheath. The dye used to stain the cells was methylene blue

E1 was about 80-100μm in diameter. E2 was slightly larger, 100-150μm in diameter, and found close to E4. E3 was approximately 80-100μm in diameter and found close to the intestinal nerve. E4 was the largest cell of this group, being 150-200μm in diameter and found at the bottom right hand corner of the ganglion. E5 was positioned above E4 and was roughly 100μm in diameter. All of these cells, with the exception of E4 which was usually silent were spontaneously active when impaled. Their resting potentials varied between -50mV and -60mV.

5-HT response in identified neurones E1, E2, E3, E4 and E5

When 5-HT was applied iontophoretically to an identified voltage-clamped neurone within the visceral ganglion a large inward current was seen (Fig. 2.5A). The effect of 5-HT was rapid in onset and 3-10nA in amplitude. The response to 5-HT seen under voltage-clamped conditions is a more realistic representation of the response because it indicates that an inward current is responsible for the excitatory response which is observed under current-clamp conditions. When 5-HT was applied iontophoretically to an identified neurone under current-clamp conditions, a depolarizing excitatory response, lasting 20-30 seconds, was observed leading to the firing of action potentials (Fig. 2.5B). The size of the response in current-clamped neurones was 3-15mV. Under current-clamp conditions the cells were usually hyperpolarised by 10-20mV in order to reduce any spontaneous activity that the cells had at their resting membrane potential which might have interfered with the observed 5-HT response.

The response to 5-HT of the identified cells within the visceral ganglion under both voltage- and current-clamp conditions was easily desensitized with

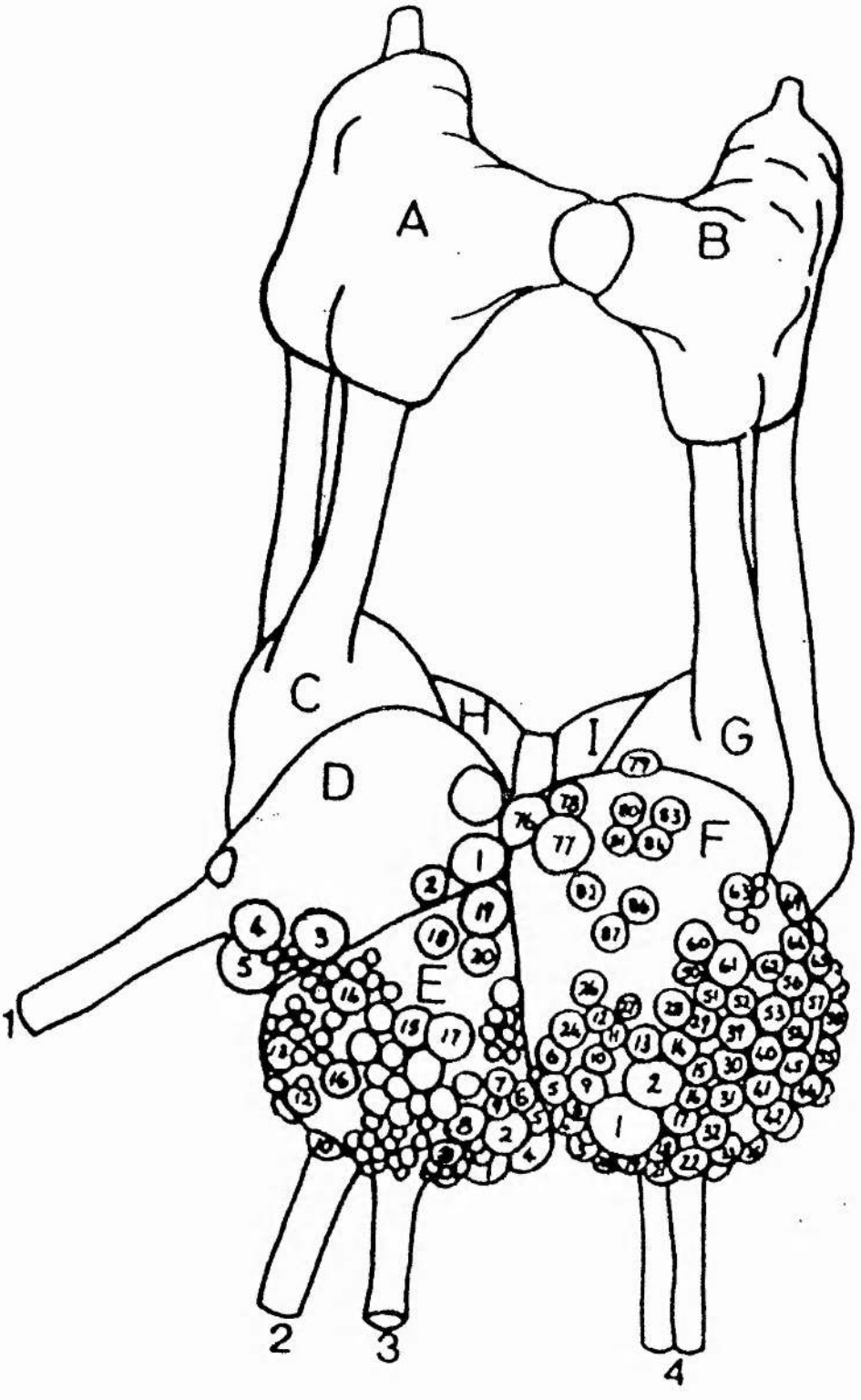


FIGURE 2.3

Diagram to show the dorsal view of the suboesophageal ganglia. The identified neurones in each ganglion are numbered. The ganglia are lettered: A, left cerebral; B, right cerebral; C, left pleural; D, left parietal; E, visceral; F, right parietal; G, right pleural; H, left pedal; I, right pedal. The nerves are numbered: 1, left pallial; 2, anal; 3, intestinal; 4, right pallial. This drawing is taken from Kerkut *et al.* (1975).

FIGURE 2.4

Photograph of the identified cells in the visceral ganglion (E1-E5) which were used in the experiments. The cells have been individually labelled but they have not been fully exposed, they are still covered by the transparent inner sheath. Methylene blue was used to dye the cells. The intestinal nerve is marked with an I, the anal nerve with an A and the right pallial with a RP. Note that the neurones in the adjacent right parietal ganglion have been fully exposed and have consequently taken up more of the dye. The scale bar indicates 100μm.

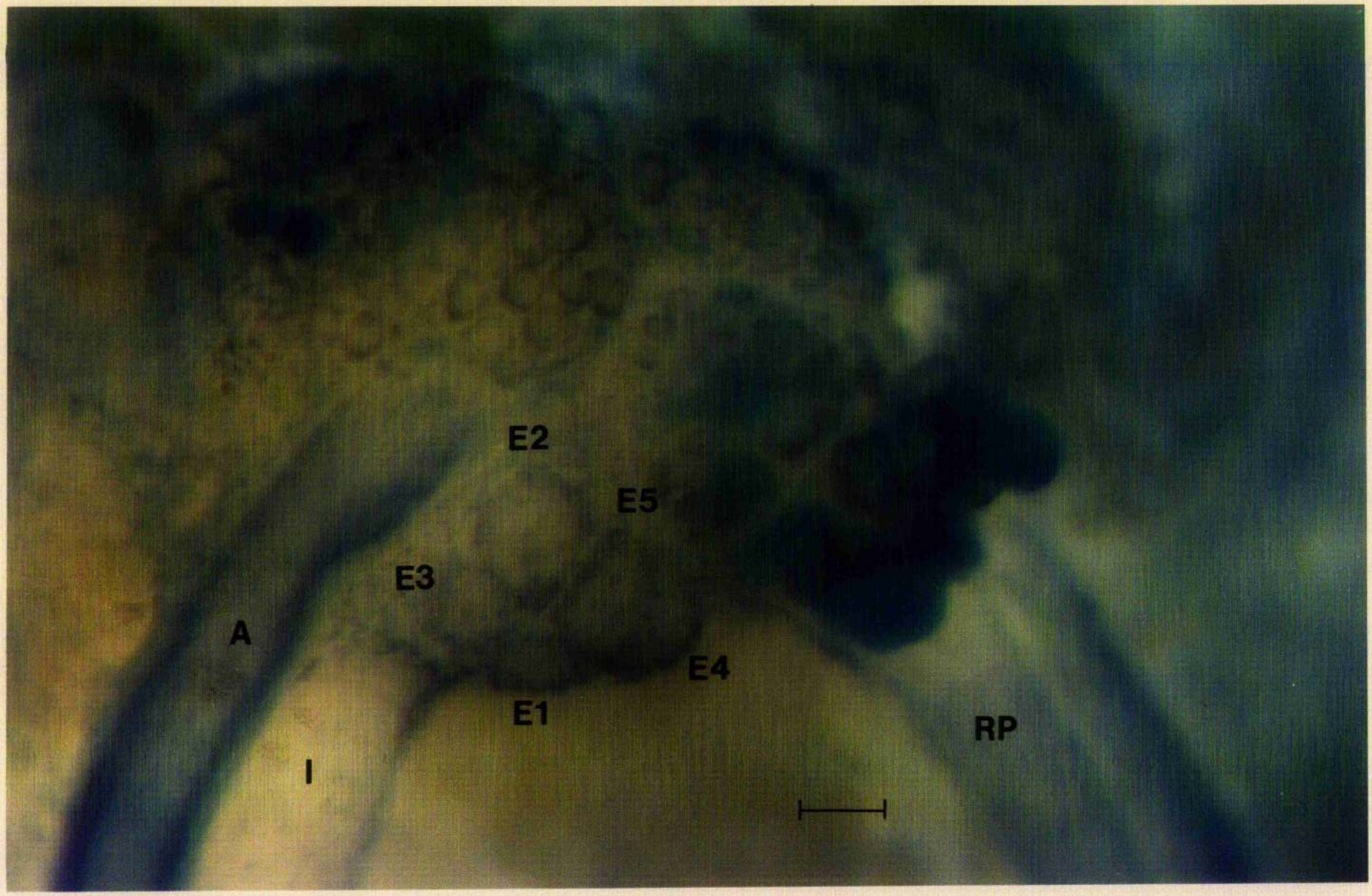
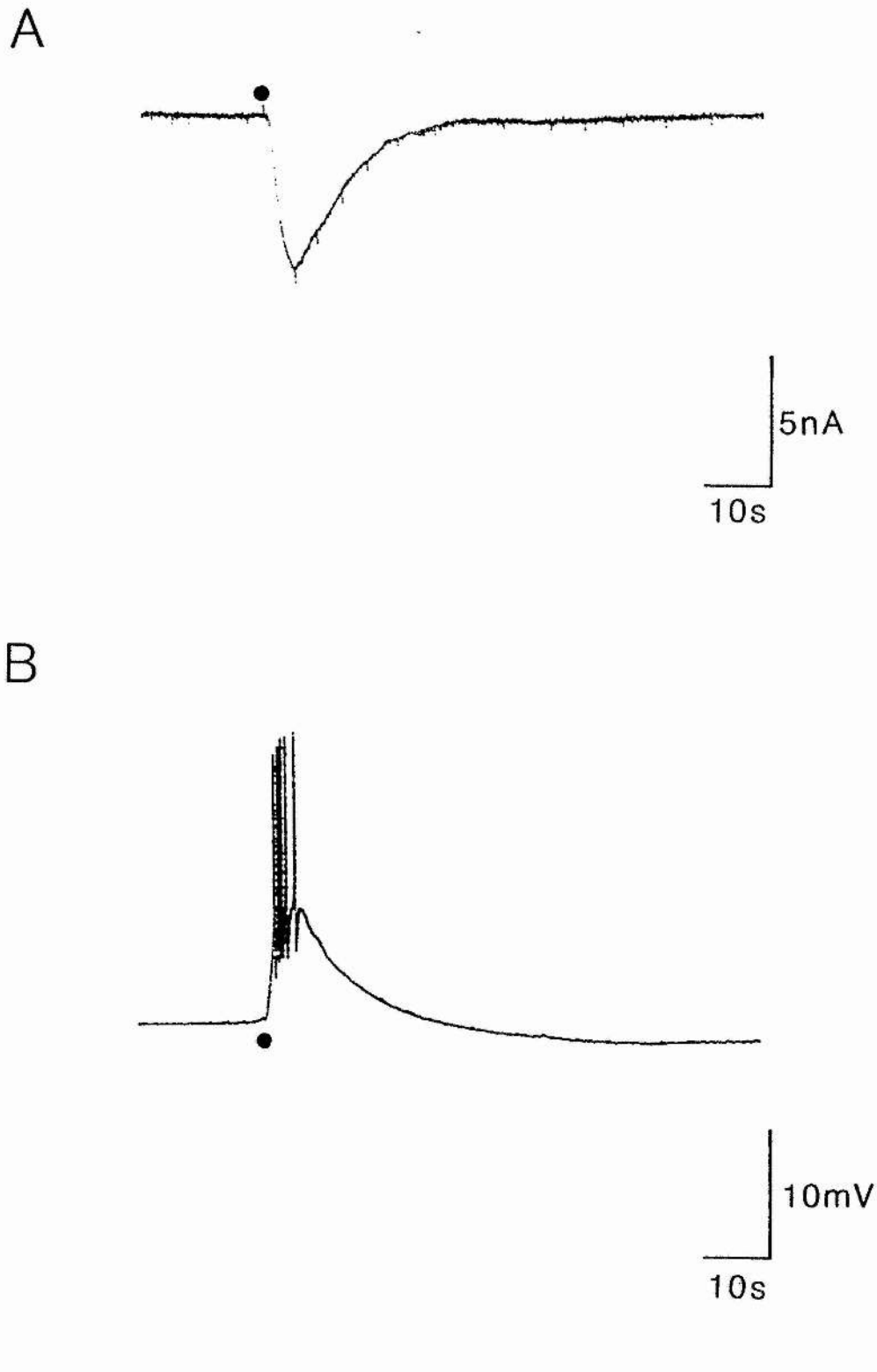

E2
E5
E3
A
E4
E1
RP
I

FIGURE 2.5

A. Response of an identified neurone, E3, in the visceral ganglion to 5-HT under voltage-clamp conditions. The cell was held at a holding potential of -70mV. The iontophoretic application (200nA for 2s) of 5-HT is indicated by the filled circle. The preparation was continuously perfused throughout the experiment.

B. Response of an identified neurone, E3, in the visceral ganglion to 5-HT under current-clamp conditions. The cell was hyperpolarised by 10mV from its resting potential in order to reduce any spontaneous activity. The resting potential of the cell was approximately -60mV. The iontophoretic application (200nA for 2s) of 5-HT is indicated by the filled circle. The preparation was continuously perfused throughout the experiment.


A
5nA
10s
B
10mV
10s

repetitive iontophoretic applications of 5-HT (Fig. 2.6A and B). For experimental purposes, to avoid desensitization, iontophoretic applications of 5-HT were made once every 3-4 minutes with continuous perfusion of physiological saline for the entire experiment. The flow of saline was 0.8-1.0ml min^{-1}. With the volume of the bath being only 0.5ml this ensured that the bath volume was changed at least once every minute.

This response seen with iontophoretic application of 5-HT is sensitive to blockade with tubocurarine at a concentration of 100μM (Fig. 2.7A and B). When the identified visceral ganglion cells were stimulated using single repeating current pulses (0.4pulses.s^{-1}) under current-clamp conditions, iontophoretic applications of 5-HT caused a reduction in the voltage deflections observed in the neurone (Fig 2.9). The reduction in the size of the voltage deflections during the 5-HT-evoked responses indicated a decrease in membrane resistance with an apparent increase in membrane conductance; i.e. current is flowing into the cell.

A second, different, 5-HT response observed within identified neurones E1 and E2

A much slower depolarizing excitatory response to iontophoretic application of 5-HT was seen in neurones E1 and E2. This response appeared similar to the one under investigation, but its time course was much slower (Fig. 2.8A). It showed no desensitization with repetitive iontophoretic applications (200nA for 2s) of 5-HT (Fig. 2.8B). No inhibition of this 5-HT response was seen when 100μM tubocurarine was perfused through the bath for 20 minutes (Fig. 2.8C). The fact that this response showed no desensitization made it easily distinguishable from the 5-HT response under investigation; by repetitively iontophoresing 5-HT onto the impaled cell under either current- or voltage-clamp conditions at the start of the experiment it was possible to immediately discriminate the 5-HT response being observed. This was ascertained at the beginning of each experiment in order to avoid confusion between the two 5-HT responses seen in the identified neurones within the visceral ganglion.

FIGURE 2.6

A. Responses of an identified neurone, E4, in the visceral ganglion to 5-HT under voltage-clamp conditions which show desensitization of the 5-HT response. The cell was held at a holding potential of -70mV. The iontophoretic application (200nA for 2s) of 5-HT is indicated by filled circles. The preparation was continuously perfused throughout the experiment. The first response of the cell is a control response to 5-HT. The second response is a response to 5-HT immediately after the recovery from the preceding response (less than 1 minute) and the third response is the response seen after adequate recovery time from the preceding 5-HT response (approximately 3 minutes).

B. Responses of an identified neurone, E3, in the visceral ganglion to 5-HT under current-clamp conditions which show a desensitization of the 5-HT response. The cell was hyperpolarised by 10mV from its resting potential. The resting potential of the cell was approximately -60mV. The iontophoretic application (200nA for 2s) of 5-HT is indicated by filled circles. The preparation was continuously perfused throughout the experiment. The first response of the cell to 5-HT is a control response to 5-HT. The second response is a response to 5-HT immediately after recovery from the preceding response (less than 1 minute) and the third response was after adequate recovery time from the preceding response to 5-HT (approximately 3 minutes).

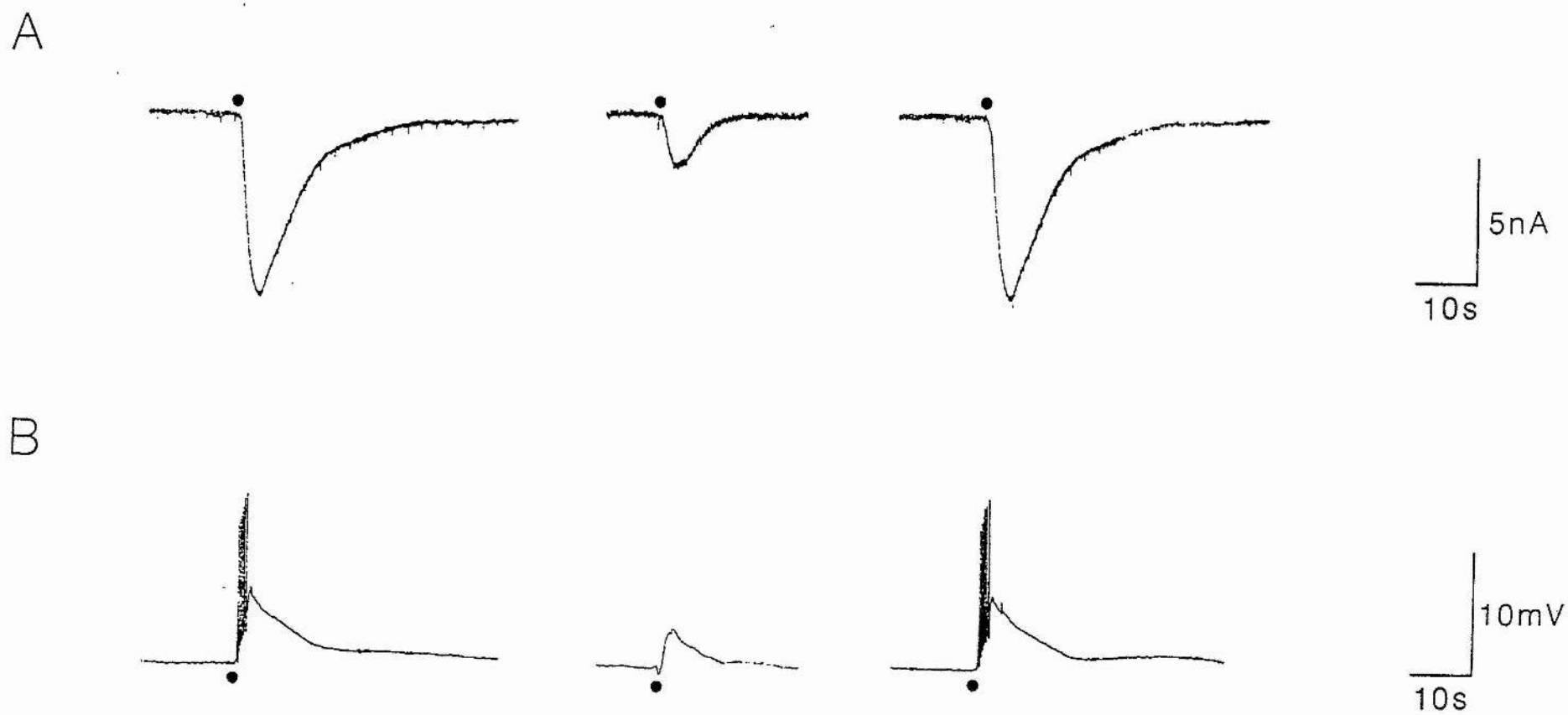

A
5nA
10s
B
10mV
10s

FIGURE 2.7

Responses of identified neurones, E4 in **A** and E5 in **B**, in the visceral ganglion to 5-HT illustrating an inhibition of the 5-HT response by 100μM tubocurarine under **A**, voltage-clamp conditions and **B**, under current-clamp conditions. In **A** the holding potential of the cell was -70mV. In **B** the cell was hyperpolarised from its resting potential by 10mV. The resting potential of the cell was approximately -60mV. In both **A** and **B** the iontophoretic application (200nA for 2s) of 5-HT is indicated by filled circles. Three responses to 5-HT are shown in both **A** and **B**. The first is under normal conditions, the second is with 100μM tubocurarine perfused through the bath while the last is after recovery and washing with fresh physiological saline for 10 minutes.

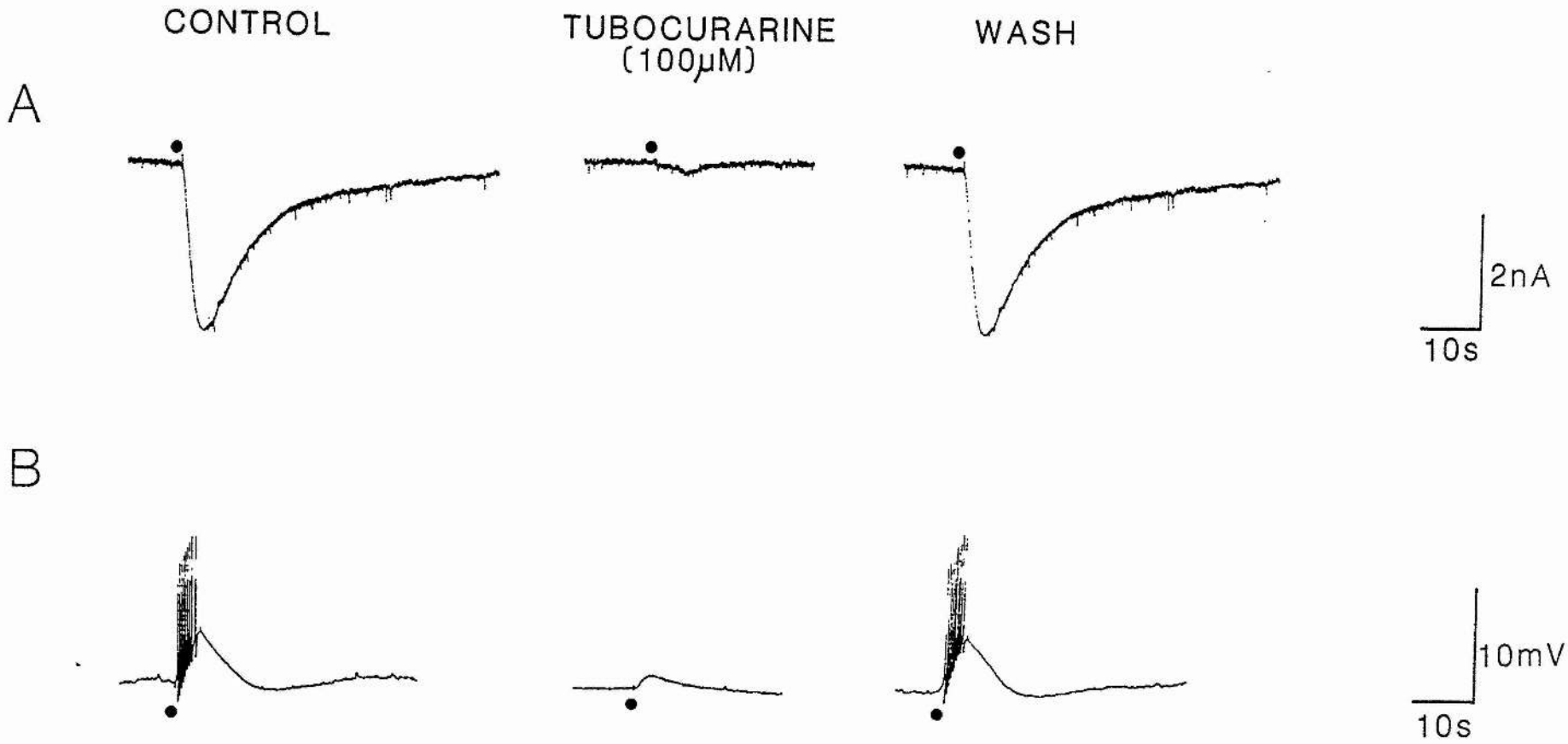

CONTROL
TUBOCURARINE
(100μM)
WASH
A
2nA
10s
B
10mV
10s

FIGURE 2.8

Responses of identified neurones, E1 and E2, within the visceral ganglion to 5-HT which show a different response to 5-HT. All responses to 5-HT are shown under current-clamp conditions with the cells hyperpolarised, by 15-20mV from their resting potential. The resting potential of the cells was approximately -55mV. Iontophoretic application (200nA for 2s) of 5-HT is indicated by filled circles. Continuous perfusion is maintained throughout the experiments.

A. Response of neurone E1 to 5-HT showing the longer time course of the response.

B. Responses of neurone E1 to repetitive iontophoretic applications of 5-HT showing lack of desensitization of the subsequent 5-HT response.

C. Responses of neurone E2 to 5-HT showing no inhibition with 100μM tubocurarine.

The first response is the control response of the cell to 5-HT, the second response is the 5-HT response in the presence of 100μM tubocurarine while the last is the response to 5-HT after a wash period.

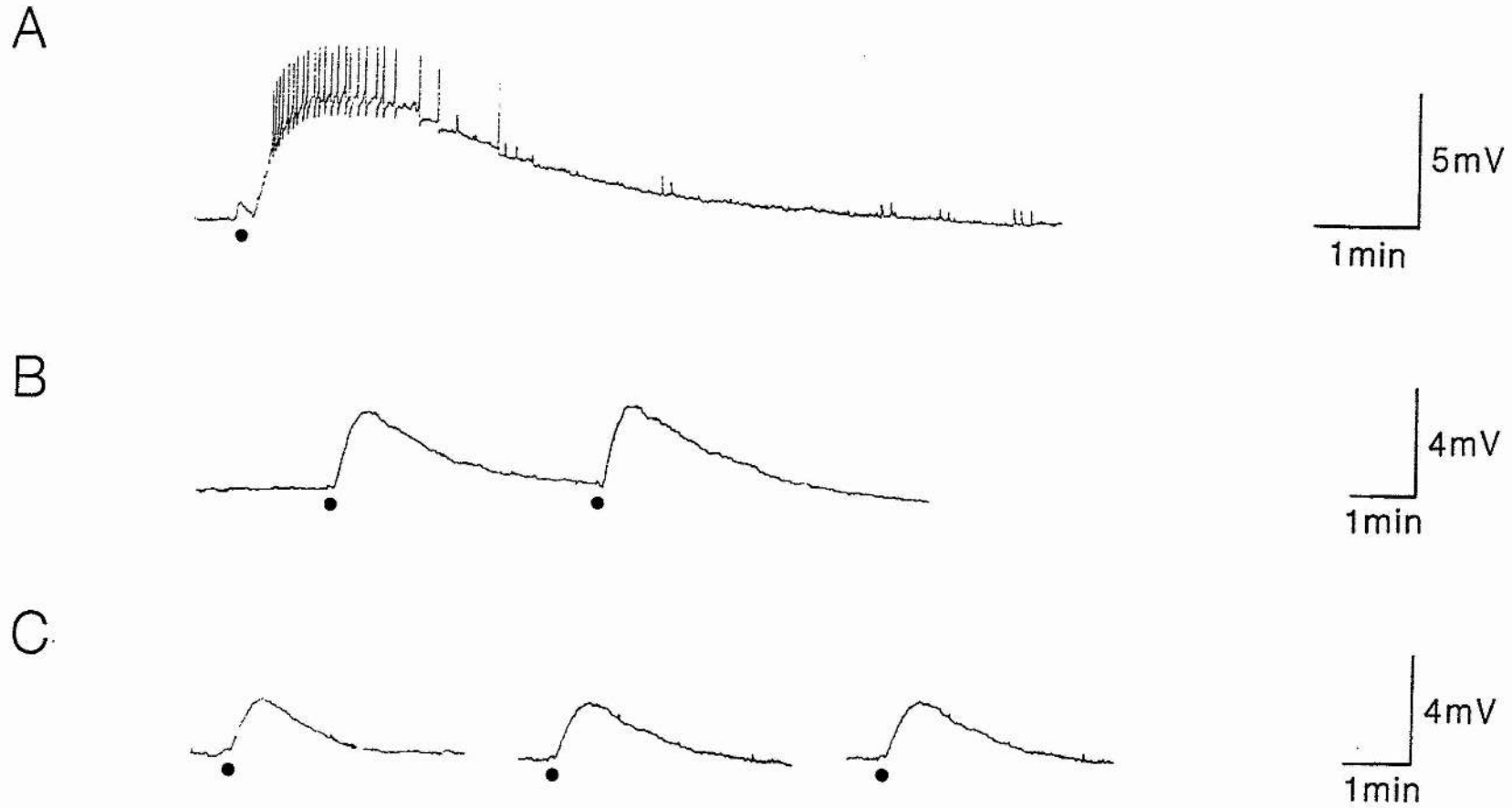
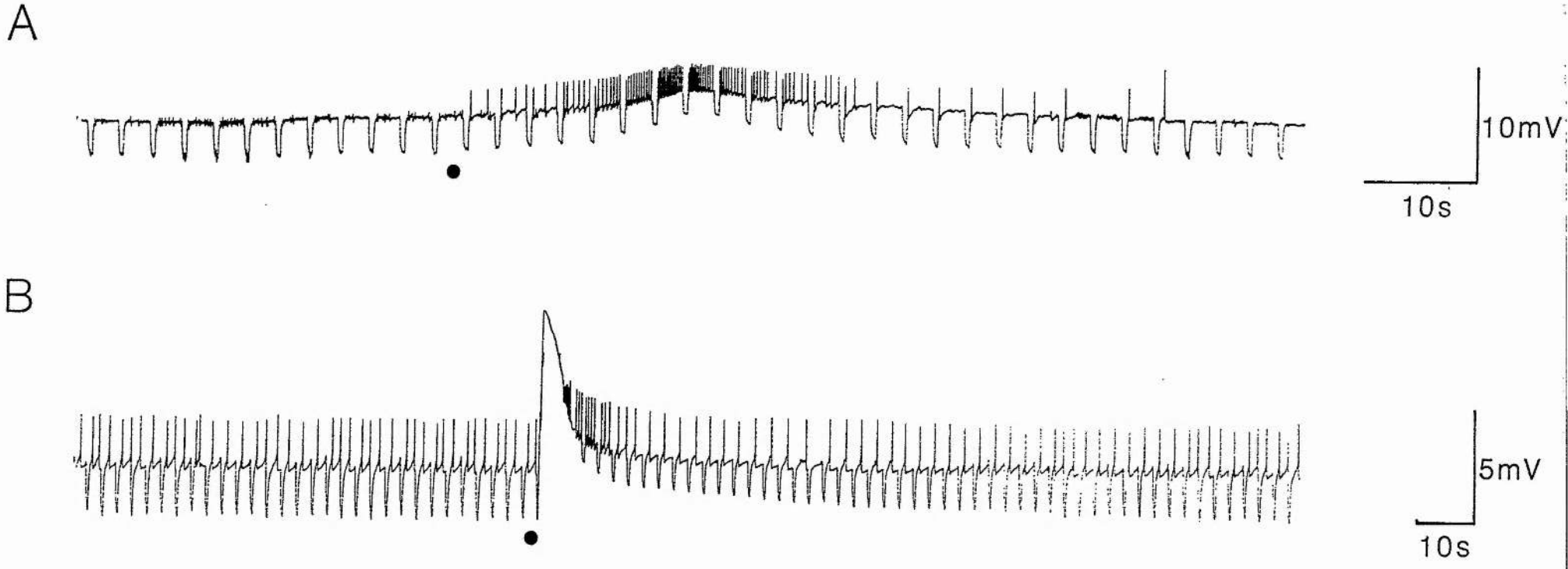

A
5mV
1min
B
4mV
1min
C
4mV
1min

FIGURE 2.9

Two examples **A** and **B**, of the response to 5-HT in identified neurones in the visceral ganglion under current-clamp conditions while the cells are being stimulated with single repeating pulses from a stimulator. In both **A** and **B** the cells were hyperpolarised from their resting potential by 10mV. The resting potential of the cells was approximately -60mV. Iontophoretic application (200nA for 2s) of 5-HT is indicated by the filled circles. Continuous perfusion was maintained throughout the entire experiments. The stimulator provided a stimulus of 3-4 volts at a rate of 0.4 pulses.s^{-1} with a duration of 400ms.


A
10mV
10s
B
5mV
10s

The reversal potential of the excitatory type A 5-HT response in the identified neurones

As already observed the 5-HT response under voltage-clamp conditions is an inward current. At hyperpolarised holding potentials, the size of the current response within the cell to 5-HT was quite large but, at more depolarized potentials, the size of the current response to 5-HT was reduced (Fig. 2.10). Evidence for the involvement of certain ions in this inward current is shown in the plot of current response against holding potential (Fig. 2.11). From -80mV to -10mV a straight line plot gave an estimated reversal potential of between -10—0mV by extrapolation. No values were obtained above a holding potential of -10mV because activation of other voltage-dependent currents at more depolarized potentials made difficult the measurement of 5-HT-induced currents, close to their reversal potential. With increased current activity at these more depolarized potentials, the holding potential of the neurone became increasingly difficult to maintain at the given level. A reversal potential of -10—0mV indicates that sodium is involved. However, a response involving only sodium ions would result in a more positive value nearer the sodium equilibrium potential of +55mV. As the reversal potential value obtained in this study was nearer the zero level, it would suggest that the fast depolarizing response to 5-HT involved an increase both in sodium and potassium conductances.

Difference in the 5-HT response in identified neurones with changes in iontophoretic eject current

The response to iontophoretic application of 5-HT increased in amplitude with increases in the size (nA) of the iontophoretic eject current (Fig. 2.12). At high values of eject current the response did not increase, indicating that the maximum response has been reached. When log eject current is plotted against percentage maximum response a typical sigmoid curve is obtained (Fig. 2.13). In order to ensure that supermaximal concentrations of 5-HT were not being used in these experiments, the eject current was set using values on the slope of the curve, usually about 200nA. In this way any effects of 5-HT agonists and antagonists could not be masked by utilising 5-HT at too high a concentration.

FIGURE 2.10

The responses of an identified neurone, E3, in the visceral ganglion to local application of 5-HT under voltage-clamp conditions. Responses were recorded at different membrane holding potentials. The holding potential of the cell is as stated. Iontophoretic application (200nA for 2s) of 5-HT is indicated by the filled circles. Continuous perfusion was maintained throughout the experiment.

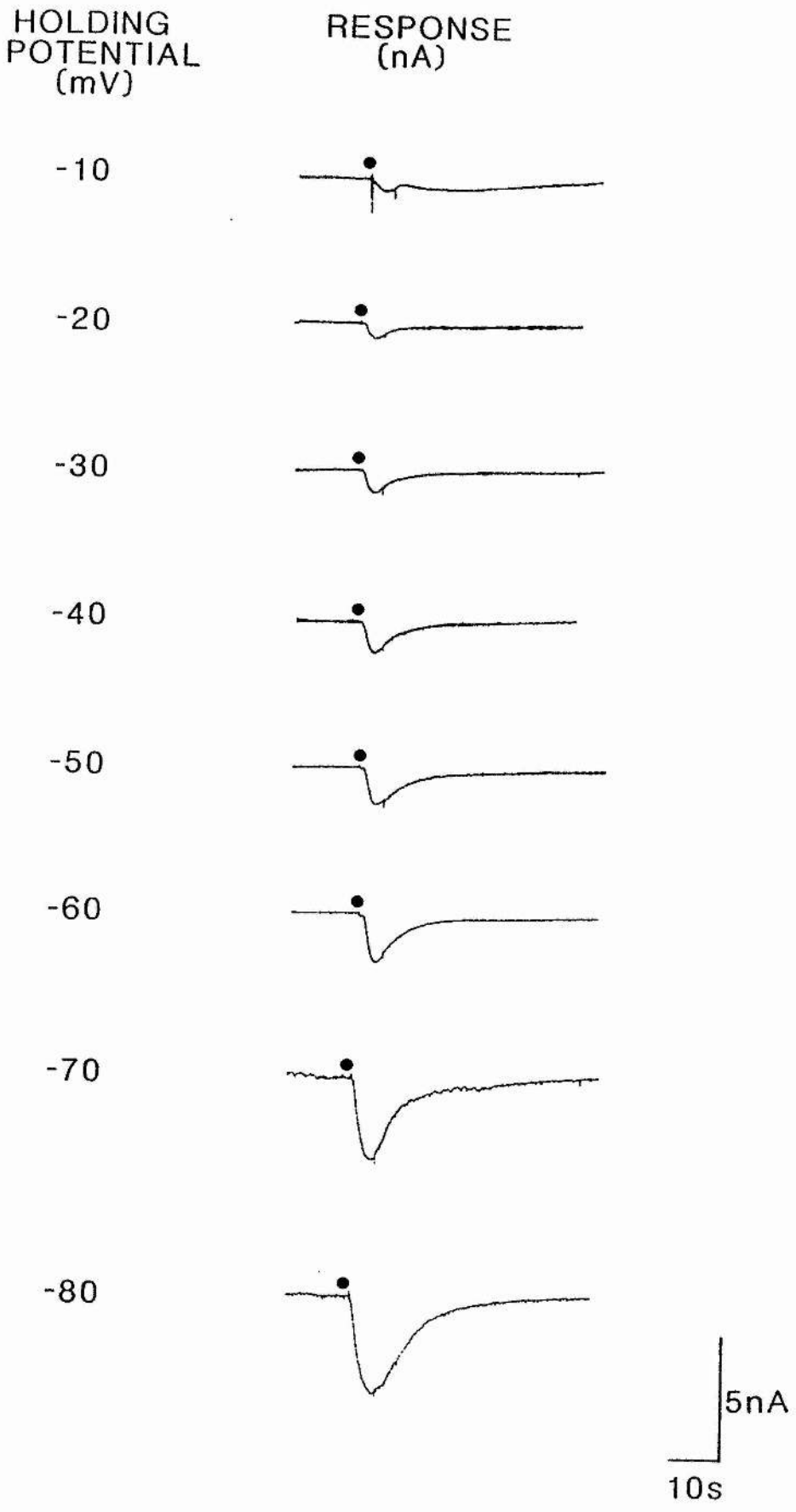

HOLDING POTENTIAL (mV)
RESPONSE (nA)
-10
-20
-30
-40
-50
-60
-70
-80
5nA
10s

FIGURE 2.11

Relationship between current response to 5-HT and holding potential of E3 neurone. The x axis gives the holding potential of the cell in mV while the y axis gives the current response in nA.

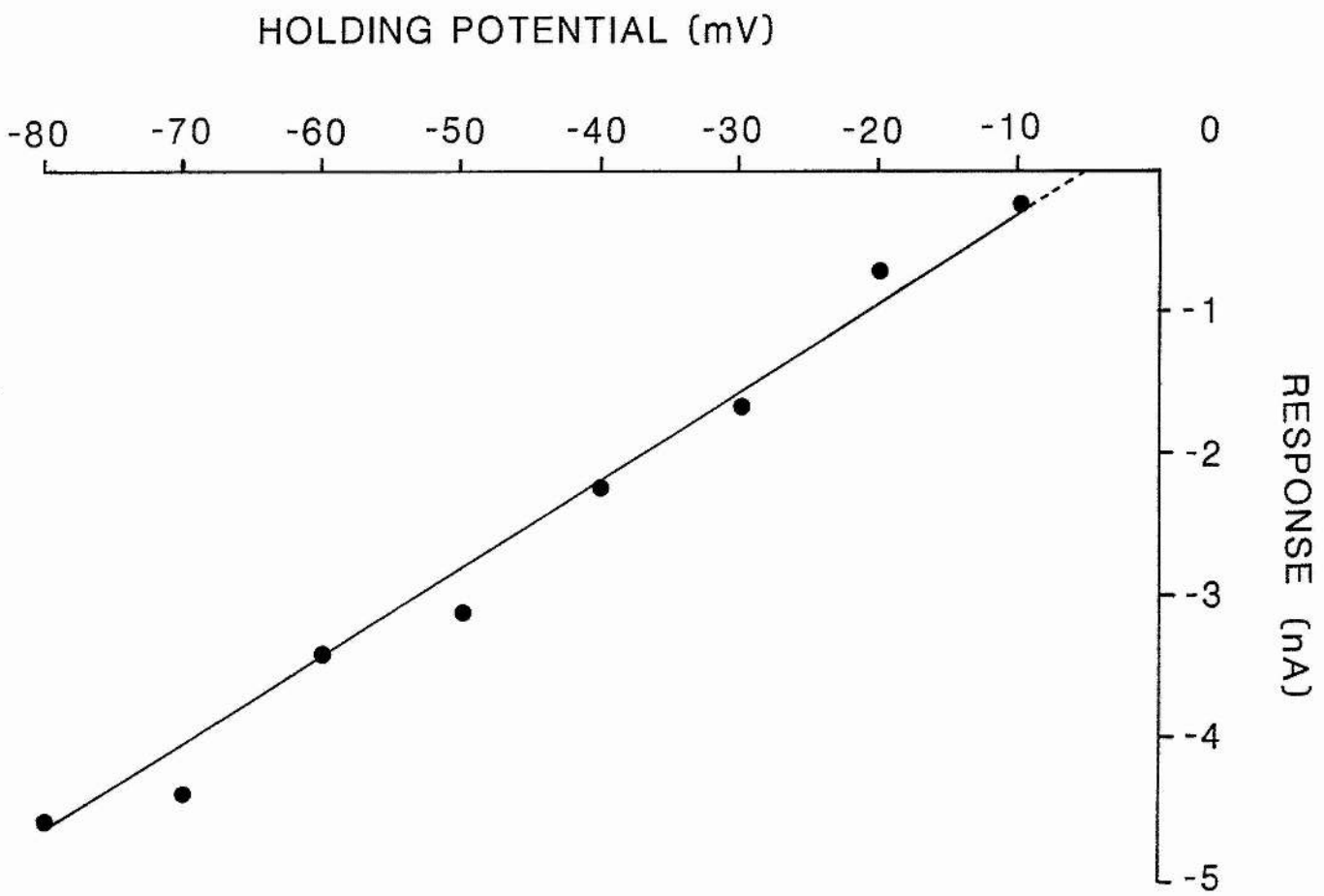
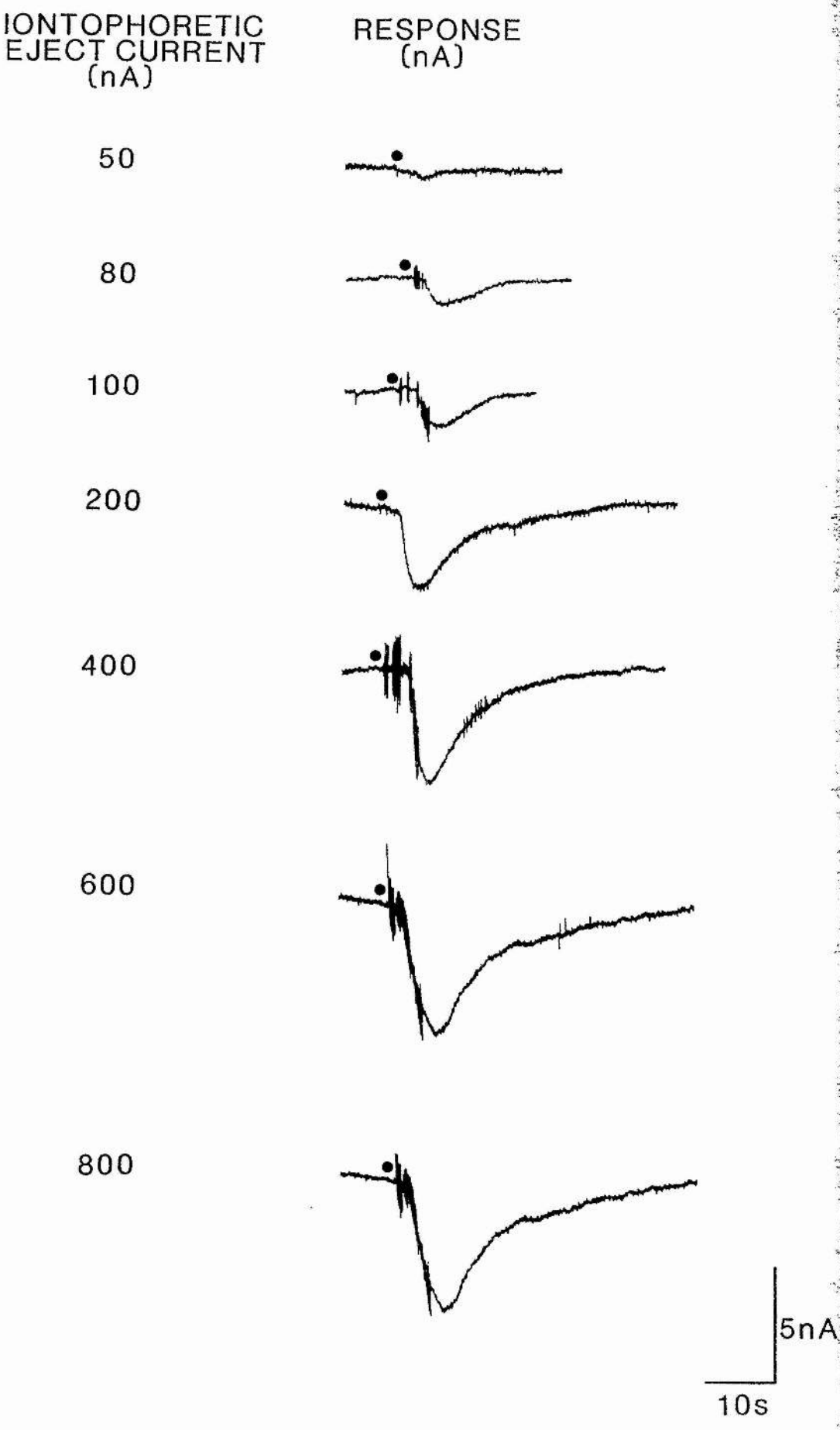

HOLDING POTENTIAL (mV)
-80
-70
-60
-50
-40
-30
-20
-10
0
-1
-2
-3
-4
-5
RESPONSE (nA)

FIGURE 2.12

The responses of E4 neurone within the visceral ganglion under voltage-clamp conditions to iontophoretic application of 5-HT at different iontophoretic eject currents. The holding potential of the cell was -80mV. The iontophoretic application of 5-HT (2s) is indicated by filled circles with the stated eject current in nA. Continuous perfusion was maintained throughout the experiment.


IONTOPHORETIC
EJECT CURRENT
(nA)
RESPONSE
(nA)
50
80
100
200
400
600
800
5nA
10s

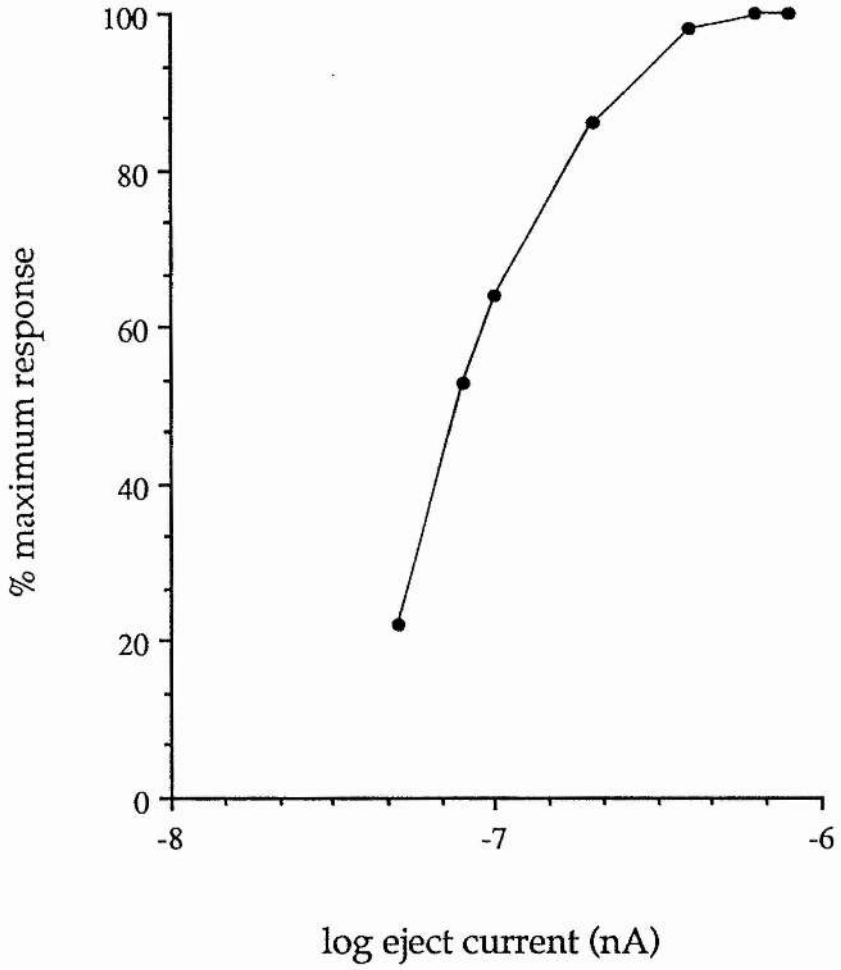


FIGURE 2.13

Plot to show the dependence of the response in E3 neurone in the visceral ganglion on the size of eject current in nA. The x axis gives log of the eject current while the y axis gives percentage maximum response.

The effects of 5-HT agonists

In looking at the effects of agonists on the identified cells within the visceral ganglion the 5-HT response was always used as a reference. Once a response to 5-HT had been obtained the iontophoretic electrode was removed and replaced with one similar but containing the 5-HT agonist under investigation. The iontophoretic electrode was placed in, as near as possible, the same position as the 5-HT iontophoretic electrode had been so that the iontophoresing of the agonist would be under similar conditions to that of 5-HT.

5-CT, the specific 5-HT_1 receptor agonist, at the same concentration (40mM) within the iontophoretic electrode and under the same retain and eject conditions as 5-HT, excited a response very similar to 5-HT under both voltage and current-clamp conditions (Figs 2.14A and B). In every case the response was either equal to, or slightly smaller than that of 5-HT. This may be explained by 5-CT having less potent actions than 5-HT on the 5-HT receptor present in the cell membrane. However this was difficult to determine definitively because the process of iontophoresis is qualitative rather than quantitative.

α-Me-5-HT, the 5-HT_2 receptor agonist, also mimicked the action of 5-HT when iontophoresed onto the identified cells of the visceral ganglion. It elicited a depolarizing excitatory response similar to 5-HT (Figs. 2.15 A,B and C) under current-clamp conditions. The response to α-Me-5-HT was not attempted under voltage-clamp conditions. Once again the response to α-Me-5-HT was the same or slightly smaller than 5-HT indicating a similar potency of α-Me-5-HT at the 5-HT receptor on the cell membrane. However, because the response was qualitative only, the potency of α-Me-5-HT for the receptor under investigation could not be established definitively.

Sumatriptan, another 5-HT_1 receptor agonist, mimicked to a certain extent the action of 5-HT by eliciting a response similar to, but much smaller than, that elicited by 5-HT (Figs. 2.16A,B and C). The depolarizing excitatory responses elicited by sumatriptan were observed only under current-clamp conditions. In contrast, 2-Me-5-HT, a 5-HT_3 receptor agonist elicited no response from the identified cells when the drug was applied

iontophoretically (Figs. 2.17A and B). Once again, the conditions of retain and eject and the concentration of the agonist in the iontophoretic electrode were the same as that for 5-HT. It could then be suggested that, because 2-Me-5-HT failed to elicit any response, 2-Me-5-HT had no affinity for the 5-HT receptor present on the cell membrane. A summary of the results obtained with the agonists is given in Table 2.3 below.

TABLE 2.3 Summary of the 5-HT agonists tested on identified *Helix* neurones. The concentration given is the concentration within the iontophoretic electrode.

Agonist	Concentration (mM)	n	Effect
5-HT	40	40	Inward current (3-10nA) under v-clamp at potentials of -70 — -80mV Depolarization (3-15mV) under c-clamp at potentials of between -60 — -80 mV
5-CT	40	12	Mimicks effect of 5-HT in v- and c-clamp. Responses same size as 5-HT or slightly smaller
α–Me-5-HT	40	10	Mimicks effect of 5-HT in c-clamp. Responses same size as 5-HT or slightly smaller
Sumatriptan	40	10	Mimicks effect of 5-HT in c-clamp. Responses much smaller than those of 5-HT
2-Me-5-HT	40	8	No effect seen with this agonist

FIGURE 2.14

Responses of identified neurones, E5 and E3, within the visceral ganglion to 5-HT and 5-CT under **A** voltage-clamp conditions and **B** current-clamp conditions. In **A** the holding potential of the cell was -70mV. In **B** the cell was hyperpolarised from its resting potential by 15mV. The resting potential of the cell was approximately -55mV. Iontophoretic application (200nA for 2s) of 5-HT is indicated by filled circles and that of 5-CT is indicated by asterisks. In both **A** and **B** the first response is a control response to 5-HT followed by a response, in the same cell, to 5-CT. The third response is the response to 5-HT after that of 5-CT. Continuous perfusion was maintained throughout the experiments. The interval between responses is four minutes.

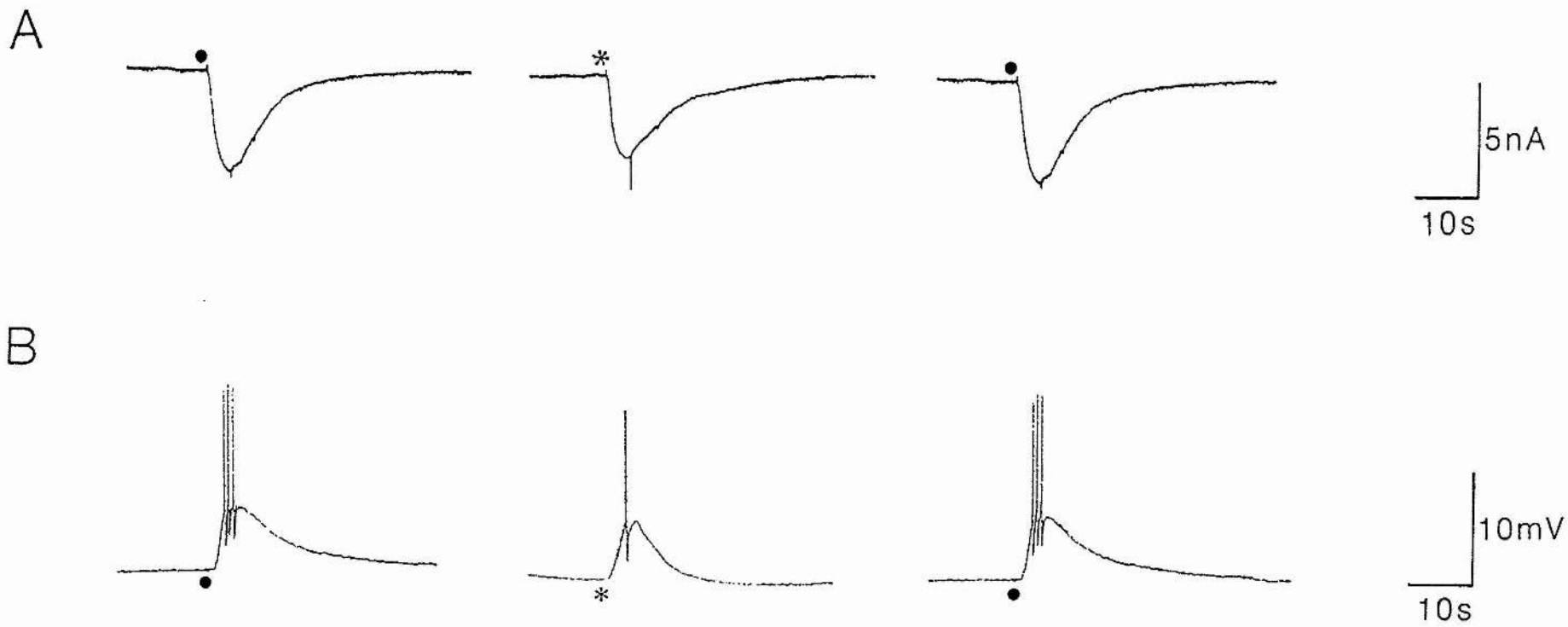

A
B
5nA
10s
10mV
10s

FIGURE 2.15

Responses of three identified neurones, E3, E4 and E2, (**A**, **B** and **C**) within the visceral ganglion to 5-HT and to α–Me-5-HT under current-clamp conditions. In **A**, **B** and **C** the cells were hyperpolarised by 10mV from their resting potentials. The resting potential of the cells was approximately -60mV. Iontophoretic application (200nA for 2s) of 5-HT is indicated by filled circles while that of α-Me-5-HT is indicated by asterisks. In **A**, **B** and **C** the first response is a control response to 5-HT. The second response, of the same cell, is to α-Me-5-HT. The third response is a response to 5-HT after that of α-Me-5-HT. The time interval between iontophoretic application was 4 minutes. Continuous perfusion was maintained throughout the experiments.

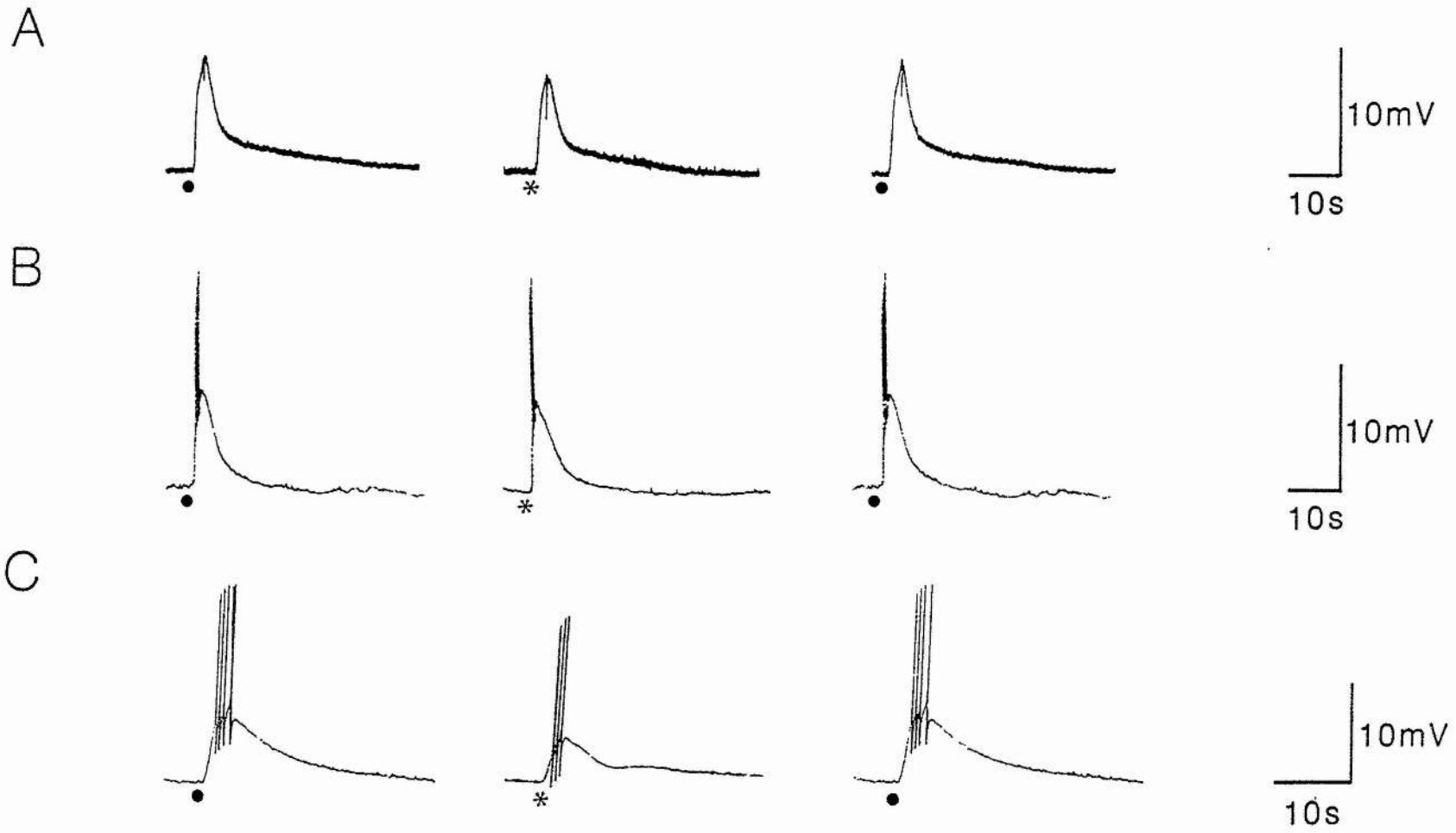

A
B
C
10mV
10s
10mV
10s
10mV
10s

FIGURE 2.16

Responses of three identified neurones, E3, E4 and E5 (**A, B** and **C**) within the visceral ganglion to 5-HT and to sumatriptan under current-clamp conditions. In **A, B** and **C** the cells were hyperpolarised by 10mV from their resting poentials. The resting potential of the cells was approximately -60mV. Iontophoretic application (200nA for 2s) of 5-HT is indicated by filled circles while that of sumatriptan is indicated by asterisks. In **A, B** and **C** the first response is a control response to 5-HT followed by the second response, in the same cell, to sumatriptan. The third response is a response to 5-HT after that of sumatriptan. The time internal between iontophoretic applications was 4 minutes. Continuous perfusion was maintained throughout the experiments.

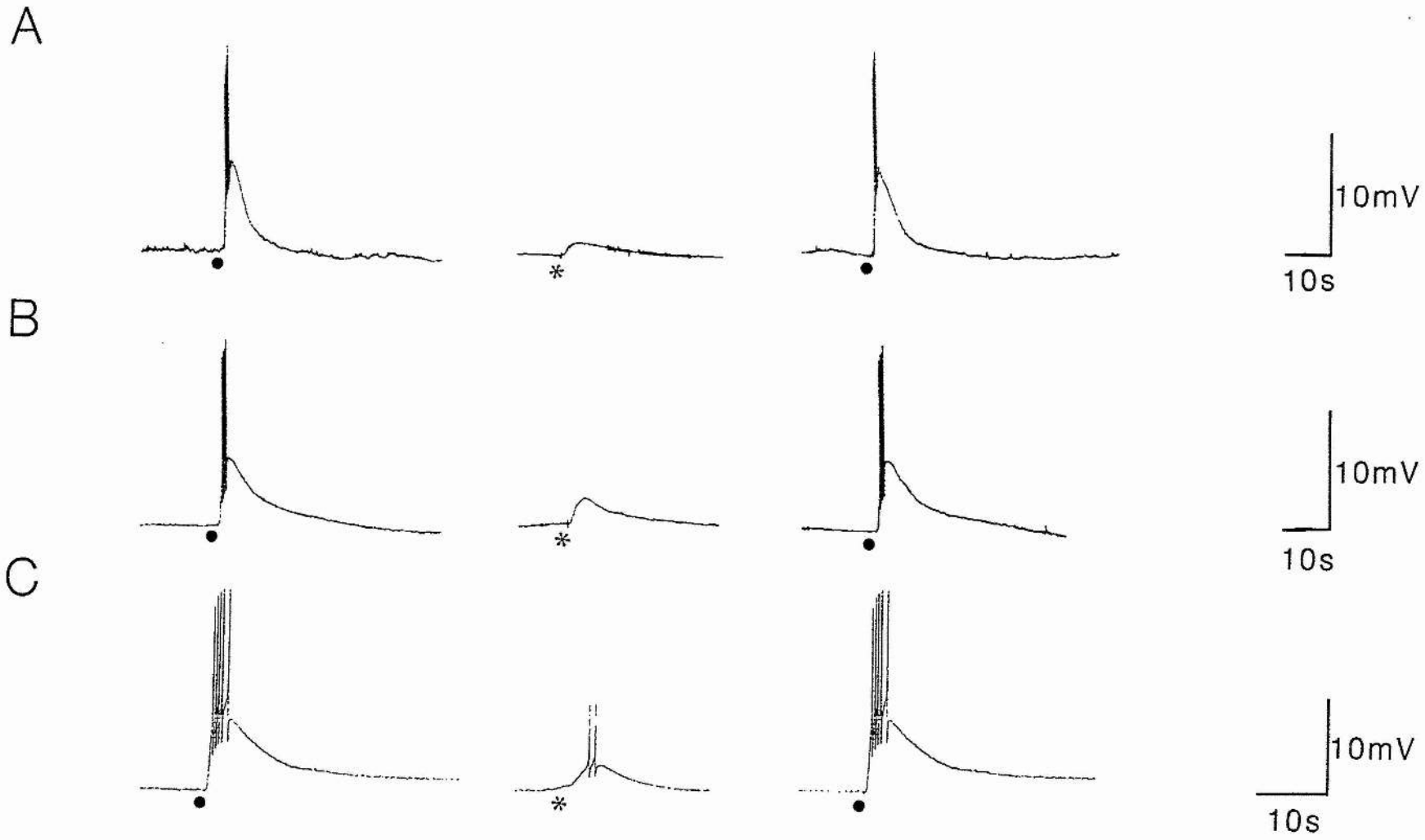
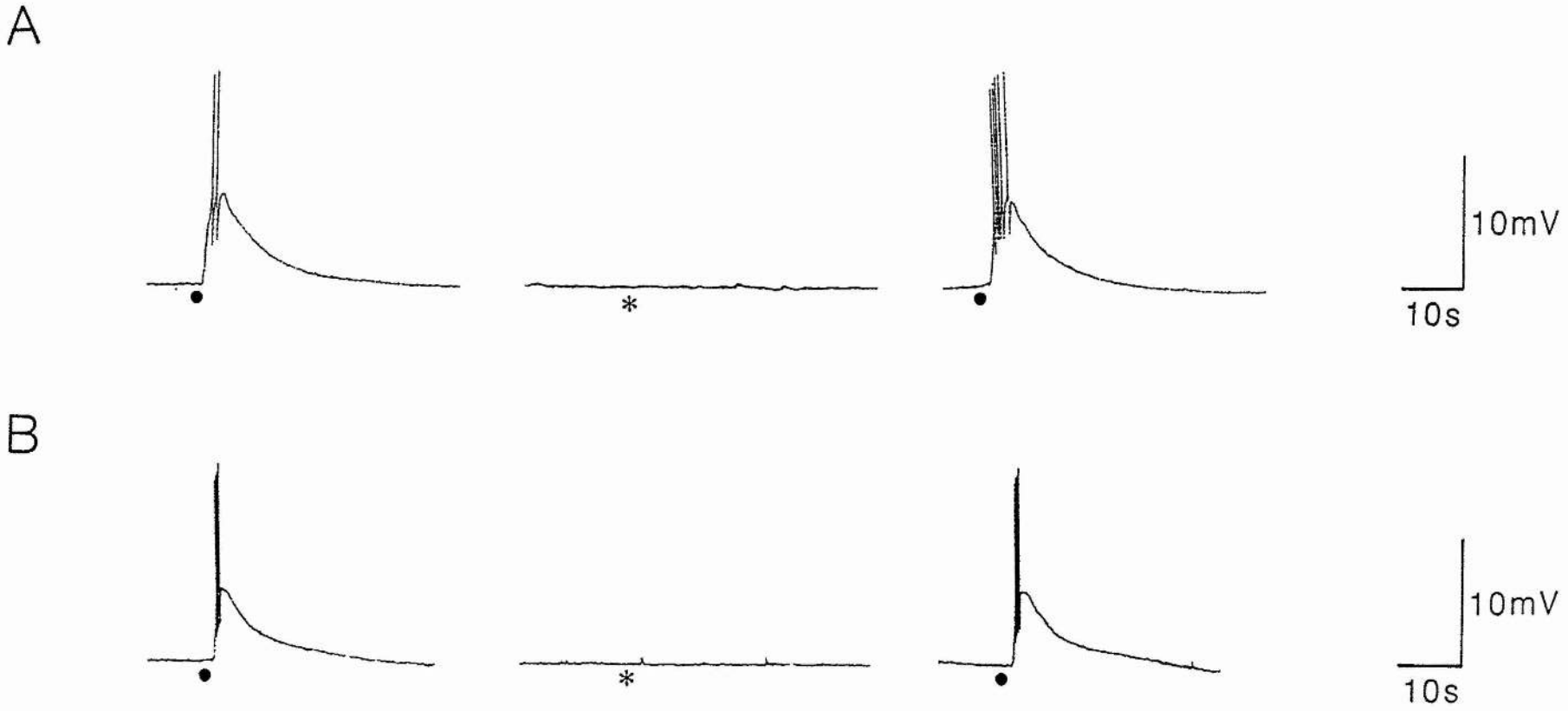

A
B
C
10mV
10s
10mV
10s
10mV
10s

FIGURE 2.17

Responses of two identified neurones, E1 and E3 (**A** and **B**), within the visceral ganglion to 5-HT and to 2-Me-5-HT under current-clamp conditions. In **A** and **B** the cells were hyperpolarised by 10mV from their resting potentials. The resting potential of the cells was approximately -60mV. Iontophoretic application (200nA for 2s) of 5-HT is indicated by filled circles while that of 2-Me-5-HT is indicated by asterisks. In **A** and **B** the first response is a control response to 5-HT followed by the second response, in the same cell, to 2-Me-5-HT. The third response is a response to 5-HT after that of 2-Me-5-HT. The time interval between iontophoretic applications was 4 minutes. Continuous perfusion was maintained throughout the experiments.


A
10mV
10s
B
10mV
10s

Effects of 5-HT antagonists

These experiments showed much similarity of the excitatory response to 5-HT to that observed in neuroblastoma cell lines, in terms of a fast depolarizing response which showed desensitization and a similar value for the reversal potential. Accordingly, the 5-HT_3 receptor antagonists which blocked the 5-HT responses in these cell lines were tested first on the 5-HT response in the identified neurones. These antagonists were ondansetron, ICS 205-930 and MDL 72222. Ondansetron was tested at concentrations ranging from 0.1μM-10μM by being perfused through the bath over a time period of 40 minutes. Even at a concentration of 10μM ondansetron had no antagonistic effect on the response to 5-HT in the identified neurones under voltage-clamp conditions (Fig. 2.18C). At lower concentrations also ondansetron had no effect on the inward current elicited by 5-HT under voltage-clamp conditions (Figs. 2.18A and B). There was a lack of antagonist effect shown by ICS 205-930 at a concentration of 1μM both under voltage- and current-clamp conditions (Figs. 2.19A and B). The response to 5-HT remained unchanged for at least 30 minutes despite the perfusion of antagonist, through the bath. The final 5-HT_3 antagonist applied, MDL 72222, also had no effect on the 5-HT response observed under both current- and voltage-clamp conditions (Figs. 2.20A and B). There was no change in the 5-HT response in the presence of 1μM MDL 72222 despite its continued perfusion over a 40 minute period.

The other specific antagonists tested were methiothepin, a 5-HT_1 receptor antagonist, and ketanserin, a 5-HT_2 receptor antagonist. In this case, methysergide, an LSD derivative, was also tested as an antagonist. No antagonistic effect was observed on the 5-HT response by methiothepin, ketanserin or methysergide, all at concentrations of 1μM. The 5-HT response both under current and voltage-clamp conditions remained unchanged even though these antagonists were perfused through the bath for 40 minutes. Because the 5-HT response with the above three antagonists remained unchanged the results are not shown as figures. Table 2.4 overpage summarizes the antagonists tested, the concentration at which they were tested and the lack of an effect seen.

TABLE 2.4 Summary of the 5-HT antagonists tested on *Helix* identified neurones

Antagonist	Concentration (μM)	n	Effect
Ondansetron	0.1, 1 and 10	8	No effect seen
ICS 205-930	1	8	No effect seen
MDL 72222	1	7	No effect seen
Methiothepin	1	8	No effect seen
Ketanserin	1	8	No effect seen
Methysergide	1	8	No effect seen

FIGURE 2.18

Responses of identified neurones, E3 and E4, within the visceral ganglion to 5-HT in the absence and presence of increasing concentrations (**A-C**) of ondansetron under voltage-clamp conditions. In **A, B** and **C** the holding potential of the cells was -75mV. In **A, B** and **C** three responses to 5-HT are shown. The first is a control response to 5-HT, the second a response to 5-HT in the presence of ondansetron and the third is a response to 5-HT after a wash period. In **A** the concentration of ondansetron was 0.1μM, in **B** it was 1μM and in **C** the ondansetron concentration was 10μM. Iontophoretic application (200nA for 2s) of 5-HT is indicated by filled circles. Continuous perfusion was maintained throughout the experiments.

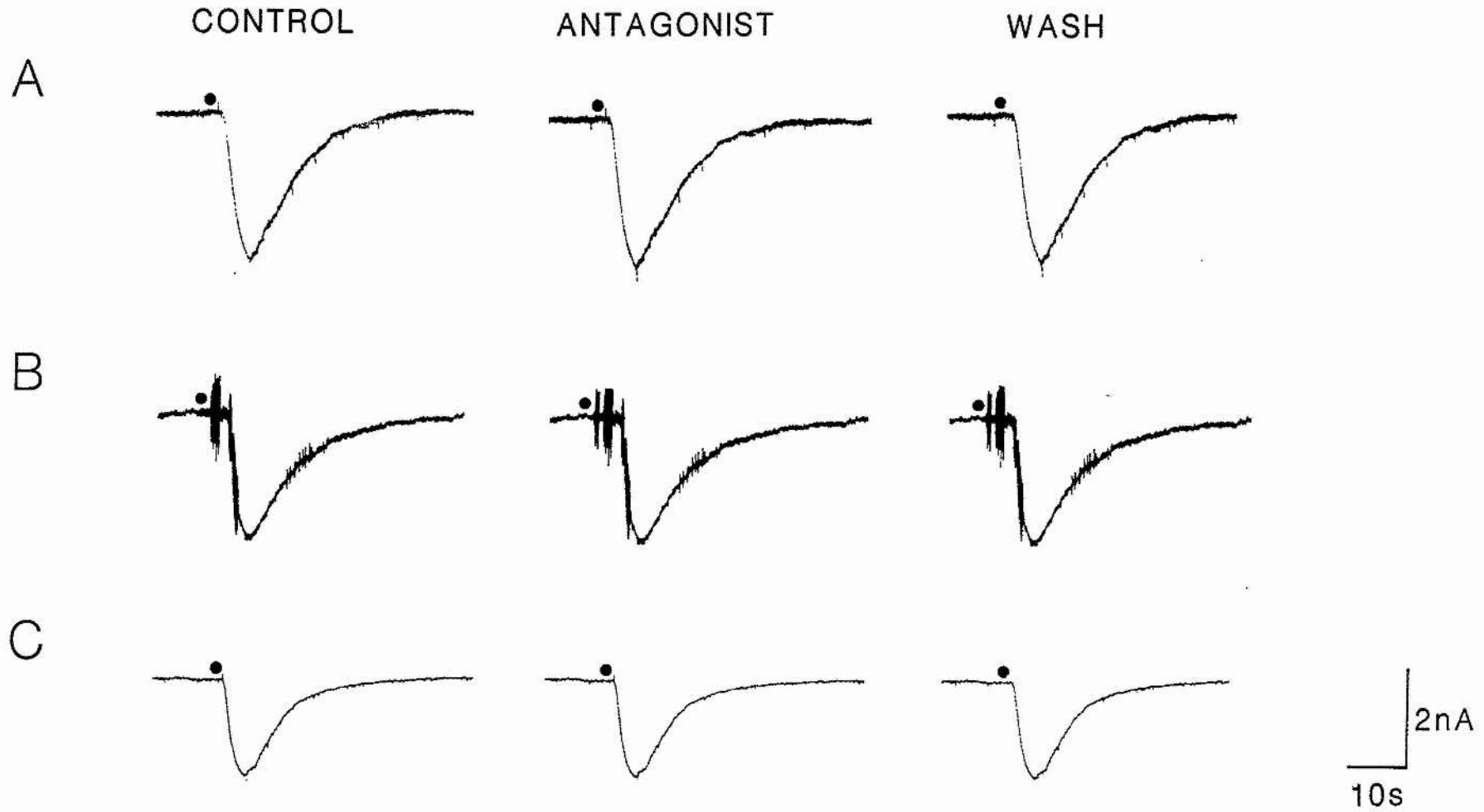

CONTROL
ANTAGONIST
WASH
A
B
C
2nA
10s

FIGURE 2.19

Responses of identified neurones, E3 and E5, within the visceral ganglion to 5-HT in the presence and absence of 1μM ICS 205-930 under **A** voltage-clamp conditions and **B** current-clamp conditions. In **A** the holding potential of the cell was -70mV. In **B** the cell was hyperpolarised by 10mV from its resting potential. The resting potential of the cell was approximately -60mV. The iontophoretic application (200nA for 2s) of 5-HT in indicated by filled circles. In **A** and **B** three responses to 5-HT are shown. The first is a control response of the cell to 5-HT, the second is a response, of the same cell, to 5-HT in the presence of 1μM ICS 205-930 while the last is the response to 5-HT after a wash period. Continuous perfusion was maintained throughout the experiments.

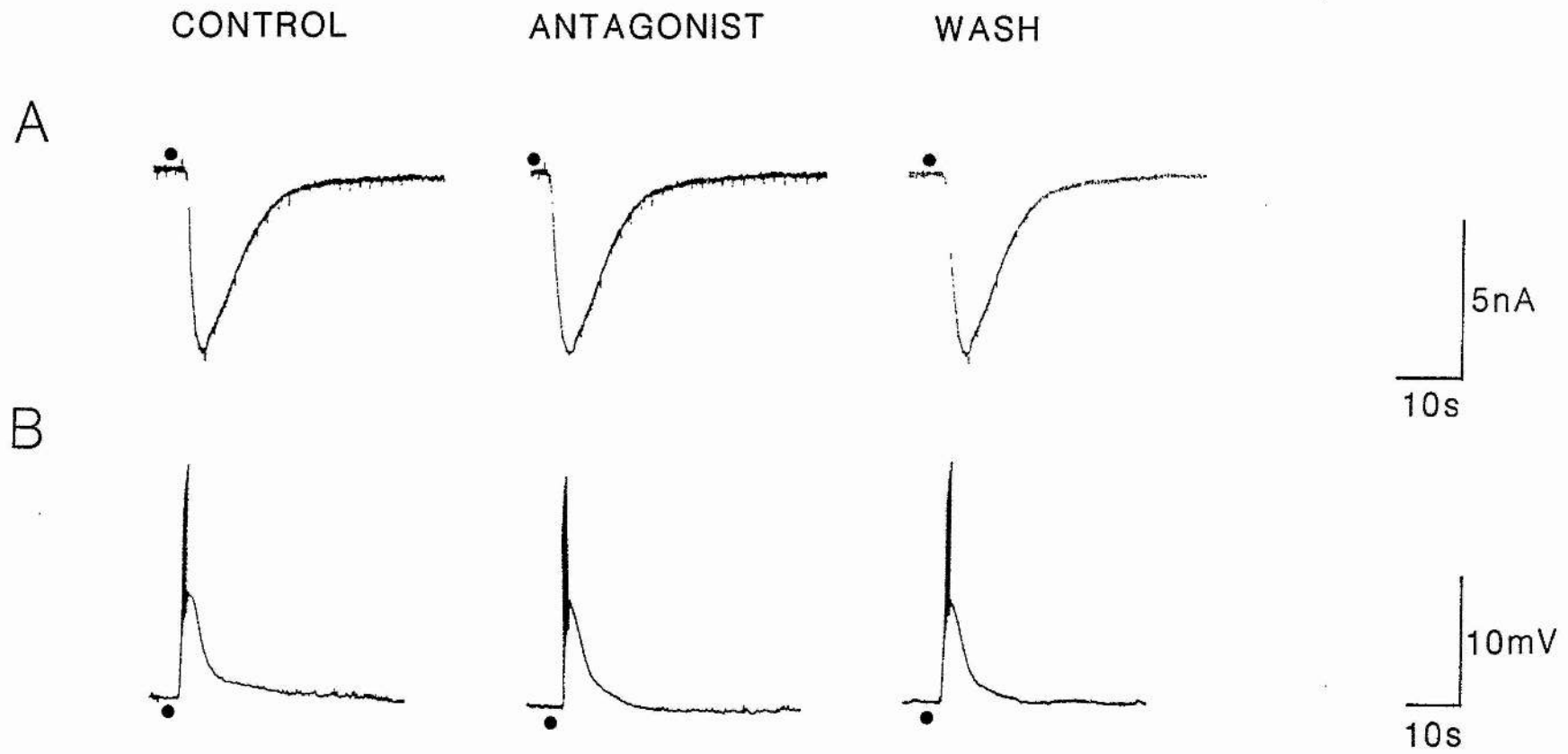
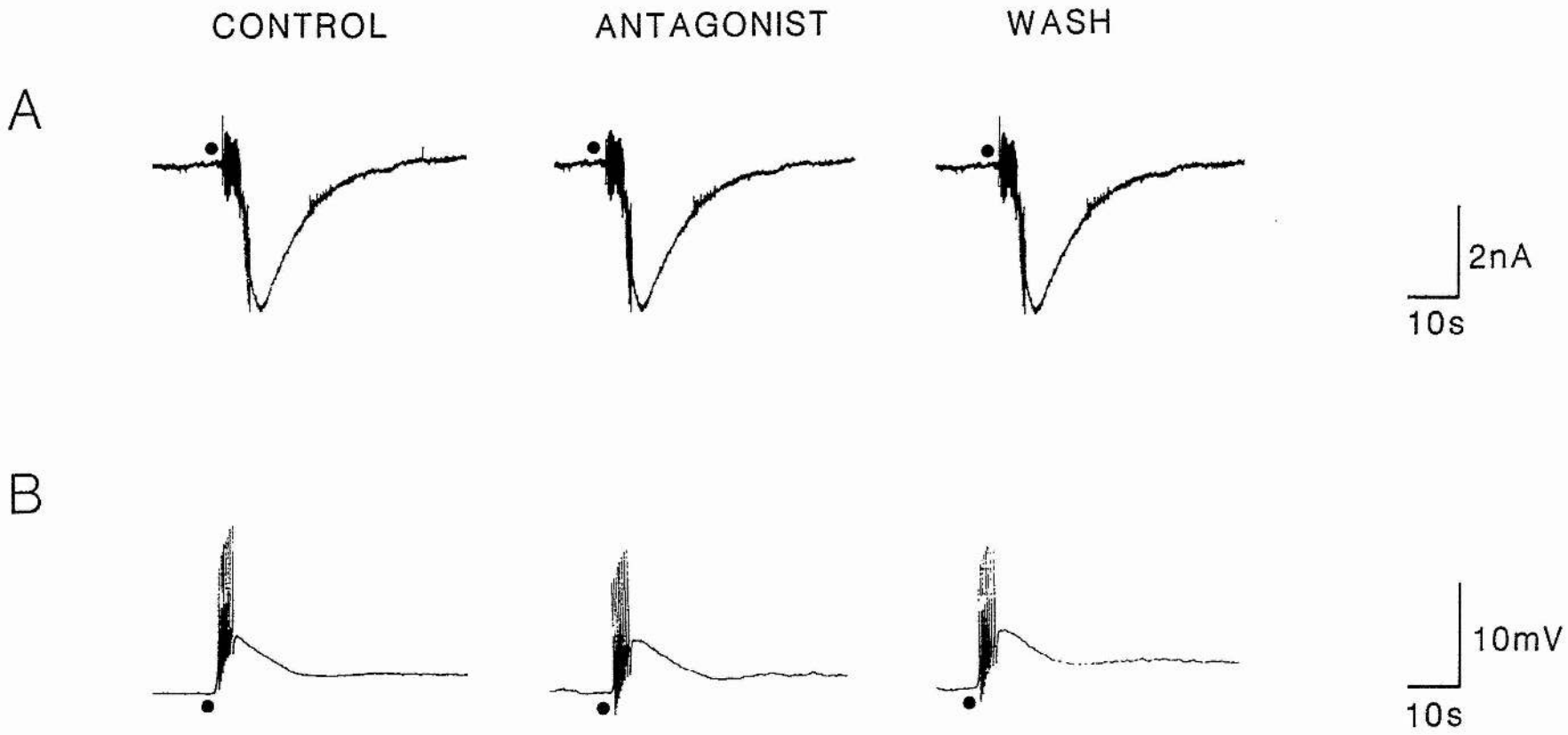

CONTROL
ANTAGONIST
WASH
A
B
5nA
10s
10mV
10s

FIGURE 2.20

Responses of identified neurone, E4, within the visceral ganglion to 5-HT in the presence and absence of 1μM MDL 72222 under **A** voltage-clamp conditions and **B** current-clamp conditions. In **A** the holding potential of the cell was -75mV. In **B** the cell was hyperpolarised from its resting potential by 15mV. The resting potential of the cell was approximately -60mV. Iontophoretic application (200nA for 2s) of 5-HT is indicated by filled circles. In **A** and **B** three responses to 5-HT are shown. The first is a control response of the cell to 5-HT, the second is a response, in the same cell, to 5-HT in the presence of 1μM MDL 72222 while the last is the response to 5-HT after a wash period. Continuous perfusion was maintained throughout the experiments.


CONTROL
ANTAGONIST
WASH
A
2nA
10s
B
10mV
10s

DISCUSSION

5-HT response for identified cells

The response seen with iontophoretic application of 5-HT onto the five identified cells (E1-E5) in the visceral ganglion of *Helix aspersa* corresponded well to the A response described by Gerschenfeld and Paupardin-Tristsch (1974a). In their investigation, the A response was rapidly depolarizing, with the potential returning to its initial level in about 20 seconds. The A response showed desensitization to successive iontophoretic applications of 5-HT and was blocked by tubocurarine. In the present study, iontophoretic application of 5-HT under voltage-clamp conditions gave an inward current which rapidly densensitized following repeated 5-HT applications: under current-clamp conditions a depolarizing excitatory response was observed. The membrane resistance of neurones, E1-E5, was reduced, with iontophoretic application of 5-HT, evoking an increase in membrane conductance. The observed reversal potential was similar to that found by Gerschenfeld *et al.* (1981) and provided further evidence that an increase of conductance to sodium ions was mediating the response seen with 5-HT. Further experiments to determine the ionic dependence of the 5-HT response under both voltage- and current-clamp conditions were not undertaken.

Block of the 5-HT response in identified neurones by tubocurarine

Tubocurarine at a concentration of 100μM reversibly antagonised the response to 5-HT in the identified cells of the visceral ganglion. Non-specific blocking actions have been shown for tubocurarine in a number of preparations. Stefani and Gerschenfeld (1969), for example, showed that D-tubocurarine reversibly blocked a fast depolarizing response of ACh and a fast depolarizing response of 5-HT, in central neurones of the snail *Cryptomphallus aspersa*. Gerschenfeld and Paupardin-Tritsch (1974a) further showed that D-tubocurarine blocked fast depolarizing and fast hyperpolarising responses of 5-HT both in *Helix* and *Aplysia* neurones. Yavari *et al.* (1979) found that D-tubocurarine antagonized 5-HT, ACh, dopamine and glutamate fast depolarizing responses in a dose-related manner. These non-specific actions of D-tubocurarine which resulted from the interaction of this antagonist and ionic channels in nerve membranes for sodium and chloride

ions was suggested by Carpenter *et al.* (1977). The interaction of drugs and ionophores has been demonstrated for a number of antagonists in nerve-cell membranes. Kehoe (1972a) showed that tetraethylammonium selectively blocked potassium channels in *Aplysia* neurones thereby preventing cholinergically- or dopamine-induced inward or outward currents. It is possible, therefore, that tubocurarine, in this instance, is achieving its blockade of the 5-HT response by a non-specific interaction with the ionic channels which contribute to the inward current seen in response to iontophoretic application of 5-HT.

Second 5-HT response observed in E1 and E2 neurones

This slower 5-HT response which showed no desensitization and no block with tubocurarine was indicative of the A' response seen in *Helix* neurones by Gerschenfeld and Paupardin-Tritsch (1974a). Pellmar and Wilson (1977) analysed some *Aplysia* neurones showing both A and A' responses using the voltage-clamp technique. Their data showed an unconventional long excitatory response to 5-HT. The response at hyperpolarized potentials was simply an increase in sodium conductance, whereas at more depolarized potentials the current was enhanced and accompanied by an apparent decrease in membrane conductance. This unusual component has now been shown to be due to 5-HT activation of a voltage-sensitive calcium conductance (Pellmar and Carpenter, 1979, 1980). This finding would account for the fact that Bokisch and Walker (1986) found that in neurones E1 and E2, the 5-HT excitatory response involved an increase both in sodium and calcium conductance. It was only in these two identified neurones that the slower 5-HT response was observed.

Characterization of 5-HT receptor on identified neurones

Since the description of six pharmacologically distinct 5-HT-induced responses in *Helix* and *Aplysia* neurones by Gerschenfeld and Paupardin-Tritsch (1974a), there has been little further data on the characterization of the four distinct 5-HT receptors which were postulated to exist in these molluscan neurones by Gerschenfeld and Paupardin-Tritsch (1974a). This is not surprising because the pharmacological tools used in the study by Gerschenfeld and Paupardin-Tritsch (1974a) to define what they suggested as

four distinct 5-HT receptors are crude by todays standards. The concentrations that were utilized in their study bear no resemblance to the concentrations of the high affinity 5-HT receptor antagonists that have been used recently to characterize the 5-HT receptor types in vertebrates. For example, there is evidence to suggest that tubocurarine has a non-specific effect by blocking ion channels rather than interacting with specific receptors (Yavari *et al.*, 1979) and neostigmine is widely used as an anti-cholinesterase. Rather than the four distinct 5-HT receptors that were suggested to exist on *Helix* neurones (Gerschenfeld and Paupardin-Tritsch, 1974a), it seems more probable that the authors were describing various components of a complex phasic physiological response elicited by 5-HT in these molluscan neurones.

From work by Bokisch *et al.* (1983) on *Helix* neurones it was postulated that the 5-HT excitatory type A receptor was similar to the mammalian 5-HT_2 receptor. However the agonists, MK 212 and RU 24969, that Bokisch *et al.* (1983) used to characterize the *Helix* excitatory receptor have been shown not to be as specific as was originally thought. MK 212 has been proved to be unselective for 5-HT_2 receptors and RU 24969 has equal selectivity for 5-HT_1 and 5-HT_2 receptors. In the present study, 1μM ketanserin lacked antagonistic activity towards the 5-HT excitatory response seen in the identified neurones in the visceral ganglion and higher concentrations of ketanserin were not tested. This lack of antagonism for the depolarizing 5-HT response by ketanserin would appear to indicate that the 5-HT receptor on *Helix* neurones does not fall into the vertebrate 5-HT_2 receptor type. In contrast, looking at the actions of the specific 5-HT_2 agonist, α-Me-5-HT, it would appear more likely than the 5-HT receptor on *Helix* neurones could be of the 5-HT_2 type. However from the structure-activity study on the excitatory receptor for 5-HT in *Helix* neurones performed by Woodruff and Walker (1972), α–methyl analogues of 5-HT were found to produce responses similar to 5-HT although the compounds were slightly less potent. This observation was in agreement with that found by Greenberg (1960) for *Mercenaria* heart, where the α-methyl analogue of 5-HT was very similar in action to 5-HT. This is also mirrored by the finding in this study that α-methyl-5-HT proved to be only 3 times less potent than 5-HT on the isolated *Helix* heart. Yet for the heart preparation, ketanserin also lacked antagonistic effect to 5-HT. It is therefore concluded that the lack of antagonist action by ketanserin indicates that a 5-HT_2 receptor

is not present on the neurones and that the agonist activity shown by α-Me-5-HT could be attributed to its affinity for *Helix* 5-HT receptor by virtue of a property, as yet unknown.

None the less the results seen with the 5-HT receptor agonists are confusing: both 5-CT and sumatriptan, which are 5-HT_1 receptor agonists, also elicited responses similar to, if not the same as, 5-HT. 5-CT certainly appeared to be more potent than sumatriptan whose responses from the same cell were always smaller than those of 5-HT.

Difficulties with iontophoretic application of agonists

The technique of iontophoresis is well established, but it necessitates a relating of the effects on neuronal activity to the current passed and to the time for which it is applied rather than to the quantity of the substance released from the electrode. Inevitably, therefore, the technique is more qualitative than quantitative. Bradley and Candy (1970) found that in the range normally used in the iontophoretic study of these compounds, the release of [^{3}H]ACh and [^{3}H]5-HT was directly proportional to the electrical charge passed. However, Kelly (1975), in his review of the principles of microiontophoretic application of drugs, concluded that the actual amount of material released by a particular current varied from microelectrode to microelectrode and from substance to substance in a quite unpredictable way. Although the effects of this variability could normally be overcome, it made the interpretation of apparently negative results extremely difficult. Nejit *et al.* (1988) suggested that in order to apply known concentrations of agonists, which was impossible with standard iontophoresis techniques, a superfusion system for whole cells be used. In their study they utilised a continuous flow system which, for adjustable periods, could be switched to solutions containing agonists or antagonists by a servo-motor operated valve. This flow could then be applied to the cell in question from a capillary of 1mm diameter located at approximately 1mm from the cell. Such a system was not employed in the present study. Nevertheless despite the qualitative nature of the results, conventional iontophoresis proved adequate for investigating the 5-HT receptor on identified *Helix* neurones.

A possible 5-HT_3 receptor

Due to the above-noted similarities with the 5-HT responses in cultured neuroblastoma cell lines and in other tissues, it was deduced that the receptor on identified *Helix* neurones mediating the A response (as described by Gerschenfeld and Paupardin-Tritsch 1974a), might be of the 5-HT_3 type. Three specific 5-HT_3 receptor antagonists, ondansetron (at 0.1, 1 and 10μM), MDL 72222 (at 1μM) and ICS 205-930 (at 1μM) were all tested against the 5-HT response in the identified *Helix* neurones. None of these compounds, at these concentrations, displayed antagonistic action towards the 5-HT response. Hales *et al.* (1988) found that 1nM ondansetron reversibly reduced whole cell currents evoked by 5-HT NCB-20 neuroblastoma hybrid cells. In NIE-115 neuroblastoma cells 0.1nM ICS-205-930 reduced the maximum evoked response to 5-HT by about 50% (Nejit *et al.*, 1988). Thus it appeared that the 5-HT_3 receptors present in these neuroblastoma cell lines are sensitive to low concentrations of these specific antagonists which show high affinity for this receptor type. Even in neurones of guinea pig submucous plexus, where there is thought to be a 5-HT_3 receptor, the effect of 5-HT was blocked by ICS 205-930 (10-500nM) and ondansetron (10-1000nM) (Derkach *et al.*, 1989). This indicates a lower affinity of these antagonists at this 5-HT_3 receptor, but a block was still observed by both compounds at 1μM. Therefore a 1μM concentration of the specific 5-HT_3 antagonists was considered sufficient to cause a block of the 5-HT response in identified *Helix* cells if a 5-HT_3 receptor were present.

Richardson and Engel (1986) proposed the existence of three subtypes of 5-HT_3 receptors termed 5-HT_{3A}, 5-HT_{3B} and 5-HT_{3C}. Their evidence for this subclassification stemmed from substantial differences in the affinities of certain antagonists for the 5-HT_3 receptors present on i)sympathetic ganglion cell somata and primary visceral afferents (3A subtype), ii)the terminals of postganglionic sympathetic neurones in the rabbit heart (3B subtype), and iii)enteric neurones of the guinea pig ileum (3C subtype). However, recent evidence suggested that species, rather than tissue, differences might at least in part have explained the reported heterogeneity of 5-HT_3 receptors (Butler *et al.*, 1990). Burridge *et al.* (1989) and Lattimer *et al.* (1989) have demonstrated that several 5-HT_3 receptor antagonists display lower affinities in guinea pig vagus nerve compared to rat vagus nerve. Furthermore, antagonist affinities

in guinea pig vagus agreed well with those that have been determined in the ileum from the same species; this suggested a common receptor type in the two tissues.

Despite the evidence from vertebrates that there could be a species difference in the 5-HT_3 receptor, it is unlikely that in *Helix* a 5-HT_3 receptor exists because of the inability of the specific 5-HT_3 receptor antagonists to inhibit the depolarizing response to 5-HT. This hypothesis, however, cannot be totally discounted: it could be postulated that the *Helix* neuronal 5-HT receptor might be a ligand-gated ion channel as the 5-HT_3 receptor has been found to be in vertebrates (Derkach *et al.*, 1989), but that it does not show the same recognition sites as the vertebrate 5-HT_3 receptor. In this way the response to 5-HT in *Helix* neurones could show many similarities, as indeed it does, with the 5-HT response mediated by 5-HT_3 receptors without being susceptible to inhibition from the specific 5-HT_3 receptor agonists and antagonists. This hypothesis would assist also in interpreting the blockade of the 5-HT depolarizing response by tubocurarine. It has been argued by Yavari *et al.* (1979) that tubocurarine exerts its blocking effect by non-specific interaction with ion channels not receptors. This is the most probable action of tubocurarine in *Helix* neurones; it exerts its blockade of the 5-HT response by blocking the ion channel, which is gated by 5-HT. This gating of the ion channel by 5-HT can be achieved in one of two ways — either the receptor is directly linked to an ion channel, like that of 5-HT_3 receptors (Derkach *et al.*, 1989) or the receptor is indirectly linked to the channel via cAMP which itself causes opening of the channel. The latter hypothesis is the more probable of the two as there is a difference in the timing of the 5-HT response — the 5-HT response in *Helix* neurones is much slower, being in the region of 20-30 seconds, whereas the 5-HT response mediated by the 5-HT_3 receptor-channel complex is considerably faster, being in the millisecond range.

A possible 5-HT_1 receptor

Bokisch *et al.* (1983) concluded that the excitatory type A 5-HT receptor on *Helix* neurones was similar to the mammalian 5-HT_2 receptor, with respect to the agonists but not the antagonistic properties of the compounds tested. RU24969, which at the time was thought to be a potent 5-HT_1 receptor agonist, was tested and had no clear agonist action although it did antagonize

the excitatory effects of 5-HT. In the present investigation other specific 5-HT_1 receptor agonists and antagonists were tested. Methiothepin, a 5-HT_1 receptor antagonist, failed to block any 5-HT response at a concentration of 1μM. Both 5-HT_1 receptor agonists (5-CT and sumatriptan) proved to be potent agonists, although from the electrophysiological results presented here a comparison of potency values is difficult. 5-CT elicited responses similar in time course and size to 5-HT whereas the responses to sumatriptan were of similar time course but smaller than those of 5-HT. It appeared, from this evidence, that 5-CT and sumatriptan were acting at the same receptor site as 5-HT.

Effect of methysergide

The LSD derivative, methysergide, at a concentration of 1μM, had no effect on the response elicited by 5-HT in identified *Helix* neurones. Having been found to be an agonist on the 5-HT receptor in the *Helix* heart and PRM methysergide was predicted to have a similar effect on the neurones. However in the present study, the action of the ergot and LSD derivatives on *Helix* heart failed to provide conclusive evidence that these compounds were exerting their effects through the 5-HT receptor. The high degree of agonism shown by these compounds on *Helix* heart and PRM tissue suggests the possibility of a separate "ergot" receptor which when activated shows similar effects to 5-HT. The lack of response of methysergide on the identified visceral ganglion neurones indicates a possible difference in the affinity of the neuronal membrane of *Helix* to the LSD and ergot derivatives.

Conclusion

In the present study, no specific 5-HT receptor antagonist was found to block the excitatory 5-HT response, thought to be the A response described by Gerschenfeld and Paupardin-Tritsch (1974a). This lack of action for any of the tested 5-HT receptor antagonists made characterization difficult. If the excitatory 5-HT receptor on *Helix* neurones was of the 5-HT_2 type as suggested by Bokisch *et al.* (1983), it would more than likely be linked to phosphatidlyinositol turnover as is the case for vertebrate 5-HT_2 receptors. This intracellular mechanism has yet to be linked to 5-HT receptors in molluscan ganglia. It is cyclic nucleotides, such as cAMP, that have been implicated in the mechanism of action of 5-HT at many sites in invertebrates.

The binding studies by Drummond *et al.* (1980a,b) provided evidence that the 5-HT receptors in *Aplysia* and *Helix* nervous tissue were linked to adenylate cyclase. Cyclic AMP appeared to mimic the action of 5-HT in identified neurones in *Aplysia* (Pellmar, 1981). In a number of *Helix* neurones, 5-HT induced an inward current which was associated with a decrease in potassium conductance (Deterre *et al.*, 1982). This response could also be induced by cAMP.

Also confusing the characterization of the neuronal 5-HT receptor in *Helix* are the results with the different specific 5-HT agonists. 5-CT and sumatriptan, both 5-HT_1 receptor agonists, elicited responses similar to 5-HT in the identified neurones. Thus in terms of agonist pharmacology, the 5-HT receptor would appear to have characteristics of both 5-HT_1 and 5-HT_2 receptors whereas for antagonist pharmacology no characterization can be made. Despite the lack of antagonism by the 5-HT_3 receptor antagonists, it is interesting to speculate that neuronal 5-HT receptors in *Helix* could be ion channels as has been shown for 5-HT_3 receptors in vertebrates. The similarities between the *Helix* neuronal 5-HT depolarizing response, under investigation, and the 5-HT response mediated by 5-HT_3 receptors in vertebrates are extensive and would appear to indicate that the ionic channels mediated by 5-HT, in each case, are the same. However the recognition sites of *Helix* neuronal receptors and vertebrate 5-HT_3 receptors must differ in structure because of the difference in affinity of the two receptors to the specific 5-HT receptor agonists and antagonists.

The present data thus imply that the 5-HT receptor on the identified *Helix* neurones is unique in that it cannot be either characterized or classified into any of the presently acknowledged vertebrate 5-HT receptor categories. In order to evaluate this hypothesis, further investigation is necessary.

CHAPTER THREE

THE 5-HT RECEPTOR ON *HELIX* HEART

INTRODUCTION

Primary studies of the action of 5-HT on molluscan hearts

The stimulatory action of 5-HT on molluscan hearts was first described by Erspamer and Ghiretti (1951). They demonstrated that enteramine, so called because of its derivation from enterochromaffin cells of the gastrointestinal mucosa (and later identified as 5-HT (Erspamer and Asero, 1952)), provoked a marked increase in the amplitude and frequency of systolic contractions of both *Octopus* and *Helix* hearts, whether isolated or *in situ*. The tonus of the hearts also rose conspicuously and with larger doses of 5-HT a persistent systolic arrest could be seen. The stimulant action extended also over the pulsatile arteries such those of the kidney and gills, with reinforcement of their contractions or renewal of their rhythmic activity if this had disappeared. The hearts of *Murex trunculus, Murex brandaris, Dolium galea* and *Aplysia limacina* were, however, found to be less sensitive than either *Helix* or *Octopus* hearts

At about the same time Welsh (1953) found that the heart of *Mercenaria mercenaria* was stimulated by low concentrations of 5-HT. The *Mercenaria* heart was believed to be singly innervated by inhibitory neurones which were thought to release acetylcholine (ACh), the most likely candidate for such an inhibitory transmitter. Antagonists of ACh, such as mytolon, were applied to the clam heart. These antagonists inhibited the ACh receptors and on stimulation of the visceral ganglion the frequency and amplitude of heart beat was increased markedly. This was strong evidence for double neuronal innervation of the clam heart. When adrenaline and noradrenaline were applied to the clam heart they were found to excite the organ, but only in high concentrations: both substances caused an increase in the rate of the heart (Welsh, 1953). Other excitatory drugs tested on the heart included certain ergot alkaloids, which were found to act at lower concentrations. The excitation produced by 5-HT mimicked closely the excitatory effects induced by indirect stimulation of the ganglion controlling the clam heart which had previously been treated with ACh inhibitors. Further studies by Welsh (1954) included

the use of several agents, such as LSD and the ergot alkaloids, that inhibited 5-HT; when these were applied to the heart the excitatory function of the nerves innervating the heart was abolished. Welsh considered this strong circumstantial evidence that a neurotransmitter substance was produced to excite the clam heart and that this substance was 5-HT.

An extensive study was carried out by Greenberg (1960 a,b) on *Mercenaria* heart. Hearts exposed to high concentrations of 5-HT or other tryptamine analogues for long periods became tachyphylactic to low doses of these substances. However high doses of 5-HT (> 20μM) still excited the tachyphylactic heart but the response was like that of the catecholamines.

Greenberg (1960b) and Bertaccini and Zamboni (1961) tested a number of tryptamine analogues on the isolated hearts of *Mercenaria* and *Helix* respectively. Of interest in Greenberg's study was the potency of LSD which was found to have an exceedingly low threshold concentration on the *Mercenaria* heart. Greenberg suggested that the LSD molecule must bind tightly to the 5-HT receptor to account for this high potency and that this gave an indication of the most likely conformation of 5-HT for maximum activity. It was an interesting observation that LSD was so potent on molluscan heart tissue. The interaction between LSD and 5-HT had previously been shown on the rat uterus where it was in fact an antagonist, giving a pA_2 valus of 8.7 (Gaddum, 1953).

Development of molluscan hearts as bioassays

Since the studies of Welsh (1953, 1954), the heart of *Mercenaria mercenaria* has been considered one of the most suitable preparations for the bioassay of 5-HT. The response of this heart showed, like that of *Helix* and *Octopus*, three components: an increase in amplitude and frequency of heart rate and an increase in the resting tone of the muscle. The relative importance of these components varied with the 5-HT concentrations. At low to moderate concentrations (1nM-1μM), a positive inotropic effect was dominant whereas at high concentrations (>10μM) a large increase in muscle tone was seen. Because of its sensitivity to low concentrations of 5-HT it was the *Mercenaria* heart that was first used as a bioassay to demonstrate the presence of 5-HT in mammalian urine and tissues (Twarog and Page, 1953).

Gaddum and Paasonen (1955) investigated the action of 5-HT on a range of molluscan hearts in an attempt to establish a convenient bioassay for 5-HT which involved molluscs living either in Britain or in its coastal waters. This had the advantage for workers in Britain in being more easily available than *Mercenaria mercenaria*, whose native habitat is the coastline of the U.S.A. The heart of *Solen siliqua* was stimulated by $1\mu g.l^{-1}$ 5-HT, and also by LSD and ergometrine. 5-HT ($1\mu g.l^{-1}$) also excited the hearts of *Cyprina islandica, Mya arenaria, Helix aspersa, Helix pomatia* and *Spisula solida* (Gaddum and Paasonen, 1955). LSD, ergometrine, dihydroergotamine and hydergine all increased the amplitude of the *Helix aspersa* heart beat, but could not be shown to block 5-HT. LSD and 6-methygramine failed to block the action of 5-HT on the heart of *Helix pomatia*.

Innervation of molluscan hearts

Most molluscan hearts were thought to receive both inhibitory and excitatory innervation, usually via the visceral ganglion and nerve. The extrinsic nerve supply of *Helix pomatia* was described by Ripplinger (1957). It was shown to consist of two branches which originated in the visceral nerve. One branch entered the heart at the pulmonary vein-atrium junction and the second joined at the ventricle-aorta junction. Later Ripplinger and Ripplinger (1975) showed that the inhibitory innervation in *Helix* terminated in the AV region. Cottrell and Osborne (1969) described a dense network of nerve fibres in the AV junction of the heart of *Helix pomatia*. Their results, both with light and electron microscopy, suggested that this system had a neurosecretory function. By means of a fluorescent histochemical technique they demonstrated the presence of 5-HT and/or one of the primary catecholamines within the nerve fibres of the *Helix pomatia* heart.

Attempt to characterize the 5-HT receptor present on the *Helix* heart

Evidence from extensive studies on vertebrate central 5-HT receptors suggested that there was more than one type of 5-HT receptor as discussed previously. As 5-HT had such potent actions on molluscan hearts which indicated the presence of a specific 5-HT receptor, an attempt to classify this receptor in terms of 5-HT_1 or 5-HT_2 was made by Boyd *et al.* (1985). The actions of a number of agonists and antagonists were studied on the 5-HT

receptor present within the heart muscle cells of *Helix aspersa*. They concluded from the agonist studies that the 5-HT receptor was possibly of the 5-HT_1-like type as RU24969 had appeared to be more 5-HT_1-like than MK212. This was supported by the finding that ketanserin, a potent 5-HT_2 receptor antagonist, was only a weak antagonist of this 5-HT receptor. However, they were unable to clearly separate the *Helix* heart excitatory 5-HT response into either the 5-HT_1-like or 5-HT_2 type of receptor.

Involvement of cAMP in the action of 5-HT on molluscan hearts

Additional evidence that implicated cAMP as an intracellular mediator of 5-HT-induced excitation was presented by Higgins (1974). 5-HT (1μM) increased adenylate cyclase activity and intracellular cAMP levels in the ventricular muscles of the bivalves *Mercenaria mercenaria* and *Macrocallista nimbosa*, but had no effect on cGMP. A positive correlation between the excitatory action of 5-HT and the simultaneous increase in intracellular cAMP, suggested that this nucleotide mediated the excitatory action of 5-HT. Cyclic AMP, dibutyryl cAMP and phosphodiesterase inhibitors had no effect on the mechanical activity of isolated bivalve hearts but methysergide (1μM) antagonised the action of 5-HT on the *Mercenaria* heart.

Higgins and Greenberg (1974) investigated the effects of cAMP, cAMP-induced phosphorylation and 5-HT on calcium uptake by bivalve heart microsomes. They found that the cAMP-mediated phosphorylation of microsomal protein augmented calcium uptake, but 5-HT and cAMP also directly induced a similar increase. Their study suggested that cAMP could mediate 5-HT action by either the activation of a cAMP-dependent protein kinase or by direct modification of the amount of membrane-associated calcium. Wolleman and S-Rózsa (1975), showed that 5-HT (1μM) stimulated the activity of adenylate cyclase prepared from heart tissue of *Anodonta* and *Helix*. Higgins (1977) found that a high concentration of 5-HT specifically desensitized the isolated ventricle of *Mercenaria* and that this 5-HT-induced tachyphylaxis was paralleled by a 5-HT-specific desensitization of the myocardial adenylate cyclases and a decrease in cAMP.

Hess *et al.* (1981) studied the effects of theophylline and IBMX on the contractility, cAMP content and phosphodiesterase activity of *M. mercenaria*

ventricle. They found that both IBMX and theophylline were relatively poor inhibitors of *Mercenaria* myocardial cAMP-dependent phosphodiesterase activity, and that this accounted for the inability of these agents to increase either myocardial intracellular cAMP levels or contractility. Later Drummond *et al.* (1985) reported the stimulation of adenylate cyclase in the heart of *Aplysia californica* by 5-HT and forskolin, a plant diterpene which had been found to have a stimulating action on membrane-bound adenylate cyclase in a variety of mammalian tissues. From their experiments it was clear that 5-HT stimulated enzyme activity in the *Aplysia* heart by a receptor-mediated process which was antagonised by 5-HT receptor blockers. 5-HT-stimulated enzyme activity was also antagonised by low calcium ion concentration and they suggested that this divalent cation exerted regulatory properties on the enzyme. Adenylate cyclase activity was augmented by forskolin. At the same time Paciotti and Higgins (1985) were studying the potentiation of the 5-HT-induced increases in myocardial contractility in *Mercenaria* ventricle by forskolin. They found that forskolin (10μM) increased the myocardial mechanical response and intracellular cAMP at all doses of 5-HT. However, forskolin alone significantly elevated cAMP levels but did not affect contractility.

Cardioactivity not associated with 5-HT in molluscan hearts

Throughout the 1960s, cardioactivity not associated with the neurotransmitters 5-HT, ACh and the catecholamines was detected in extracts of molluscan ganglia (Greenberg *et al.*, 1973). Frontali *et al.* (1967) had previously used gel filtration to resolve this activity into five cardioexcitatory peaks. The number of peaks varied with the species and three of the peaks, designated A, B and C, from the ganglia of *Mercenaria* were shown to be peptides.

By 1980 only peak C from the pooled ganglia of the sunray clam, *Macrocallistra nimbosa*, had been fully purified, identified and synthesized. It proved to be a tetrapeptide with a blocked carboxyl terminal, Phe-Met-Arg-Phe-NH_2, which subsequently became known as FMRFamide (Price and Greenberg, 1977). Higgins *et al.* (1978) tested both FMRFamide and 5-HT on the ventricle of the bivalve *Mercenaria mercenaria*: both agents increased myocardial contractility, intracellular cAMP concentration of intact hearts and

adenylate cyclase of a myocardial membrane fraction. In the bivalve, they found FMRFamide was 5-10 times more potent than 5-HT. All the effects of 5-HT, but none of those of FMRFamide, were blocked by methysergide. This effect of methysergide proved that FMRFamide and 5-HT activated separate receptors, but that they shared the same common intracellular mechanism involving adenylate cyclase and cAMP. Moreover, Higgins (1977) had shown that *Mercenaria* hearts made tachyphylactic to 5-HT could yet respond to FMRFamide, showing increased contractile force, raised levels of cAMP and increased adenylate activity. In *Helix* heart, FMRFamide was found to increase the myocardial contractility, although it proved to be only a weak agonist compared to 5-HT (Greenberg and Price, 1980).

FMRFamide itself appears now to be ubiquitious among molluscs. In some gastropods, however, related peptides also are known. In particular, pulmonate ganglia were found to have activity not attributable to FMRFamide. In *Helix aspersa*, the ganglia contained not only FMRFamide but also related peptides distinguishable by their chromatographic behaviour and biological activity (Cottrell *et al.*, 1981). Price *et al.* (1985) identified a novel FMRFamide-like peptide in *Helix* ganglia, pQDPFLRFamide, which was found to be 100 times more potent than FMRFamide on the isolated *Helix* heart: this suggested it acts as a cardioregulatory hormone.

Evidence for other transmitter action in molluscan hearts

From evidence in vertebrates, ACh acting on nicotinic receptors on the cardiac sympathetic nerves in the isolated rabbit heart was shown to be excitatory causing an increase both in atrial and ventricular tension. It was with this preparation that some 5-HT-like agonists were observed to have a dual mechanism of stimulant action which involved not only receptors sensitive to 5-HT but also nicotinic receptors (Fozard and Mobarok Ali, 1978). The action of ACh on the cardiac nerves in vertebrates was in contrast to its action on invertebrate hearts, particularly molluscan hearts. ACh had been found to be the most potent compound for inhibition of the heart in molluscs (Prosser, 1940). Welsh (1943) had used the heart of *Mercenaria* as a bioassay for the quantitative estimation of ACh in tissue extracts.

It had been suggested also that catecholamines might act as neurotransmitters in molluscs. Catecholamines were certainly found to be present in the nervous system of some molluscs (Osborne and Cottrell, 1970), and were usually excitatory on hearts, causing increases both in amplitude and rate of beat. Noradrenaline was generally much less active than dopamine on the hearts, with adrenaline being virtually inactive. Thus, receptors for the catecholamines are clearly present on molluscan hearts but whether they fall into the same categories as those described for vertebrates remains to be seen. Evidence for a possible interaction between 5-HT and noradrenaline on isolated vertebrate hearts emanated from a study on the isolated rabbit heart, in which 5-HT caused noradrenaline release from noradrenergic nerve terminals by a receptor mechanism (Fozard and Mwaluko, 1976).

The final compound thought to have possible action on the *Helix* heart is histamine. By far the most convincing evidence for a transmitter role for histamine in invertebrates was in the gastropod CNS. Histamine had a powerful action on *Helix* neurones and this action was antagonised by the H_1 antagonist mepyramine (Kerkut *et al.*, 1968): histamine excited some cells while inhibiting others. Studies attempting to classify the histamine receptors into H_1 and H_2 on *Helix aspersa* neurones have been undertaken (Caine, 1978). The data showed that there were differences between the histamine receptors of different neurones and so there was difficulty in classifying them in terms of H_1 and H_2. Evidence for histamine receptors on the *Helix* heart is decidedly lacking but their presence in the heart and the cardiovascular system in vertebrates suggested the possibility of their presence on invertebrate hearts.

Dependence of molluscan heart on calcium ions

It has long been known that the isolated superfused ventricle of *Helix aspersa* is dependent on external calcium for force generation in isometric contractions induced by AC stimulation (Burton and Mackay, 1970). Intracellularly recorded action potentials from molluscan hearts have a typical cardiac form, in that a slowly rising prepotential appears to trigger a spike which gives way to a prolonged plateau during repolarisation (Irisawa *et al.*, 1967; Kiss and S-Rózsa, 1973). The resting potential in *Helix pomatia* ventricular muscle cells was dependent mainly upon external potassium, but

was diminished by the sodium diffusion potential, as shown by a 20% hyperpolarisation in the absence of external sodium. The action potentials of the ventricular cells were abolished in zero external sodium or in zero external calcium substitutions being made by Tris and sucrose respectively. Thus both sodium and calcium participated in the generation of the *Helix* action potential. A relationship between plateau duration and force of contraction was found to be evident in molluscs which showed this type of cardiac action potential. The higher the plateau, the larger the force of contraction. 5-HT was found to increase the plateau height (Hill, 1974a; Hill and Yantorno, 1979). Thus it would appear that calcium was intimately involved in the generation of the cardiac action potential and could contribute to the excitatory effect seen with 5-HT in molluscan hearts.

Aims

From the wealth of evidence presented here, it is apparent that 5-HT has a potent cardioactive effect on molluscan hearts. From this evidence, 5-HT is presumed to be the excitatory transmitter, particularly on *Mercenaria* and *Helix* hearts. The aim of this study was to attempt to characterize the excitatory 5-HT receptor, present on the heart of *Helix aspersa*, into one of the existing 5-HT receptor types already identified in vertebrates. This has been achieved by using a standard organ bath technique and isolated *Helix* hearts. The method involved monitoring the positive inotropic effect of bath-applied 5-HT. In this way the effects of the specific 5-HT agonists and antagonists could also be clearly monitored.

From evidence already given, it would appear that cAMP was involved in the effect of 5-HT in molluscan hearts. It was therefore decided to test this hypothesis by measuring the effect of 5-HT on cAMP levels in *Helix* heart tissue. In order to establish that 5-HT was not causing the release of other supposed transmitters by stimulation of receptors other than those of 5-HT, a series of suitable antagonists were tested against the action of 5-HT in the *Helix* heart. The action of the novel molluscan cardioactive peptides was observed so that a comparison could be made not only of the utility of the *Helix* heart as a preparation but also of the methods here employed as opposed to those employed in other studies. Though the main aim of this study was to characterise the 5-HT receptor mediating the inotropic effect of 5-HT on the

isolated *Helix* heart, other factors were taken into consideration. These included looking at the method of inactivation of 5-HT in the isolated heart and looking at the possible role played by calcium in the inotropic effect of 5-HT in the preparation.

METHODS AND MATERIALS

Animals

Helix aspersa were purchased from Blades Biological. They were kept in a humid environment at room temperature in tanks enclosed with mesh wire and fed with lettuce at regular intervals. All snails used in this study were kept in the active state for at least one week before use.

Dissection

With the aid of sharp scissors, the snail's shell was totally removed by cutting round the spiral of the shell. The whole snail was then pinned by the head and mantle in a wax-bottomed dissecting dish with the head facing forwards. The heart is located in one of the grooves of the spiral and could easily be identified from (i) the darker colouration which delineated the heart area and (ii) the vessels leading to and from the heart, which like the heart itself, were outlined by darker colouration. Fig. 3.1 shows the position of the heart in *Helix*. The pericardium was carefully cut open to avoid damage either to the heart or to the nephridium, which lies to the left hand side. Both perfused and non-perfused heart methods were assessed before a decision was made as to which one was to be used for the experiments. These two methods will be described separately below.

Perfused heart method

In this method, once the heart had been exposed, a small hole was cut in the atrium and a fine glass cannula inserted into the atrium and through to the ventricle. Using thread, the cannula was tied off as near to the AV junction as possible. The cannula was then lifted out of the animal and the rest of the atrium cut away so as to free the atrium end of the heart. A small wire hook was then attached to the aorta and the aorta cut distal to the insertion of the hook, thus freeing the whole ventricle. The ventricle was then mounted via the cannula onto a closed loop system originally devised by Payza (1987), but modified by Lesser (1990). The experimental apparatus is shown in Fig. 3.2. The hook in the aorta was threaded to a force transducer (L.V.D.T. type SM1 Sangano transducer) which acted via a transducer amplifier onto a JJ two channel CR452 type pen recorder. The heart was

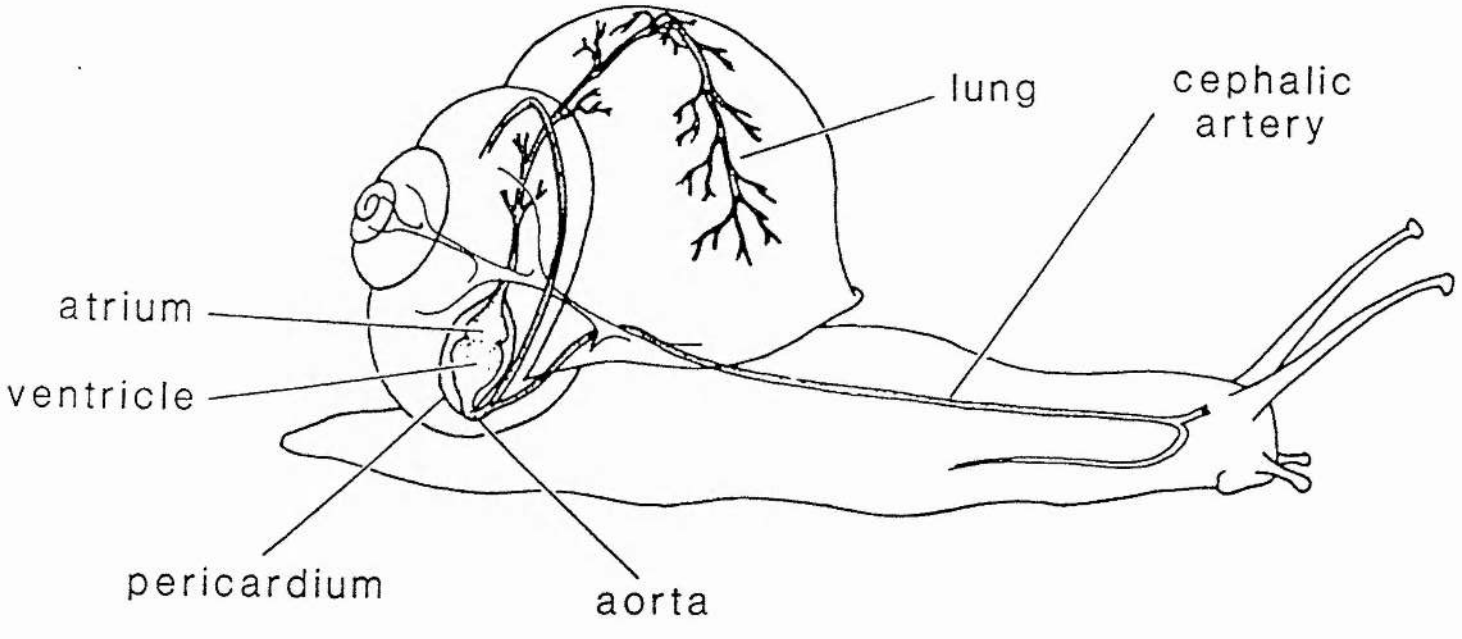


FIGURE 3.1

Diagram of the snail, *Helix aspersa*, to show the position of the heart and its associated major blood vessels. The drawing was modified from Leake and Walker (1980).

perfused with physiological saline (80mM NaCl, 5mM KCl, 7mM $CaCl_2$, 5mM $MgCl_2$, 20mM Hepes; pH 7.5) which flowed out of the aorta to bathe the external surface of the heart. This waste was collected in a beaker beneath the cannulated heart. The flow rate of saline was adjusted by two clips to provide a rate of 1-2 ml per minute: this varied from animal to animal, dependent on the intrinsic heart beat of each animal. In this way the heart preparations were spontaneously active and could remain beating for several hours. After setting up the heart, it was perfused for 30 minutes. to allow it to equilibrate and to wash away any free proteins or enzymes. Aliquots of 5-HT solutions of known concentration, made up in physiological saline, were applied with a needle and syringe, via an injection cap. The response to a single dose of 5-HT was measured as the percentage increase in myocardial contractile force above the control amplitude. 5-HT was the only drug to be tested in this method over the range 1nM-100μM. This method was not used for the other experiments, the reasons for which are discussed later

Non-perfused heart method

Once the heart had been exposed, a piece of thread was placed underneath the AV node and the junction tied off, leaving a small thread loop. The atrium was then cut away. A small wire hook was placed in the area where the aorta joins the ventricle and while the ventricle was still attached to the animal. Once the hook was firmly in place the aorta was cut distal to the hook's point of insertion. The isolated ventricle was then mounted in a 10ml organ bath suspended between an L-shaped stainless steel wire support and the small wire hook, which was attached to a Dynamometer (UF1) isometric transducer. This transducer was in turn connected to a Lectromed 8-channel pen recorder. The experimental apparatus is shown in Fig. 3.3. As with the perfused hearts, the response to 5-HT was measured by the increase in myocardial contractile force above the control amplitude and expressed as a percentage relative to the maximum response achieved with 5-HT in that preparation. The preparations were maintained at room temperature (18-20^{0}C) in a gassed (95% 0_2 5% Co_2) physiological saline (80mM NaCl, 5mM KCl, 5mM $MgCl_2$, 7mM $CaCl_2$, 20mM Hepes; pH 7.5). Once again, when set up, the hearts were spontaneously active and remained beating for several hours. Initially, a series of force-tension curves were devised for the hearts in order to indicate the optimum resting tension of the isolated hearts.

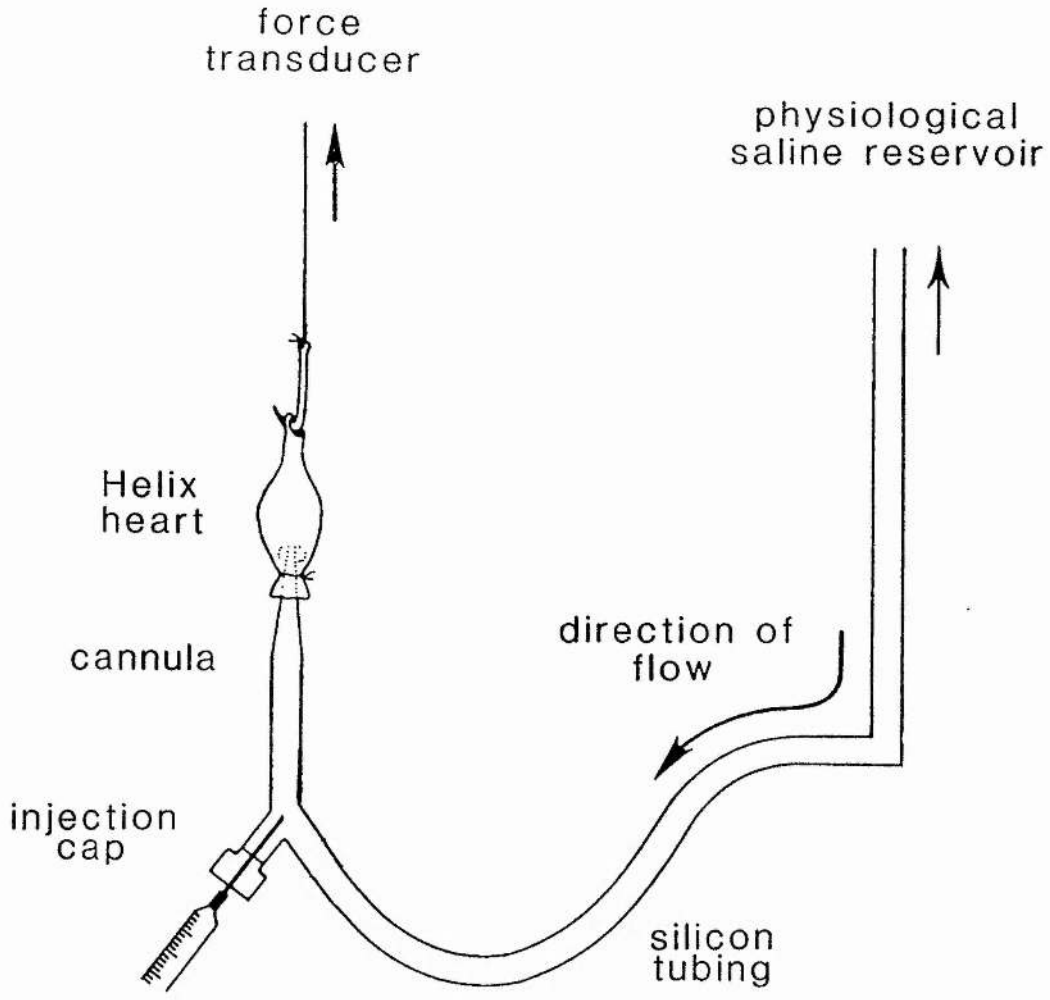


FIGURE 3.2

Diagram of the experimental apparatus used in the perfused heart method. The physiological saline reservoir was kept above the height of the heart with the level of saline constantly monitored in an attempt to keep the pressure constant. The silicon tubing was clamped at various points to control the flow of saline to the perfused snail heart. The saline overflowed at the tip of the ventricle to bathe the outside of the heart. The cannula, inserted into the ventricle of the snail heart was attached to a small Y- shaped tube which was joined in turn to the silicon tubing. A small injection cap on the Y-shaped tube allowed for the injection of known quantities of drugs. The heart was kept in place and its movement monitored by a small wire hook positioned in the ventricle-aorta region. This in turn was connected to a transducer via a thread loop. Any movement monitored by the transducer was amplified and recorded on a pen recorder.

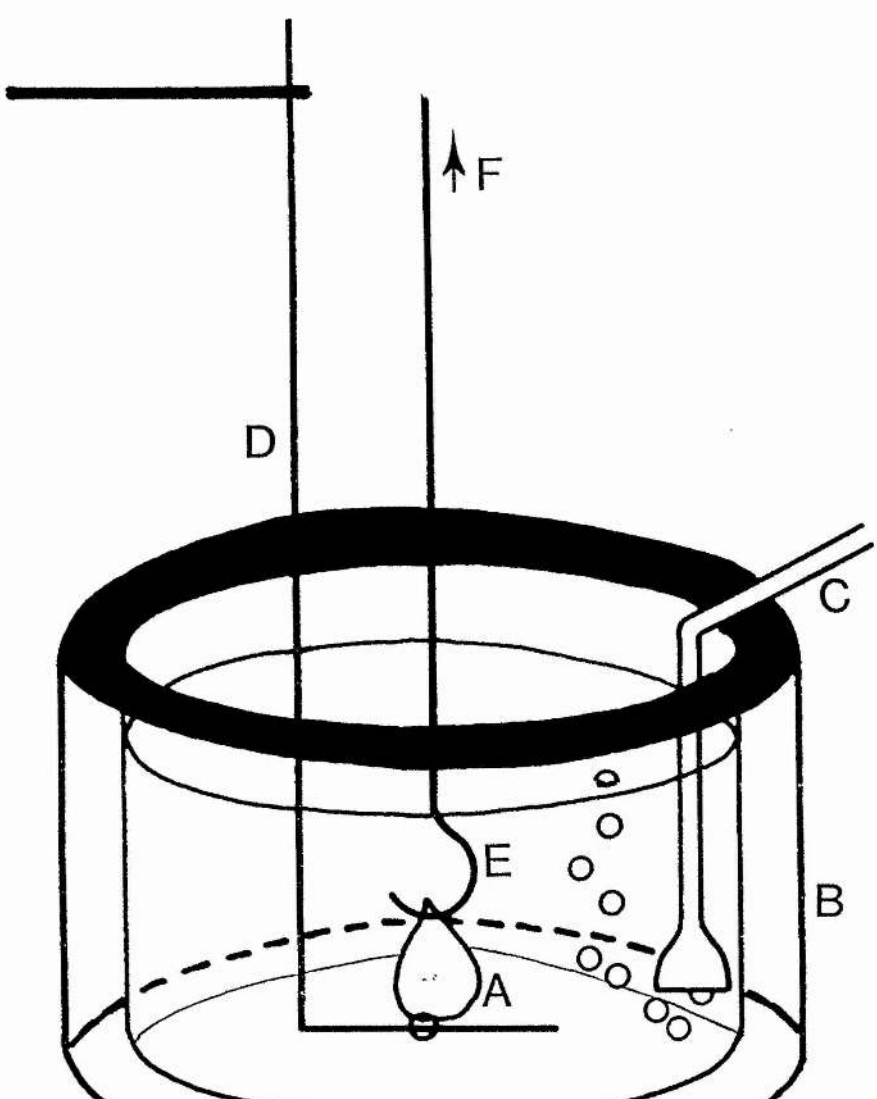


FIGURE 3.3

Diagram of the experimental apparatus used in the non-perfused heart method. The isolated ventricle (A) was mounted in a 10ml jacketed organ bath (B) in physiological saline at room temperature aerated by a 95% O_2, 5% CO_2 mixture (C). The heart was fixed between an L-shaped stainless steel wire support (D) which was kept rigid in a clamp present above the bath. A wire hook (E) was attached to a transducer (F). The transducer in turn, was connected to an 8-channel pen recorder.

This was achieved by increasing the tension on the heart by stepwise increase of the amount of stretch on the heart. This value could be read off the chart recorder which was fully calibrated daily. With each increase in tension a submaximal concentration of 5-HT (1μM) was added to the bath. The response to 5-HT was seen as an increase in heart beat amplitude giving a value for force involved in the response. Between each 5-HT application, the preparation was washed to remove any residual 5-HT before the next increase in tension. The curves varied from heart to heart but a range of optimum resting tensions was obtained and used in the subsequent experiments.

After an initial 30 minute equilibration period, a cumulative concentration-effect curve to 5-HT was established. Successive concentrations from 10nM-100μM were applied to the heart. The response of the heart to increasing concentrations of 5-HT was seen as increases in heart beat amplitude. Once the response, in amplitude, to the previous concentration had reached a plateau, the subsequent concentration of 5-HT was added without washing. When two consecutive concentrations of 5-HT produced no further amplitude in heart beat, the tissues were washed with fresh physiological saline to remove the 5-HT. This cumulative concentration-effect curve to 5-HT was established in all preparations; first, to test the viability of the ventricle and its sensitivity to 5-HT and, second, to provide a control curve before the application of test agonists, antagonists and other drugs under investigation.

Determination of agonist potency

The effect of agonists on the myocardial contractile force was tested by constructing a cumulative concentation-effect curve to the agonist up to a maximum concentration of 100μM This was done once the amplitude of the heart had returned to normal after the washing period which followed the initial cumulative concentration-effect curve to 5-HT. In control preparation, the cumulative concentration-effect curve to 5-HT was repeated to assess changes in sensitivity of the tissue to 5-HT. Agonist responses were measured as the increase in myocardial contractile force above the control amplitude. Each response was expressed as a percentage of the maximal effect. From log concentration-effect curves, the concentration producing half-maximal effect (EC_{50}) was obtained. The equipotent concentration ratio (EC_R) for the test

compound relative to 5-HT (5-HT = 1) was calculated by dividing the EC_{50} value for the test compounds by the EC_{50} value for 5-HT. Where quoted the pEC_{50} value was the negative $logarithm_{10}$ of the EC_{50} value. Arithmetic mean EC_{50} values for each agonist were calculated from each individual curve. The 5-HT receptor agonists tested on the isolated heart were 5-CT, sumatriptan and 8-OH-DPAT (all 5-HT_1 receptor agonists), α-Me-5-HT (a 5-HT_2 receptor agonist), 2-Me-5-HT (a 5-HT_3 receptor agonist) and tryptamine.

Determination of antagonist potency

Likely antagonists of this myocardial contractile response were tested by examining their ability to antagonise the increase in amplitude produced by 5-HT. In all preparations, cumulative concentration-effect curves to 5-HT were obtained. After washing, all but one of the preparations (which served as a control and thus remained untreated) were exposed to a known concentration of vehicle or antagonist. After 30 minutes a cumulative concentration-effect curve to 5-HT was repeated in all preparations. If antagonistic action was established and after washing, the preparations were then exposed to increasing concentrations of the antagonists before another concentration-effect curve to 5-HT was generated. In this way, the agonist concentration-ratio could be calculated for each antagonist concentration (Apperley *et al.*, 1976). This was achieved by dividing the concentration of 5-HT required to produce a response at that point in the presence of antagonist, by that required for 5-HT in the absence of antagonist. These estimates could then be used to calculate pA_2 and slope values by linear regression analyses of Schild plots (Arunlakshana and Schild, 1959). The 5-HT receptor antagonists tested on *Helix* heart were methiothepin (a 5-HT_1 receptor antagonist), ketanserin and ritanserin (both 5-HT_2 receptor antagonists), cocaine, ondansetron and ICS 205-930 (all 5-HT_3 receptor antagonists) and finally metoclopramide (an M receptor antagonist).

Statistics

Values were expressed as the arithmetic mean ± s.e. mean of n, n being the number of experiments

Effects of Other Drugs

Other compounds which did not fall into the categories of either 5-HT agonists or antagonists were treated in the same way as the 5-HT receptor antagonists, being allowed to equilibrate in the bath for 30 minutes before the 5-HT cumulative concentration effect curve was repeated. There were three exceptions which were treated as agonists, the ergot derivatives, the molluscan cardioactive peptides and isoprenaline. A low calcium saline was also tested on the isolated heart preparation. The calcium concentration in the physiological saline was reduced from 7mM to 3mM with substitition by sucrose to maintain isotonicity. The 4mM calcium was replaced by 12mM sucrose in order to replace each of the three ions into which $CaCl_2$ dissociates in solution. These other drugs included a muscarinic blocker (atropine), a nicotinic blocker (hexamethonium), an adrenergic β blocker (propranolol), an adrenergic α blocker (phentolamine), a histamine (H_1) blocker (mepyramine), a histamine (H_2) blocker (ranitidine), the cardioactive peptides (FMRFamide and pQDPFLRFamide), a MAO inhibitor (pargyline), two 5-HT uptake inhibitors (fluvoxamine and zimelidine), a phosphodiesterase inhibitor (IBMX), a cAMP activator (forskolin), an adrenergic β agonist (isoprenaline) and finally the ergot derivatives (ergotamine, methylergometrine, LSD and methysergide). All the drugs tested and their full names and sources are listed below.

Drugs used

5-HT creatinine sulphate (Sigma), methiothepin maleate (Hoffman La Roche), phentolamine mesylate (Ciba), atropine sulphate (Sigma), mepyramine maleate (May and Baker), methysergide hydrogen maleate (Sandox), metoclopramide monohydrochloride (Sigma), ketanserin tartrate (Janssen), IBMX (Sigma), isoproterenol (isoprenaline) bitartrate (Sigma), forskolin (Calbiochem), FMRFamide and pQDPFLRFamide (Peninsula Laboratories), ergotamine tartrate (Sigma), methylergometrine hydrogen maleate (Sandoz), pargyline hydrochloride (Sigma), fluvoxamine maleate (Duphar), zimelidine hydrochloride (Astra), verapamil hydrochloride (Sigma), ritanserin tartrate (Janssen), tryptamine hydrochloride (Sigma), ICS 205-930 (Sandox), hexamethonium bromide (Koch Light Laboratories), propanolol

hydrochloride (Sigma) and D-lysergic acid diethylamine tartrate (Sandoz). Ranitidine hydrochloride, ondansetron, sumatriptan, α-Me-5-HT, 2-Me-5-HT and 8-OH-DPAT were all synthesised by the Chemistry Research Department at Glaxo Group Research.

All drugs were dissolved in physiological saline with the exception of ketanserin, ondansetron and ritanserin which were dissolved in 0.1M tartaric acid and forskolin which was dissolved in DMSO with subsequent serial dilutions, in both cases, in physiological saline. Isoprenaline was always made up with 100μM ascorbic acid to retain its stability. All drug concentrations quoted are final bath concentrations.

Measurement of cAMP formation

Hearts were dissected from the snails as previously described with as much of the atrium removed as possible, leaving only the AV node attached to the ventricle. Also at the ventricular end as much of the aorta was removed as possible. These were then incubated in gassed (95% 0_2 5% CO_2) physiological saline (80mM NaCl, 5mM KCl, 7mM $CaCl_2$, 5mM Mg Cl_2, 20mM Hepes, pH 7.5) at room temperature for two hours. This depleted any endogenous 5-HT (Wolleman and S-Rózsa, 1975) and allowed adequate recovery time from the traumatisation of surgery. Following incubation the hearts were transferred to fresh physiological saline and incubated for a further 30 minutes in normal physiological saline or physiological saline containing the cAMP phosphodiesterase inhibitor IBMX (100μM) and test antagonists, where appropriate at appropriate concentrations. The hearts were then exposed to vehicle control or 5-HT for a further two minutes, after which they were rapidly frozen in liquid nitrogen to terminate cAMP formation.

The cAMP was extracted by homogenisation of the hearts in absolute ethanol using a glass-glass tissue grinder (Jencons Scientific). The extracts were then centrifuged at 15,000g for two minutes and the supernatants contained in the ethanol removed and dried under a gentle stream of nitrogen gas. When dry, the ethanol extracts were redissolved in a buffer (pH 7.5) comprising Tris-HC1 (50mM) and EDTA (4mM). These samples were then assayed for cAMP content using a commercially available competitive binding-assay kit (Amersham International plc). The assay kit combined the

high specificity and affinity for cAMP of a highly purified and stabilised binding protein with a charcoal separation step. The assay was based on the competition between unlabelled cAMP and a fixed quantity of the tritium labelled compound for binding to a protein which has a high specificity and affinity for cAMP. The amount of labelled protein cAMP complex that had formed related inversely to the amount of unlabelled cAMP present in the assay sample. Measurement of the protein-bound radioactivity enabled the amount of unlabelled cAMP in the sample to be counted. Separation of the protein bound cAMP from the unbound nucleotide was achieved by absorption of the free nucleotide onto coated charcoal followed by centrifugation. An aliquot of the supernatant was then removed for liquid scintillation counting. The concentration of unlabelled cAMP in the sample was then determined from the linear standard curve.

The remaining extracted pellets, after centrifugation, were dissolved in sodium hydroxide (1M) for the subsequent determination of protein contents by the method of Lowry *et al.* (1951). The procedure was based on the measurement of proteins with Folin-Ciocalteu phenol reagent after alkaline copper treatment which led to the development of a colour change whose optical density could then be determined. Standard solutions of BSA ranging from 25-100$\mu g.ml^{-1}$ in 0.1M sodium hydroxide, unknown samples diluted 1:10 with distilled water to give samples in 0.1M sodium hydroxide and blanks were added to appropriately labelled tubes. 2.5ml of alkaline copper tartrate was then added to each tube, mixed and left to stand at room temperature for 10 minutes. 250µl of diluted (1:1 with distilled water) Folins reagent was added to each tube, mixed and left to stand for 30 minutes at room temperature. The optical density of each sample was then determined at 750nm on a Pye Unicam SP-6-500 UV spectrophotometer. Using a plot of protein concentration (BSA standards) versus absorbance at 750nm, a standard curve could be generated. By the use of linear regression analysis, the unknown protein concentrations could then be determined from their absorbance values. Results were expressed as pmol cAMP formed.mg^{-1} tissue protein. These values were the arithmetic mean ± s.e. mean of n, n being the number of experiments.

Histology

Several hearts were dissected out and placed in 10% buffered formalin. The slides were then prepared by the Histology Department, Glaxo Group Research. The hearts were embedded in wax before cutting longitudinal and transverse sections. A dye, Erlich's haematoxylin, was used to stain the cells. The slides were then viewed under an optical microscope with attached camera to produce photographic slides which were then developed by the photographic department at St. Andrews University.

RESULTS

Structure of the *Helix* heart

The *Helix* heart is composed of a muscular ventricle attached to a smaller, less muscular atrium (Fig. 3.4). In *Helix* there is a pair of valves between the atrium and the ventricle which are shown and clearly marked with an arrow in Fig. 3.4. However the valve situated in the aorta is not visible in this section. An asterisk marks the place where this valve should be present (Fig. 3.4). The myocardial fibres in the *Helix* heart are arranged in a complex network of branching and anastomosing trabeculae (Fig. 3.5). The muscle bundles are arranged with the loose, large trabeculae towards the lumen of the ventricle and the smaller, more closely knit trabeculae towards the exterior. This is shown in Fig. 3.5. The *Helix* heart was capable of beating spontaneously and rhythmically in isolation. Cardiac action potentials could be spontaneously initiated within the more or less unmodified myocardial cells which made up the trabeculae. In this way the *Helix* heart was said to be myogenic (see Jones, 1983). An indication of the size of the isolated heart is given by the scale bars shown on the relevant sections.

Differences observed between the two previously described methods

Two different methods have been described previously for measuring the isolated heart's response to 5-HT. The experimental apparatus for the two methods was given in Figs. 3.2 and 3.3. Several sets of results were obtained with each method before a decision was made as to which method would be more suitable. These results are demonstrated in Figs. 3.6 and 3.7 and show the traces obtained from each method. Analysis of these traces led to the log concentration-effect curves shown in Figs. 3.8 and 3.9. The method finally chosen was the non-perfused heart method due to various underlying problems with the perfused heart method.

The system used in the perfused heart method is closed (see Fig. 3.2): any change in the liquid level in the reservoir therefore changed the pressure which in turn had an effect on the rate of beating of the perfused heart. Another persistent problem was that of dead space within the tubing. It was not possible with this method to tell i) how much of the injected bolus of 5-HT

FIGURE 3.4

Photograph of a histological longitudinal section of the *Helix* heart. Note the larger, more muscular ventricle, attached to the much smaller, less muscular atrium. An arrow indicates the presence of a pair of valves between the atrium and the ventricle. The valve situated in the aorta is not so clearly delineated.in this section. An asterisk marks the area in which the valve is usually located in the whole animal. The small black dots indicate the position of the nuclei within the myocardial cells of the *Helix* heart. The scale is equivalent to 1mm.

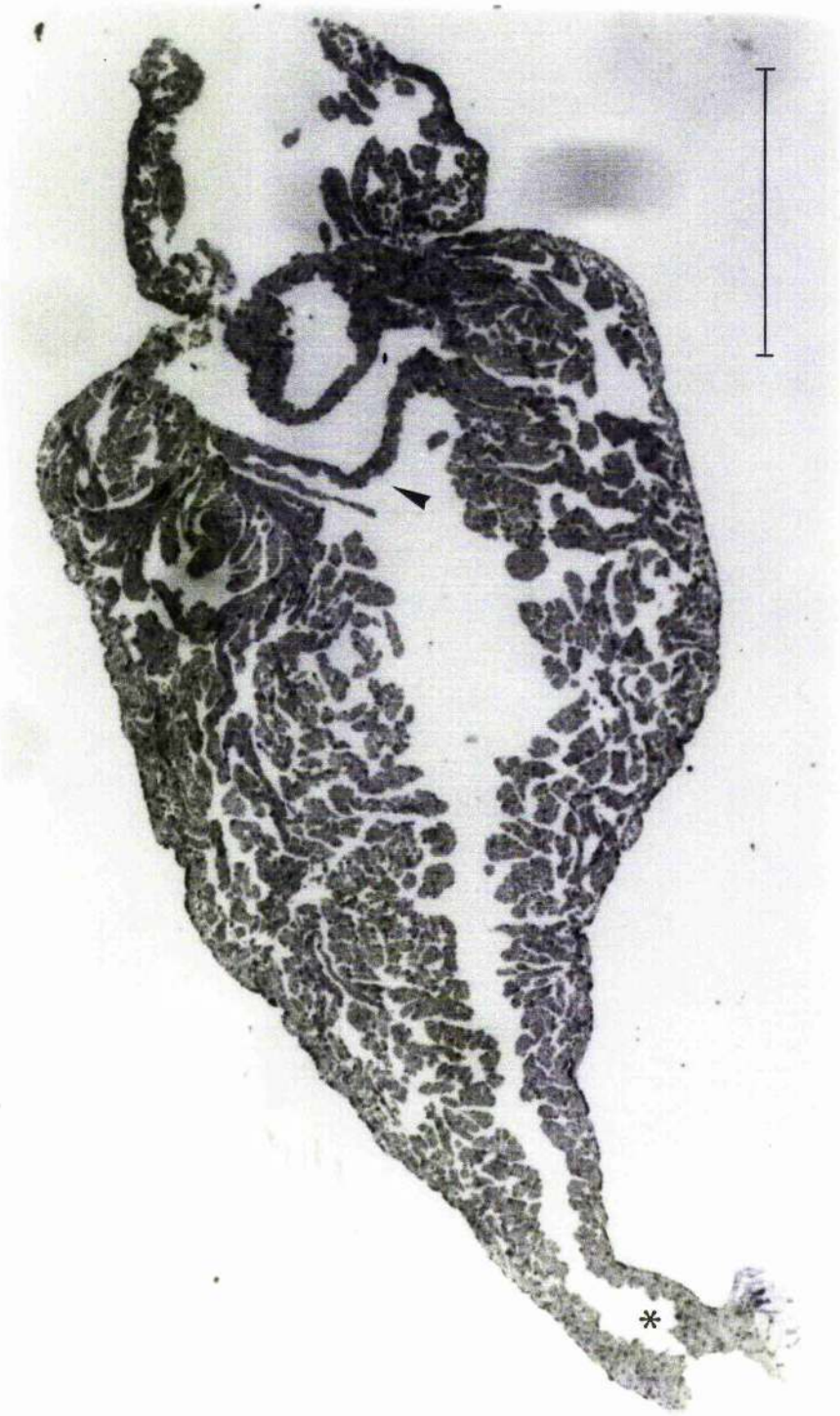
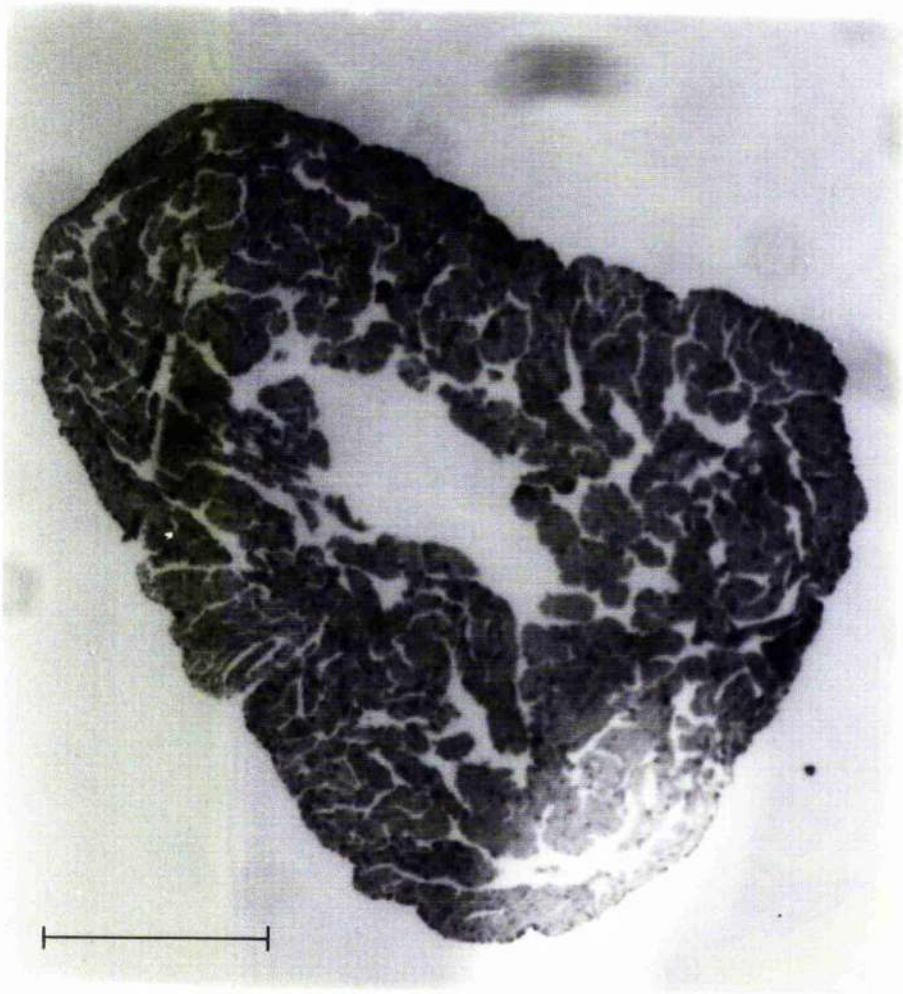


FIGURE 3.5

Photograph of a histological transverse section of *Helix* ventricle. This section clearly demonstrates the complex network of beam-like projections into which the myocardial fibres are arranged within the ventricle. The small black dots indicate the position of the nuclei in the myocardial cells of the ventricle. The scale is equivalent to 0.5mm.



reached the heart and ii) at what concentration the 5-HT reached the heart. There was also the problem concerning tachyphylaxis in the perfused heart method. This method was more sensitive to the high concentrations of 5-HT and the heart became tachyphylactic to 5-HT concentrations above 10μM; this can be seen in Figs. 3.6 and 3.9 as a lowering of the inotropic response to 5-HT within the heart. Consequently, the maximum inotropic response to 5-HT seen in this method was difficult to measure accurately and the response (Fig. 3.6) could be quantified only as a percentage increase in response above the control value (Fig. 3.9) Because the response of each heart to 5-HT varied markedly with this method, standardization of the data was impossible, leading to four different sets of results rather than just one set showing the arithmetic mean ± s.e. mean of n=4. Despite the fact that the perfused heart method showed greater sensitivity to 5-HT, with a threshold of approximately 1nM (Fig. 3.9) in contrast to a threshold of 10-30nM (Fig. 3.8) with the non-perfused method, it was the latter method that was chosen for the remainder of the experiments. The significance of this difference in sensitivity between the two methods will be discussed in detail later.

Response of *Helix* heart to 5-HT

From the recording traces in Figs. 3.6 and 3.7 it can be seen that 5-HT has potent positive inotropic effects. Positive chronotropic effects on *Helix* heart were observed only with the non-perfused heart method (Fig. 3.7). However, for experimental purposes only the inotropic effect of 5-HT was monitored because the chronotropic effects were variable and only occurred at high concentrations of 5-HT. In the non-perfused heart, 5-HT had a threshold value of between 10-30nM, reaching a maximum inotropic response at a concentration of 30μM. From Fig. 3.8 it can be seen that the 5-HT inotropic response on the isolated *Helix* heart was reproducible as indicated by the extent of overlap of the two 5-HT curves obtained consecutively with the same preparation. Even if a third 5-HT concentration-effect curve was performed on the same preparation, the curve was identical to those already obtained from that heart (result not shown here). This demonstrated not only good reproducibility of the 5-HT response on the same preparation but also indicated a lack of decrease in sensitivity of the heart tissue to 5-HT during the time course of the experiments. A control curve to 5-HT similar to that seen in Fig 3.8 was obtained for every experiment although the control 5-HT curve

FIGURE 3.6

The response of the perfused *Helix* heart to increasing concentrations of 5-HT is shown in this figure which is a trace recording obtained from one preparation. The black arrows represent the administration of 0.1ml of increasing 5-HT concentration which are as stated. The response of the perfused heart to 5-HT was not measured cumulatively and each successive concentration of 5-HT was allowed to wash out before the next concentration was administered. Note that the perfused heart exhibited tachyphylaxis at a 5-HT concentration above 10μM.

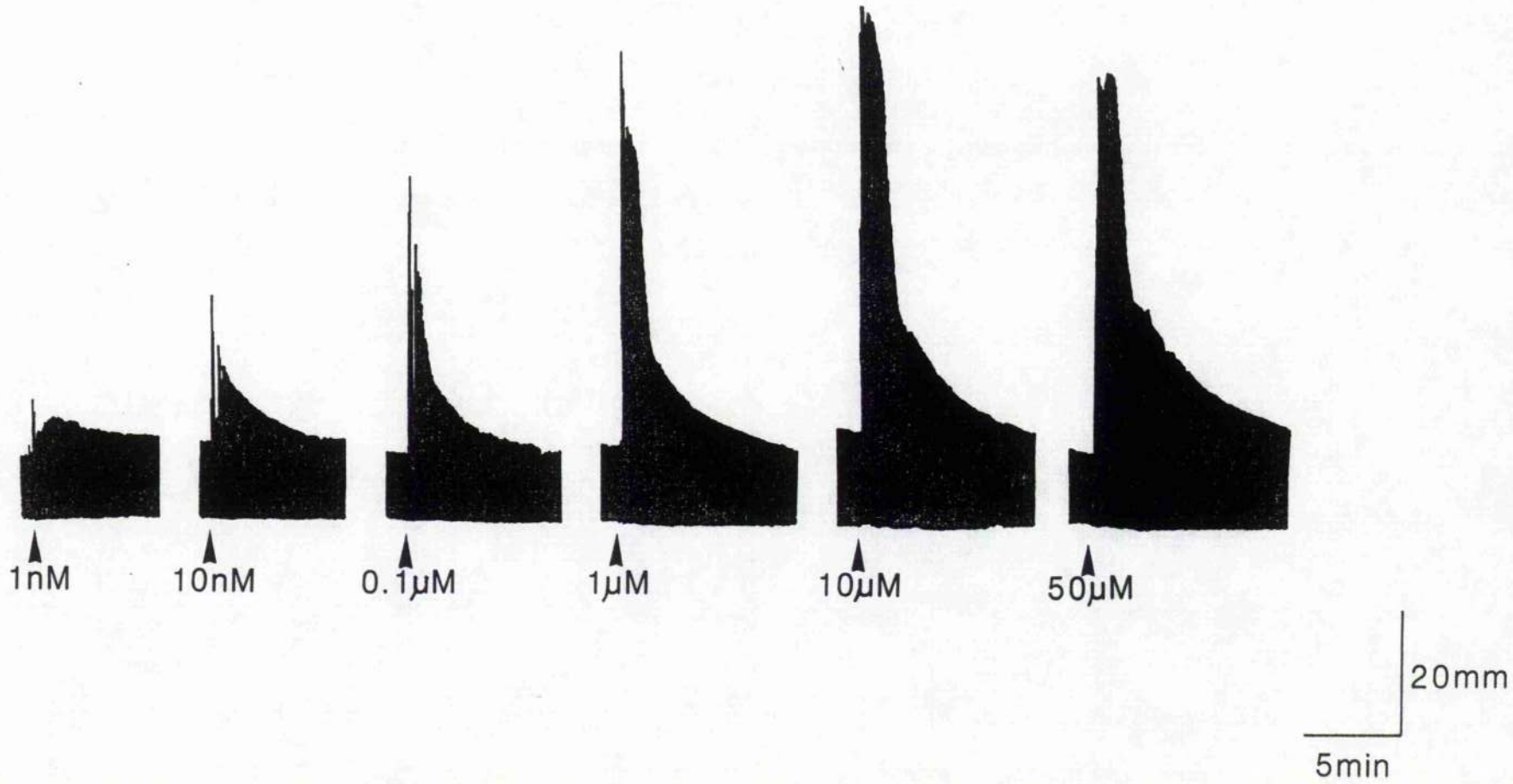
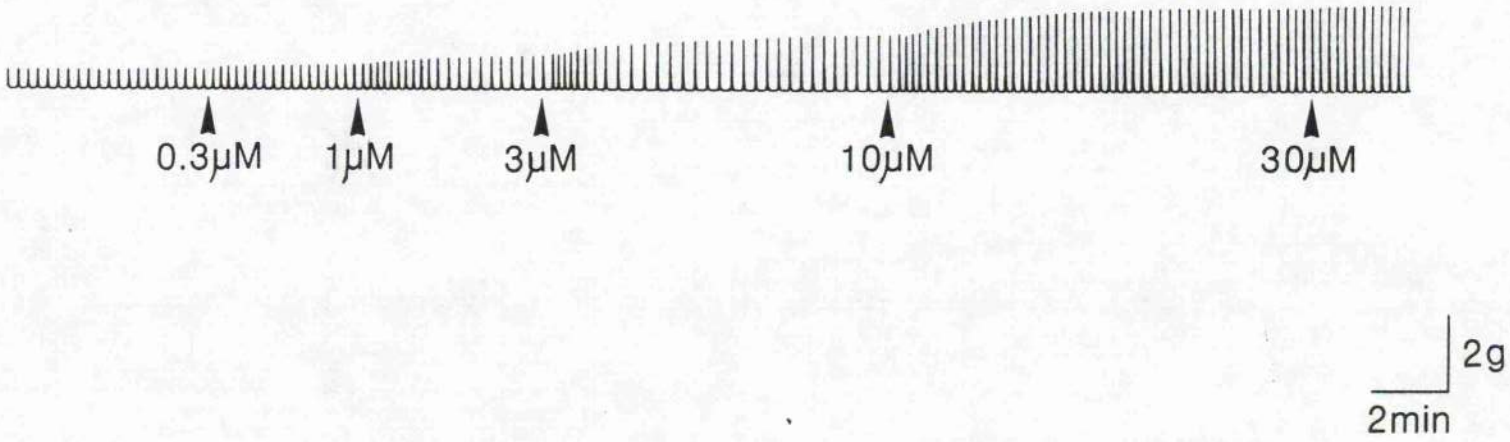

1nM
10nM
0.1μM
1μM
10μM
50μM
20mm
5min

FIGURE 3.7

The response of non-perfused *Helix* heart to increasing concentrations of 5-HT. The trace recording obtained from one preparation is shown. The black arrows indicate the addition of 5-HT to the bath. The stated 5-HT concentration being the final bath concentration to which the *Helix* heart was exposed. In this method the concentration-effect of 5-HT on the isolated snail heart was cumulative as can be seen from the trace. The scale is as shown. In this method even at a 5-HT concentration of 30μM, the *Helix* heart failed to exhibit tachyphylaxis.


0.3μM
1μM
3μM
10μM
30μM
2g
2min

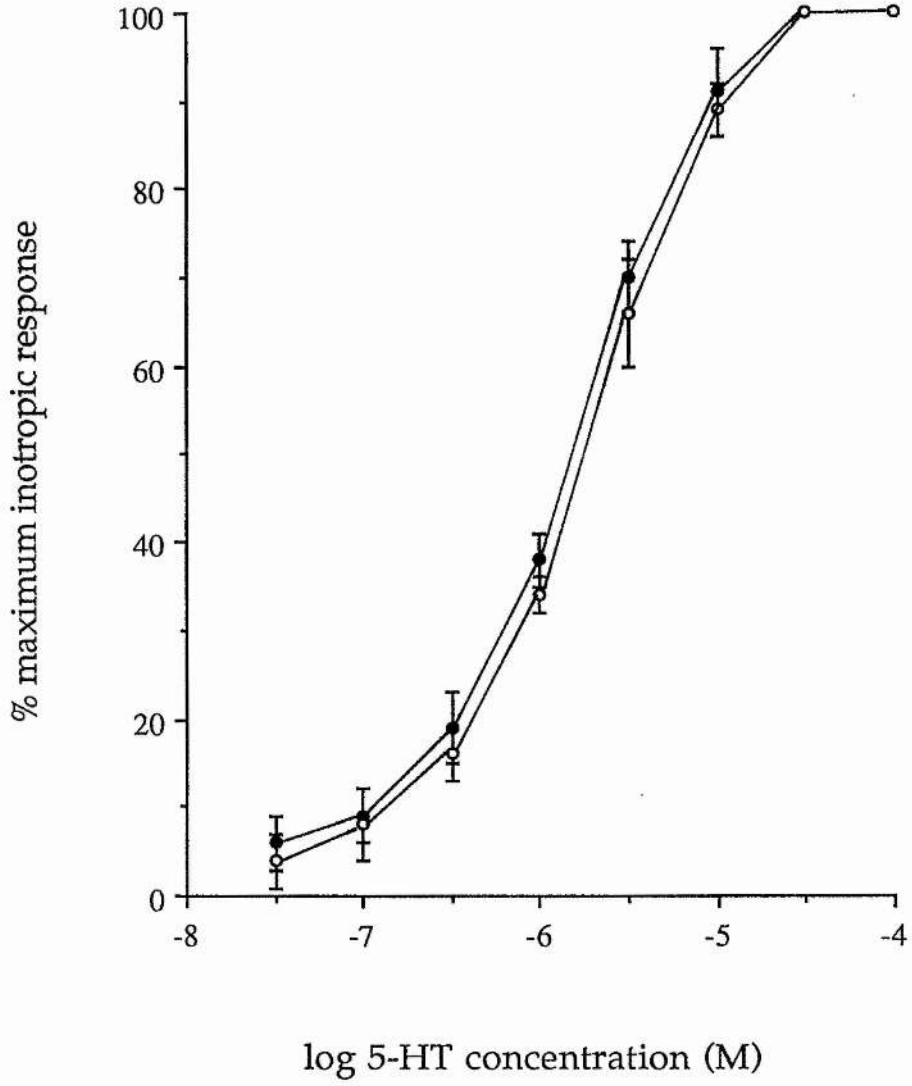


FIGURE 3.8

The concentration-effect curve for 5-HT on the isolated *Helix* heart using the non-perfused heart method is shown in this figure by closed circles. The x axis represents log of the 5-HT concentration while the y axis represents the percentage maximum inotropic response. A control concentration-effect curve for 5-HT on the same preparation, is also shown in the figure with open circles. Vertical bars represent the arithmetic means ± s.e. mean of n=22.

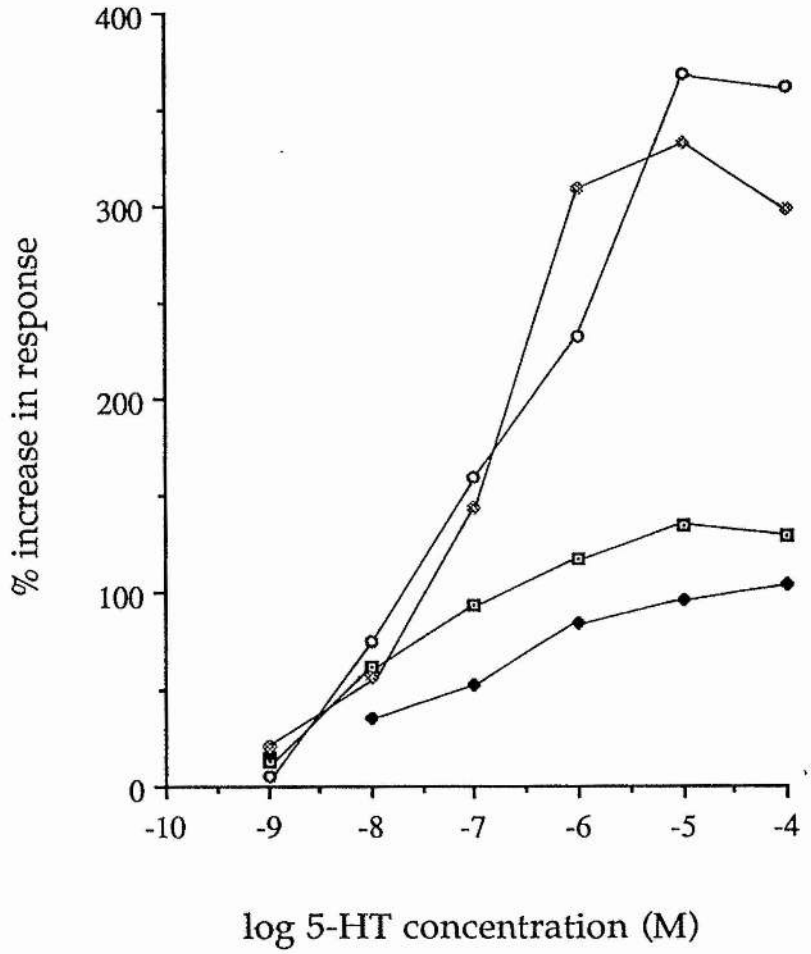


FIGURE 3.9

The concentration-effects of 5-HT on the isolated *Helix* heart using the perfused heart method are shown in this figure. The different symbols represent values obtained from n=4 experiments. The y axis gives the percentage increase in response above the control value while the x axis gives log of the 5-HT concentration. Note that at 5-HT concentrations >10μM the perfused heart exhibited tachyphylaxis.

is not shown on all the figures in order to be able to see the effect of the other drugs more clearly.

Force-tension relationship in the isolated heart

After a decision was made as to which method was to be used in investigating the 5-HT receptor present in the *Helix* heart, the force-tension relationship in the heart was investigated. Force-tension curves were obtained from four separate preparations (Fig. 3.10). A value for the optimum resting tension of *Helix* heart could then be ascertained from these curves at a point where the greatest rate of rise of force is seen for a small increase in tension. The value was found to be in the range 0.5-1g depending on the individual heart.

Effect of other transmitter antagonists on the inotropic response to 5-HT in the heart

Figure 3.11 relates to a series of experiments performed on the isolated *Helix* heart using antagonists other than those which were known to specifically block the different 5-HT receptor types. The cholinergic antagonist atropine, which blocks muscarinic receptors, at a concentration of 1μM, had no effect on the inotropic response to 5-HT in the isolated *Helix* heart. This is indicated by the extent of overlap of the two 5-HT curves, one in the absence and the other in the presence of atropine, which is shown in Fig. 3.11. The extent of overlap of the 5-HT concentration-effect curves was measured, in every case, by the dose-ratio value. Likewise these results, or lack thereof, were mimicked by the nicotinic antagonist, hexamethonium and the adrenergic antagonists propranolol and phentolamine. Neither hexamethonium, propranolol or phentolamine, at a concentration of 1μM, had any effect on the 5-HT inotropic response in *Helix* heart. Because the figures all proved to be similar to Fig. 3.11 it was decided to use Fig. 3.11 as the example and consequently none of the others are shown. Finally, the histaminergic antagonists, mepyramine and ranitidine, at a concentration of 1μM, also showed no effect on the 5-HT inotropic response in *Helix* heart. These results are summarized in Table 3.1 overpage.

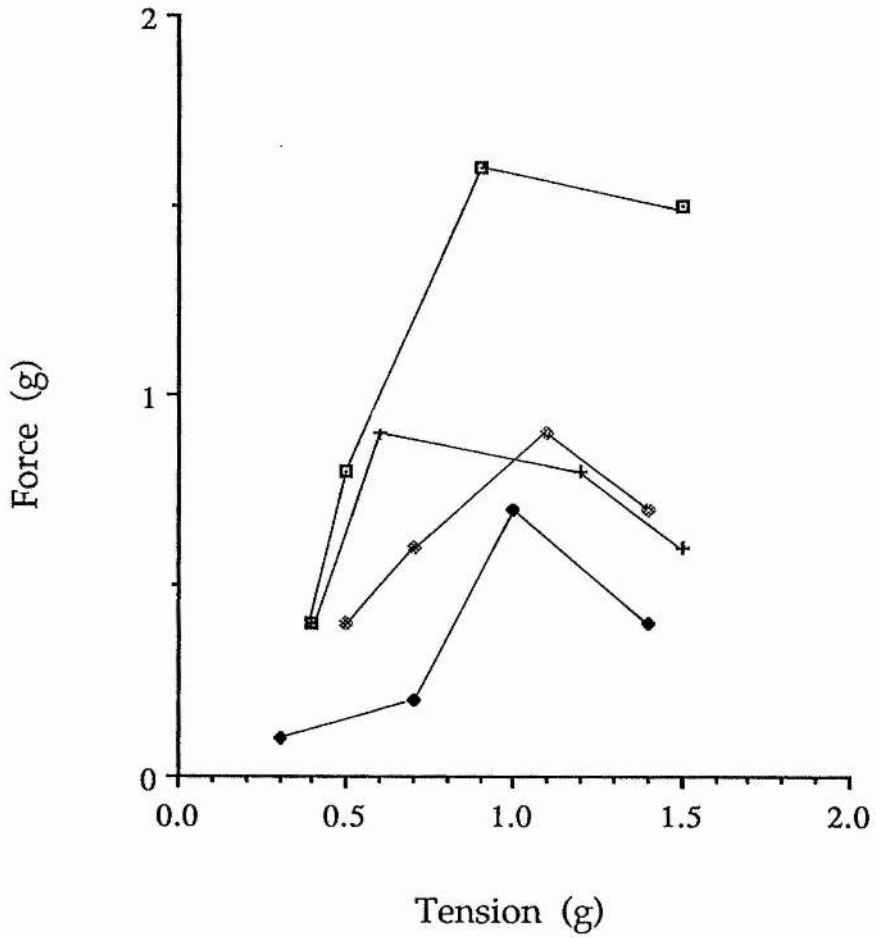


FIGURE 3.10

The relationship between the force generated by the isolated *Helix* heart in response to 5-HT and the tension applied to the heart is demonstrated in this figure which shows the results from four preparations. The tension applied to the heart is given by the x axis, while the y axis gives the force generated by the heart in response to a submaximal concentration of 5-HT. The concentration of 5-HT used was 1μM. The force-tension curves obtained from the four isolated hearts varied from animal to animal as can be seen from this figure.

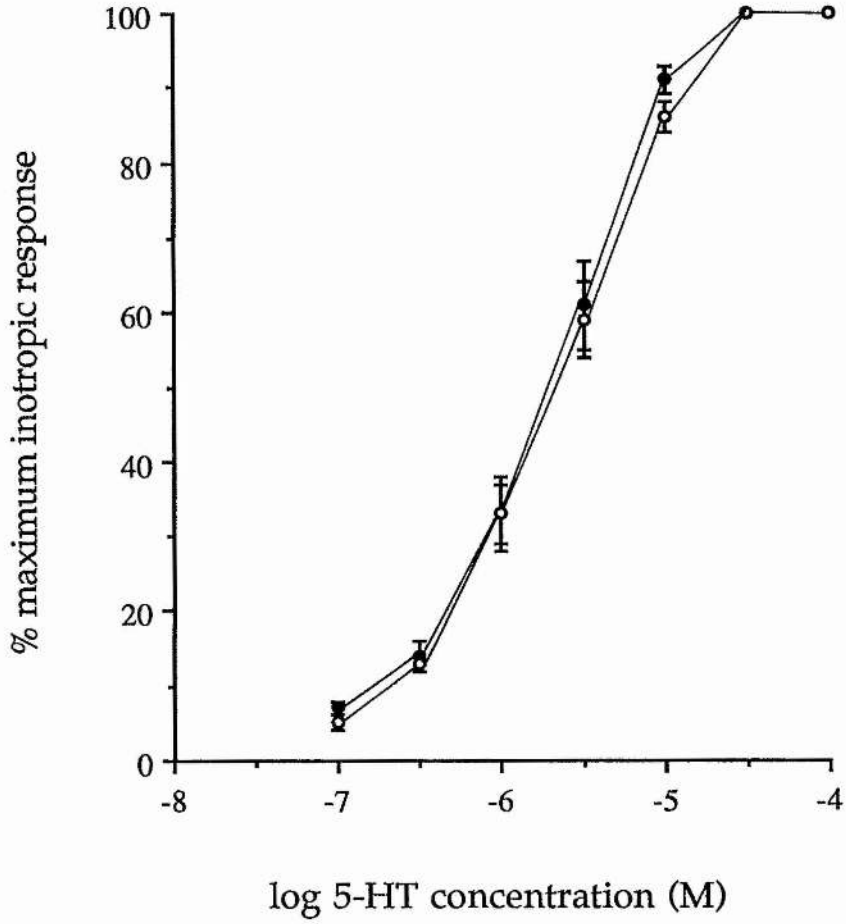


FIGURE 3.11

The concentration-effect curves for 5-HT on the isolated *Helix* heart in the absence (closed circles) and in the presence (open circles) of atropine at a concentration of 1μM are shown in this figure. The x axis gives log of the 5-HT concentration while the y axis gives the percentage maximum inotropic response. Vertical bars represent the arithmetic mean ± s.e. mean of n=6.

TABLE 3.1 Summary of the other antagonists tested on *Helix* heart.

Antagonist	Concentration tested (μM)	n	Effect
Atropine	1	6	dose-ratio = 1.0
Hexamethonium	1	6	dose-ratio = 1.0
Propranolol	1	6	dose-ratio = 1.0
Phentolamine	1	6	dose-ratio = 1.0
Mepyramine	1	6	dose-ratio = 1.0
Ranitidine	1	6	dose-ratio = 1.0

Effect of 5-HT agonists on the inotropic response of the heart

The specific 5-HT receptor agonists tested on the isolated *Helix* heart were 5-CT, sumatriptan, 8-OH-DPAT, α-Me-5-HT, 2-Me-5-HT and tryptamine. 5-HT was the most potent agonist on *Helix* heart (pEC_{50} value of 5.7). 5-CT and α-Me-5-HT both mimicked the effects of 5-HT (Fig. 3.12), but were less potent (pEC_{50} values of 4.9 and 4.7 respectively). Sumatriptan, 2-Me-5-HT, tryptamine and 8-OH-DPAT behaved like partial agonists by eliciting a maximum inotropic response, of only 66% in the case of sumatriptan (Fig. 3.13), of only 56% in the case of 2-Me-5-HT (Fig. 3.13), 54% in the case of tryptamine (Fig. 3.14) and finally of only 47% in the case of 8-OH-DPAT (Fig. 3.14). 5-CT was 6 times less potent than 5-HT on *Helix* heart. α-Me-5-HT and sumatriptan showed a potency value of approximately 9 times less potent than 5-HT. 2-Me-5-HT was 29 times less potent than 5-HT, tryptamine was 32 times less potent than 5-HT and 8-OH-DPAT being 43 times less potent than 5-HT on the isolated *Helix* heart. The above results are summarised in Table 3.2 overpage. The rank order of potency for the specific 5-HT receptor agonists was found to be 5-HT > 5-CT > α-Me-5-HT = sumatriptan > 2-Me-5-HT = tryptamine > 8-OH-DPAT.

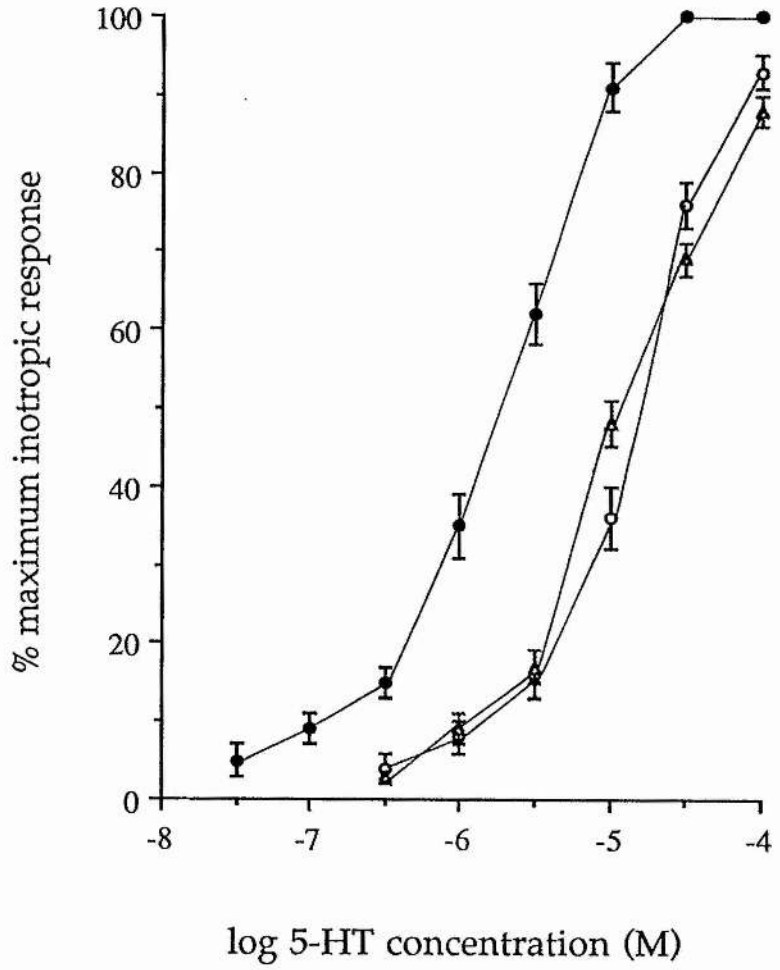


FIGURE 3.12

The concentration-effect curves for 5-HT (closed circles), 5-CT (open circles) and α-Me-5-HT (triangles) on the isolated *Helix* heart are shown in this figure. The x axis gives log of the 5-HT concentration while the y axis gives the percentage maximum inotropic response. Vertical bars represent the arithmetic mean ± s.e. mean of n=8.

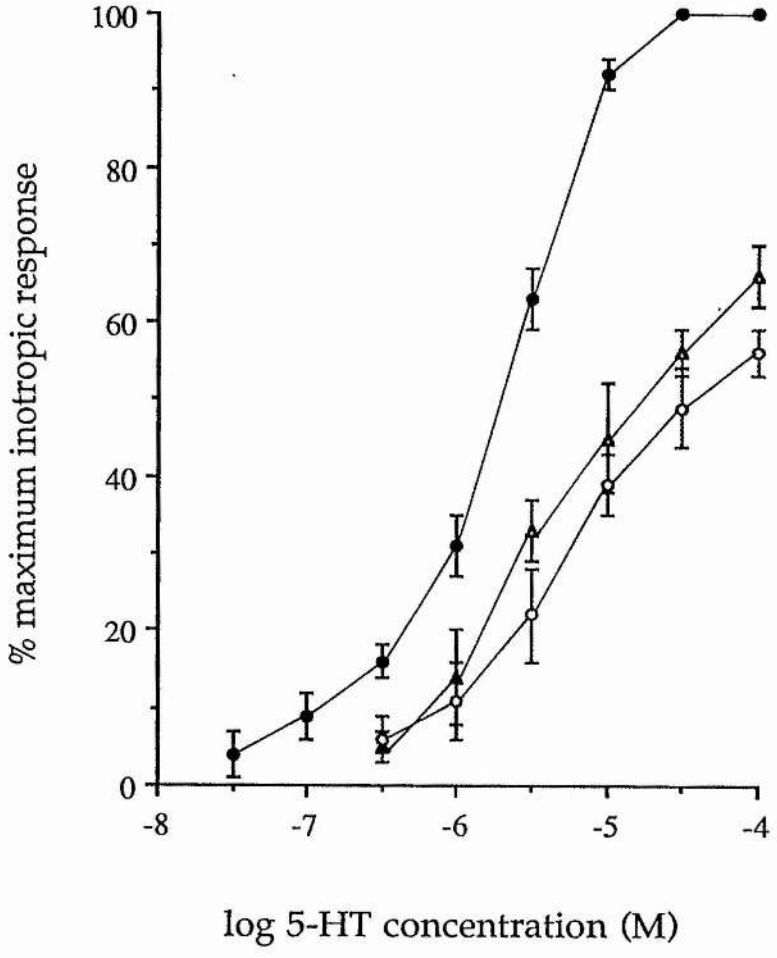


FIGURE 3.13

The concentration-effect curves for 5-HT (closed circles), 2-Me-5-HT (open circles) and sumatriptan (triangles) on the isolated *Helix* heart are shown in this figure. The x axis gives log of the 5-HT concentration while the y axis gives the percentage maximum inotropic response. Vertical bars represent the arithmetic mean ± s.e. mean of n=8.

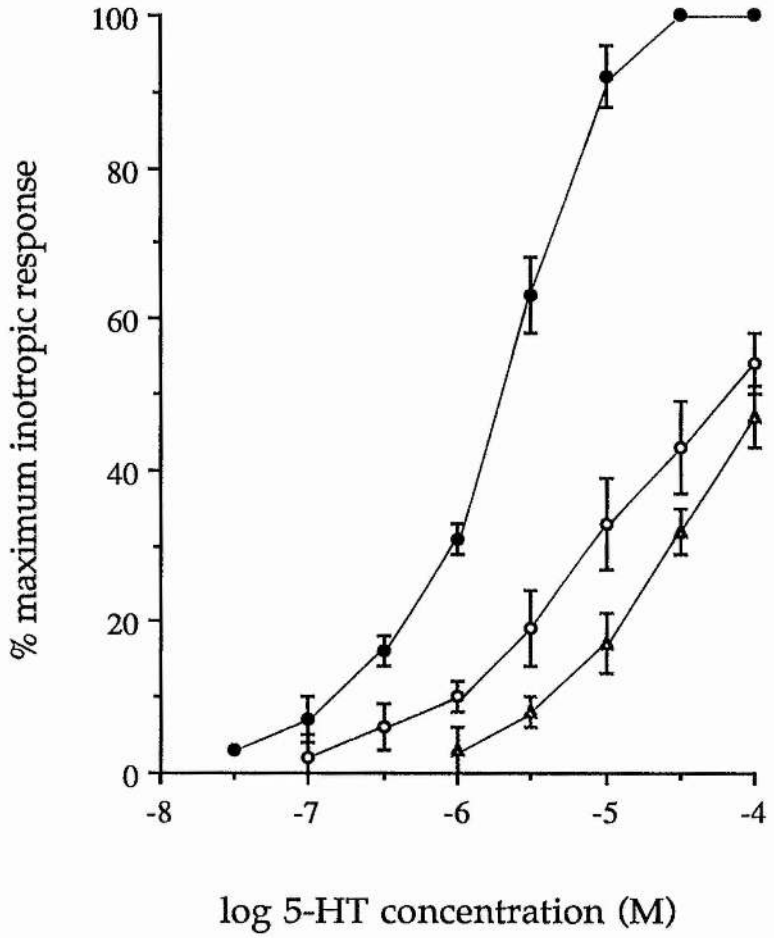


FIGURE 3.14

The concentration-effect curves for 5-HT (closed circles), tryptamine (open circles) and 8-OH-DPAT (triangles) on the isolated *Helix* heart are shown in this figure. The x axis gives log of the 5-HT concentration while the y axis gives the percentage maximum inotropic response. Vertical bars represent the arithmetic mean ± s.e. mean of n=8.

TABLE 3.2 Summary of the 5-HT agonists tested on the *Helix* heart. The EC_{50} and EC_R values are molar values.

Agonist	n	EC_{50}	EC_R	% maximum response
5-HT	22	5.7 ± 0.1	1	100
5-CT	8	4.9 ± 0.8	6.0 ± 0.8	93 ± 3
α-Me-5-HT	8	4.7 ± 0.1	9.2 ± 2.0	88 ± 2
Sumatriptan	8	4.7 ± 0.1	9.5 ± 1.0	66 ± 3
2-Me-5-HT	8	4.2 ± 0.3	29.0 ± 3.0	56 ± 5
Tryptamine	8	4.2 ± 0.3	32.0 ± 5.0	54 ± 8
8-OH-DPAT	8	4.0 ± 0.1	43.0 ± 6.0	47 ± 4

Effects of the ergot derivatives on the inotropic response in the heart

The ergot derivatives ergotamine and methylergometrine and the lysergic acid derivatives methysergide and LSD all had an agonist action on the inotropic response in the isolated *Helix* heart. Fig. 3.15 shows the concentration-effect curves for methysergide, ergotamine and methylergometrine. From this figure it would appear that methysergide, ergotamine and methylergometrine behaved like partial agonists because they elicited a maximum response of 58% for methysergide, of 70% for ergotamine and 73% for methylergometrine. When a 5-HT concentration-effect curve was obtained in the presence of 100μM methysergide (Fig. 3.16), there was only a modest reduction in the inotropic response to 5-HT in the isolated *Helix* heart. Only a very modest reduction was seen in the 5-HT concentration-effect curve after exposure to 100μM ergotamine (Fig. 3.17) and to 100μM methylergometrine (Fig 3.18). The effect of LSD on the isolated *Helix* heart is shown in Fig. 3.19. It is interesting to note that even at a maximum concentration of 1μM, LSD would appear to be as potent if not more so than 5-HT on the isolated *Helix* heart; LSD did show, nonetheless, a much slower

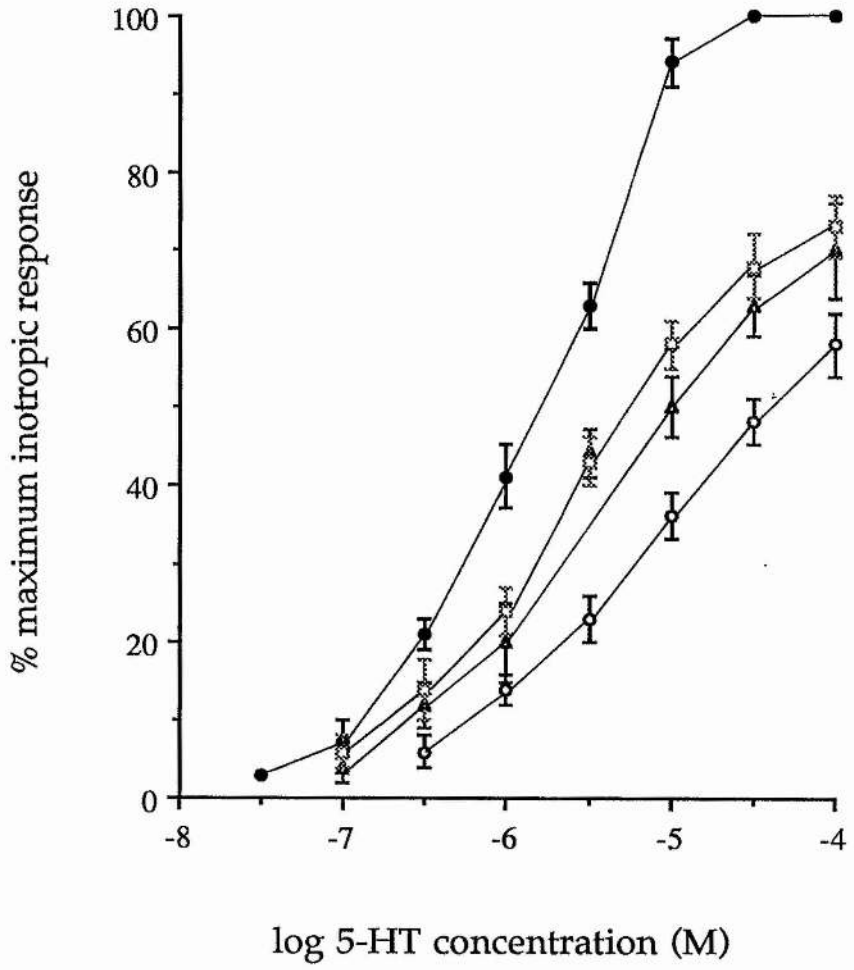


FIGURE 3.15

The concentration-effect curves for 5-HT (closed circles), methysergide (open circles), ergotamine (triangles) and methylergometrine (squares) on the isolated *Helix* heart are shown in this figure. The x axis gives log of the 5-HT concentration while the y axis gives the percentage maximum inotropic response. Vertical bars represent the arithmetic mean ± s.e. mean of n=9.

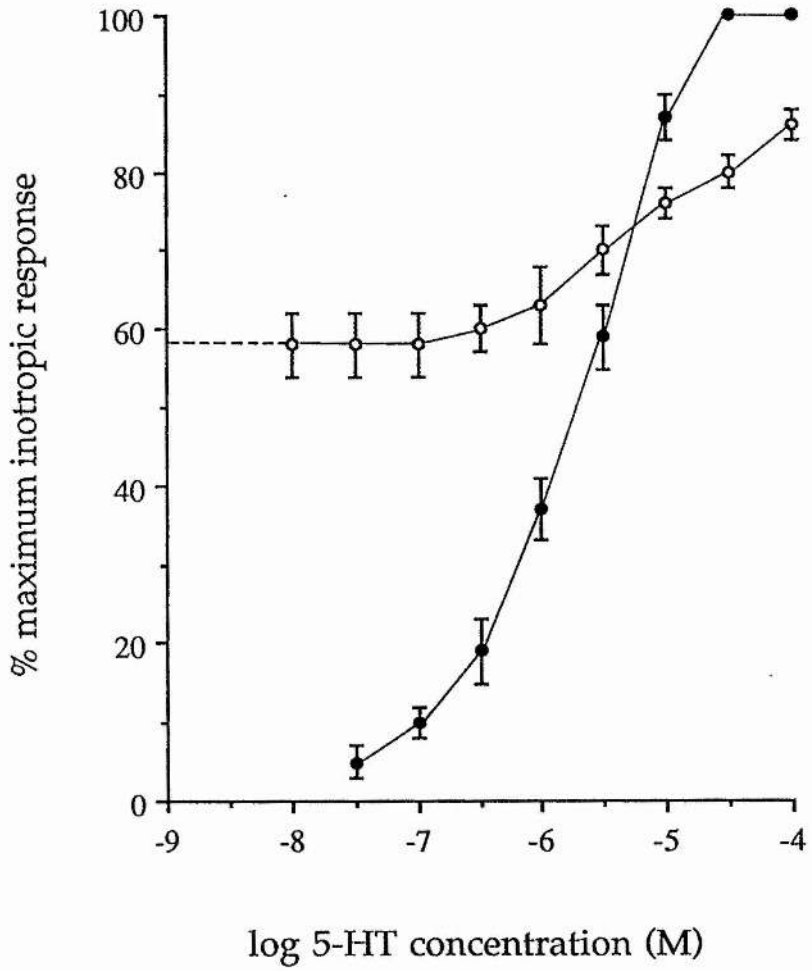


FIGURE 3.16

The concentration-effect curves for 5-HT on the isolated *Helix* heart in the absence (closed circles) and in the presence (open circles) of methysergide at a concentration of 100μM are shown in this figure. The x axis gives log of the 5-HT concentration while the y axis gives the percentage maximum inotropic response. The 5-HT curve obtained in the presence of methysergide takes into account the agonist inotropic effect of 100μM methysergide and hence the inotropic response to 5-HT is measured on top of that already observed with methysergide. Vertical bars represent the arithmetic mean ± s.e. mean of n=9.

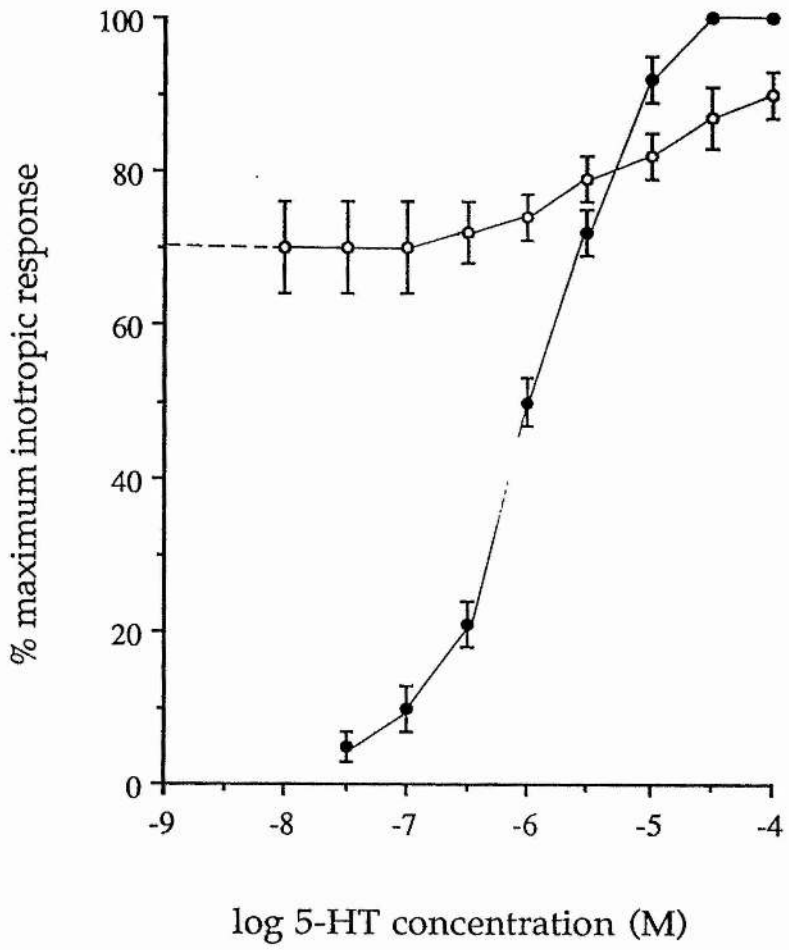


FIGURE 3.17

The concentration-effect curves to 5-HT on the isolated *Helix* heart in the absence (closed circles) and the presence (open circles) of ergotamine at a concentration of 100μM are shown in this figure. The x axis gives log of the 5-HT concentration while the y axis gives the percentage maximum inotropic response. The 5-HT curve obtained in the presence of ergotamine takes into account the agonist inotropic response of 100μM ergotamine and hence the inotropic response to 5-HT is measured on top of that already observed with ergotamine. Vertical bars represent the arithmetic mean ± s.e. mean of n=9.

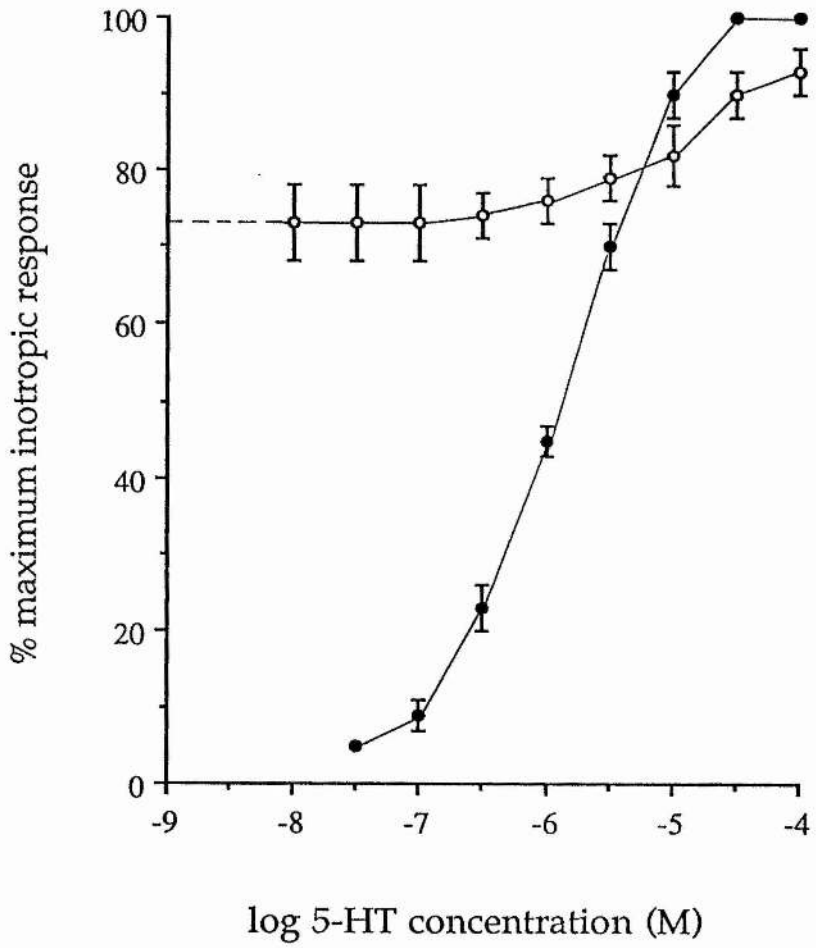


FIGURE 3.18

The concentration-effect curves to 5-HT on the isolated *Helix* heart in the absence (closed circles) and in the presence (open circles) of methylergometrine at a concentration of 100μM are shown in this figure. The x axis gives log of the 5-HT concentration while the y axis gives the percentage maximum inotropic response. The 5-HT curve obtained in the presence of methylergometrine takes into account the agonist inotropic response of 100μM methylergometrine and hence the inotropic response to 5-HT is measured on top of that already observed with methylergometrine. Vertical bars represent the arithmetic mean ± s.e. mean of n=9.

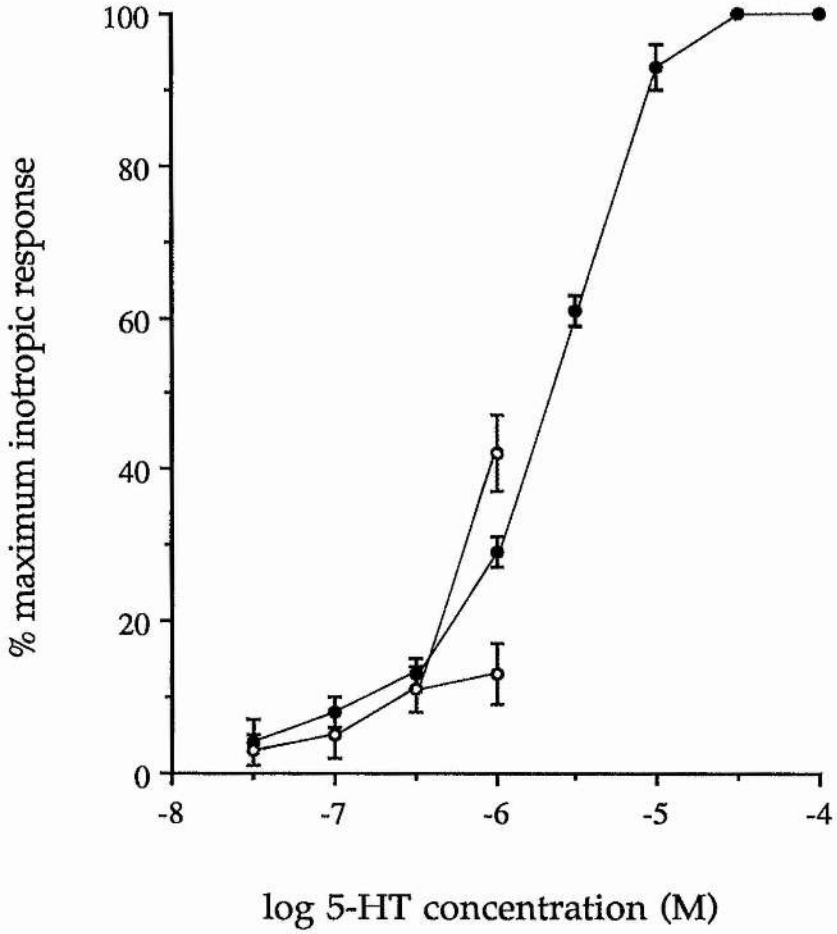


FIGURE 3.19

The concentration-effect curves to 5-HT (closed circles) and to LSD (open circles) on the isolated *Helix* heart are shown in this figure. The x axis gives log of the 5-HT concentration while the y axis gives the percentage maximum inotropic response. The full range of concentration for LSD was limited by the molarity of the initial stock solution which only allowed a final bath concentration of 1μM. At a concentration of 1μM LSD is seen to have two values of maximum response. The difference in these two values is temporal. The lower value, at an inotropic response of 14%, was obtained after 3 minutes whereas the higher value, at 42% maximum inotropic response, was obtained after 30 minutes. Vertical bars represent the arithmetic mean ± s.e. mean of n=6.

reaction time, taking at least 30 minutes to exert a maximum response. Methylergometrine and ergotamine were approximately equipotent as agonists, being 3.5 times less potent than 5-HT: methysergide was less potent still, being 28 times less potent than 5-HT. The results are summarised in Table 3.3 below.

TABLE 3.3 Summary of the ergot derivatives tested on the *Helix* heart. The EC_{50} and EC_R values are molar values.

Ergot derivative	n	EC_{50}	EC_R	% maximum response
Methyl-ergometrine	9	5.2 ± 0.2	3.3 ± 0.7	73 ± 8
Ergotamine	9	5.0 ± 0.3	5.0 ± 0.8	70 ± 6
Methysergide	9	4.2 ± 0.2	28.0 ± 3.0	58 ± 4

Effects of the 5-HT receptor antagonists on the inotropic response to 5-HT in the heart

The specific 5-HT receptor antagonists tested were methiothepin, ketanserin, ritanserin, ondansetron, ICS 205-930, metroclopramide and cocaine. ICS 205-930 at concentrations higher than 10μM is also thought to be a novel 5-HT_4 receptor antagonist; hence in this study it was tested up to a concentration of 100μM.

The extent of overlap of the two 5-HT concentration-effect curves seen in the presence and absence of 1μM methiothepin (Fig. 3.20) indicate a complete lack of antagonism by this drug to the 5-HT inotropic response in *Helix* heart. Ketanserin, even at a concentration of 10μM only, exhibited a small shift of the 5-HT concentration-effect curve to the right giving a mean dose ratio of 1.4 (Fig.3.21). This again indicates that ketanserin has no antagonist action on the inotropic response seen with 5-HT on the isolated *Helix* heart. Ritanserin, on the other hand, caused a dose-dependent lowering of the maximum inotropic response elicited by 5-HT (Fig. 3.22). The effect of ondansetron is shown in Fig. 3.23. No antagonistic action was observed; in

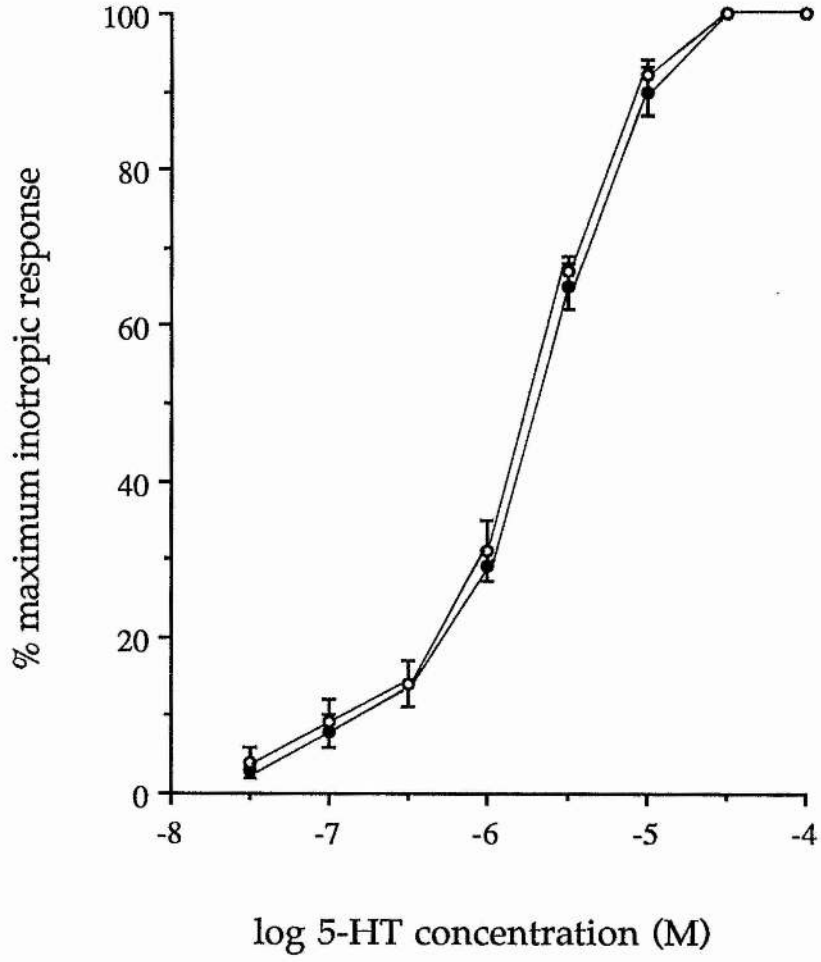


FIGURE 3.20

The concentration-effect curves for 5-HT on the isolated *Helix* heart, in the absence (closed circles) and in the presence (open circles) of 1μM methiothepin are shown in this figure. The x axis gives log of the 5-HT concentration while the y axis gives the percentage maximum inotropic response. Vertical bars represent the arithmetic mean ± s.e. mean of n=8.

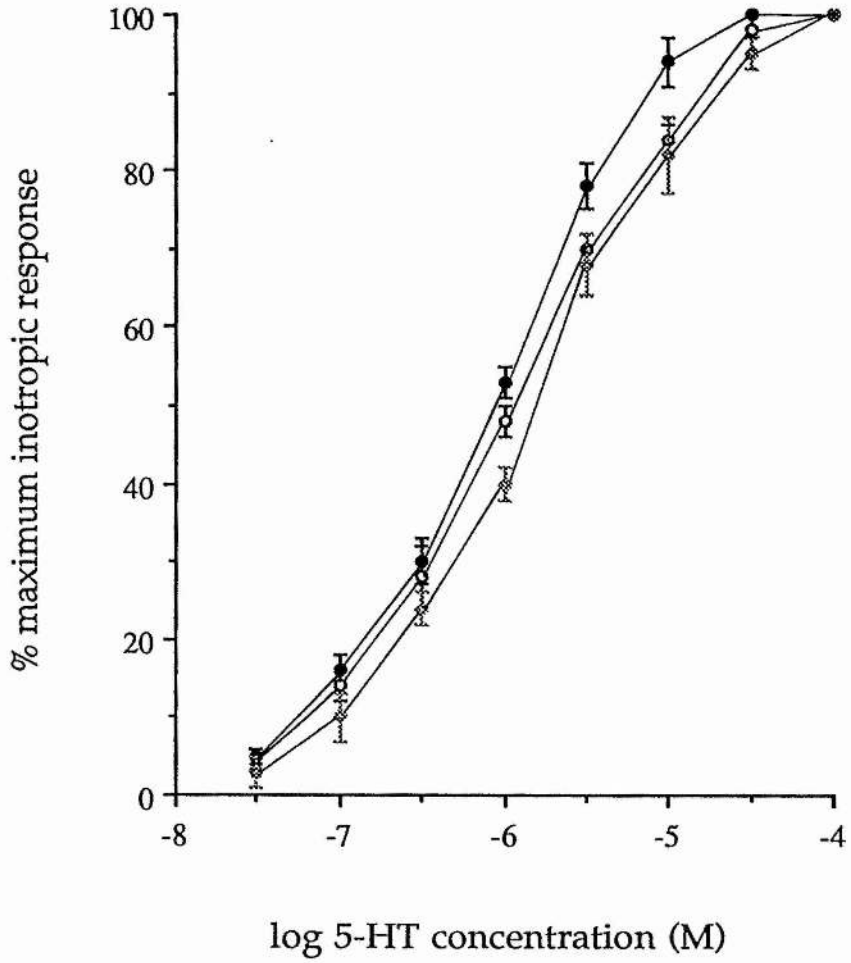


FIGURE 3.21

The concentration-effect curves to 5-HT on the isolated *Helix* heart in the absence (closed circles) and in the presence of 1μM ketanserin (open circles) and 10μM ketanserin (triangles) are shown in this figure. The x axis gives log of the 5-HT concentration while the y axis gives the percentage maximum inotropic response. Verticals bars represent the arithmetic mean ± s.e. mean of n=8.

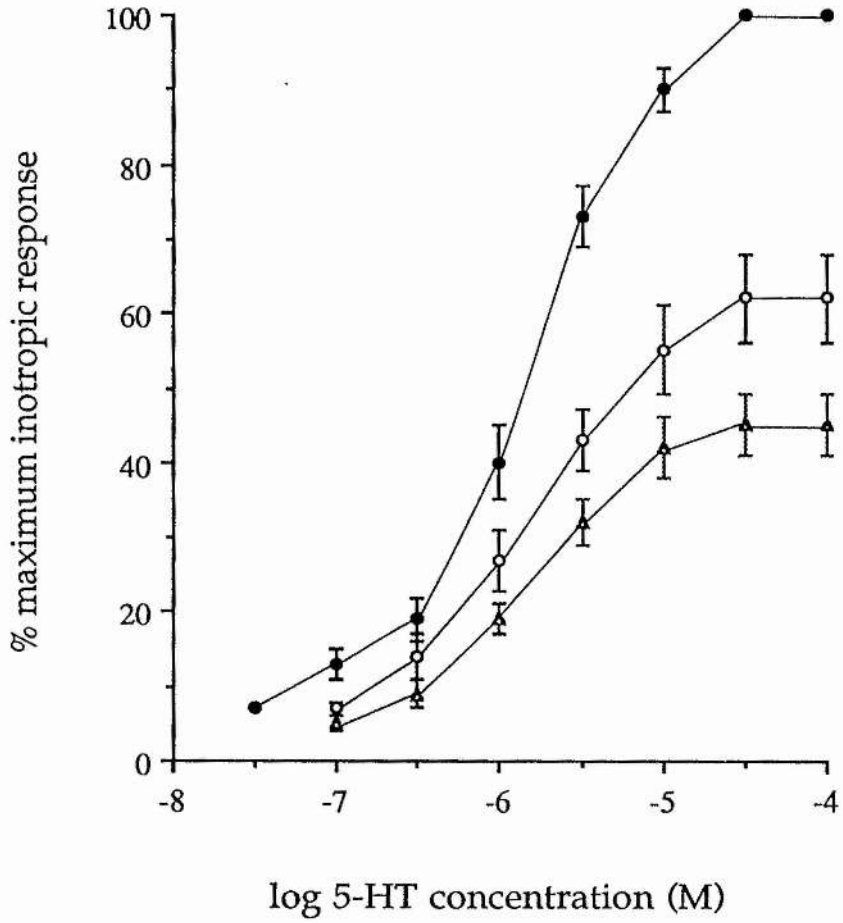


FIGURE 3.22

The concentration-effect curves to 5-HT on the isolated *Helix* heart in the absence (closed circles) and in the presence of 1μM ritanserin (open circles) and 10μM ritanserin (triangles) are shown in this figure. The x axis gives log of the 5-HT concentration while the y axis gives the percentage maximum inotropic response. Vertical bars represent the arithmetic mean ± s.e. mean of n=8.

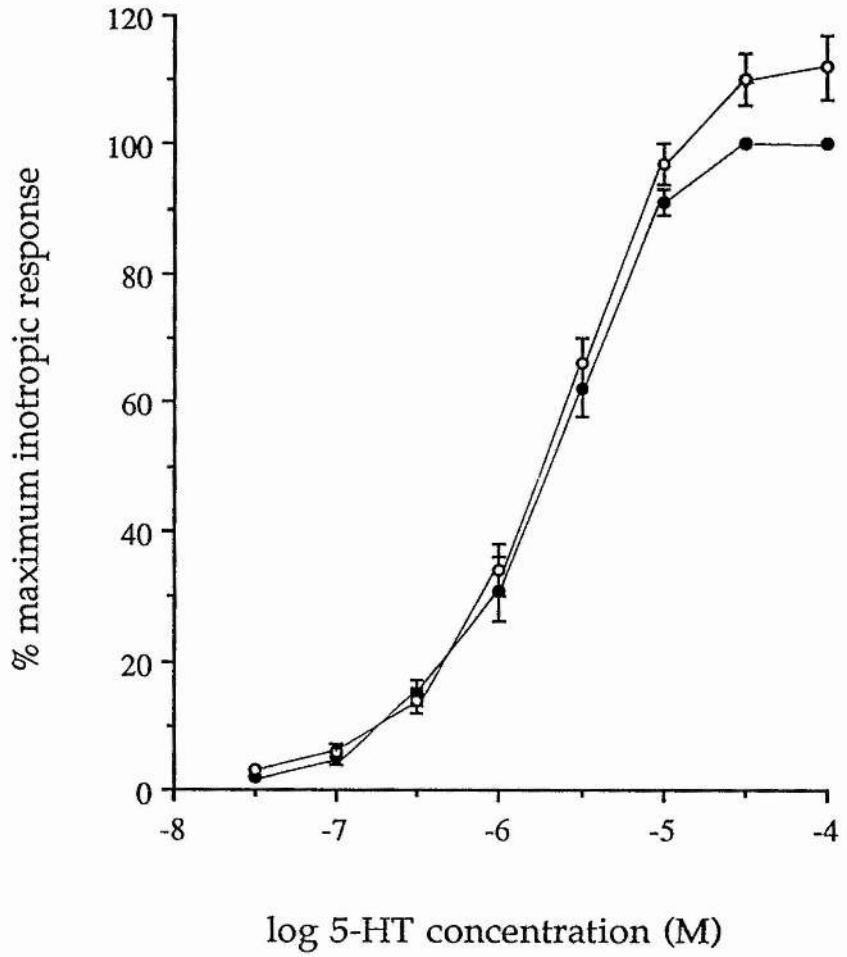


FIGURE 3.23

The concentration-effect curves for 5-HT on the isolated *Helix* heart in the absence (closed circles) and in the presence (open circles) of 1μM ondansetron are shown in this figure. The x axis gives log of the 5-HT concentration while the y axis gives the percentage maximum inotropic response. Vertical bars represent the arithmetic mean ± s.e. mean of n=8.

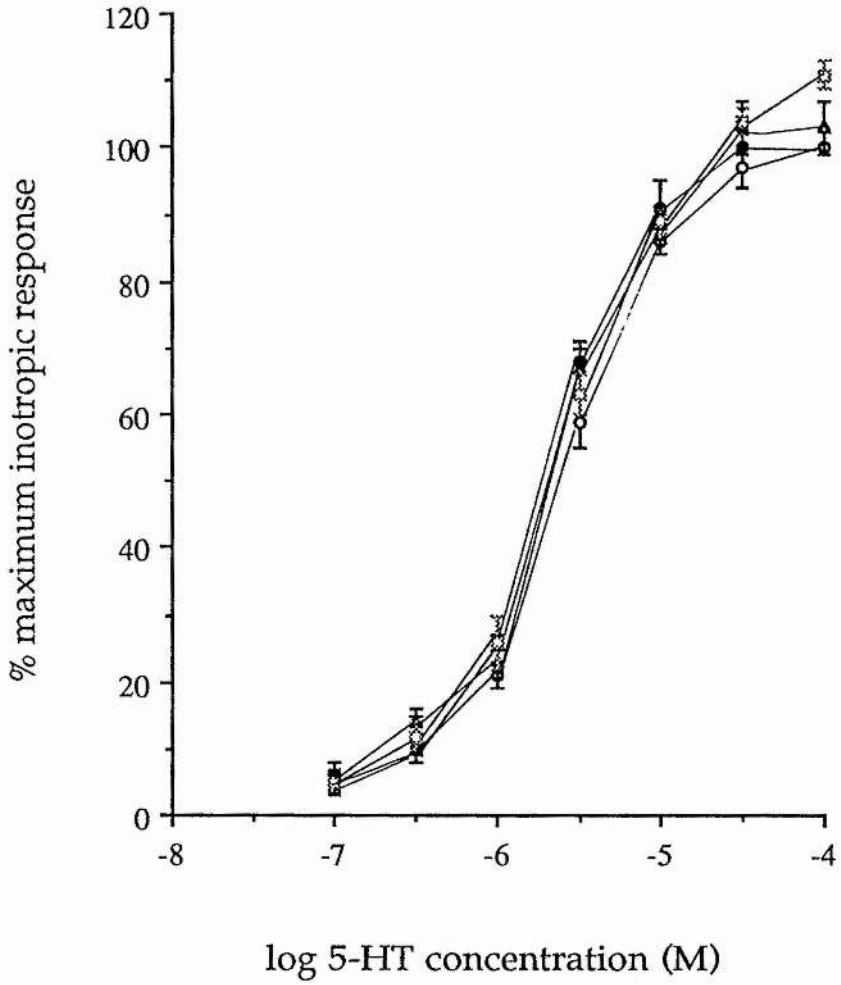


FIGURE 3.24

The concentration-effect curves to 5-HT on the isolated *Helix* heart in the absence (closed circles) and in the presence of 1μM ICS 205-930 (open circles), 10μM ICS 205-930 (triangles) and 100μM ICS 205-930 (squares) are shown in this figure. The x axis gives log of the 5-HT concentration while the y axis gives percentage maximum inotropic response. Vertical bars represent the arithmetic mean ± s.e. mean of n=8.

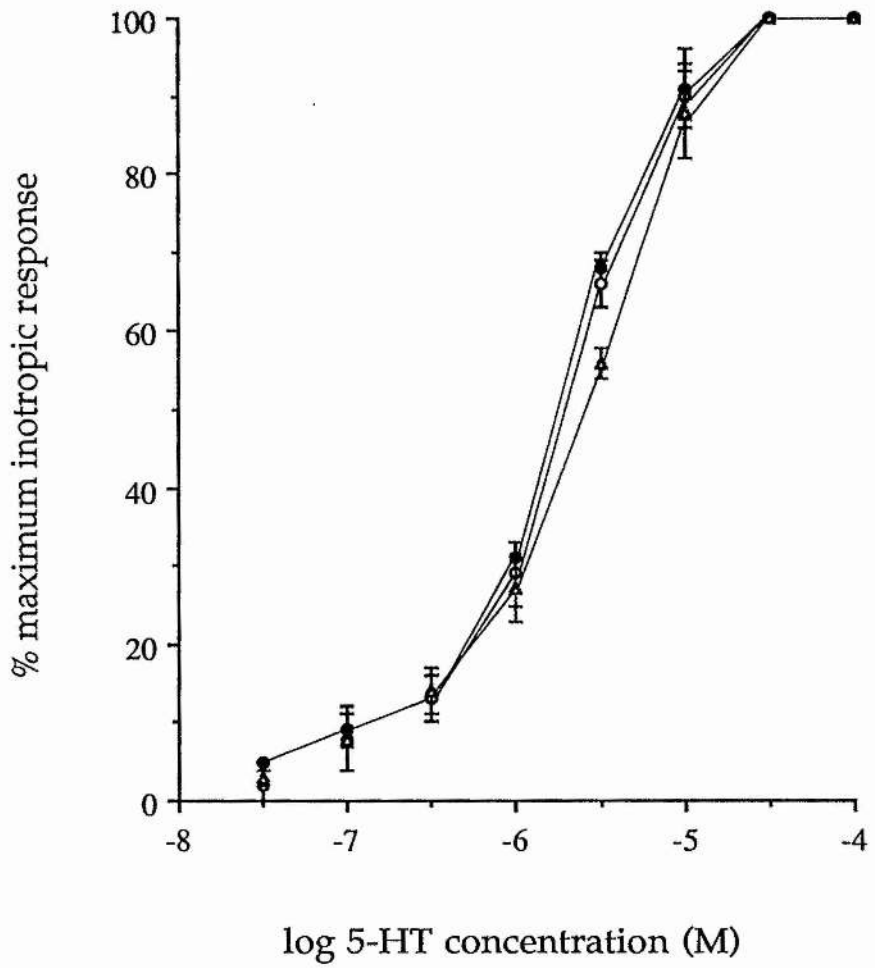


FIGURE 3.25

The concentration-effect curves to 5-HT on the isolated *Helix* heart in the absence (closed circles) and in the presence of 1μM metoclopramide (open circles) and 10μM metoclopramide (triangles) are shown in this figure. The x axis gives log of the 5-HT concentration while the y axis gives percentage maximum inotropic response. Vertical bars represent the arithmetic mean ± s.e. mean of n=8.

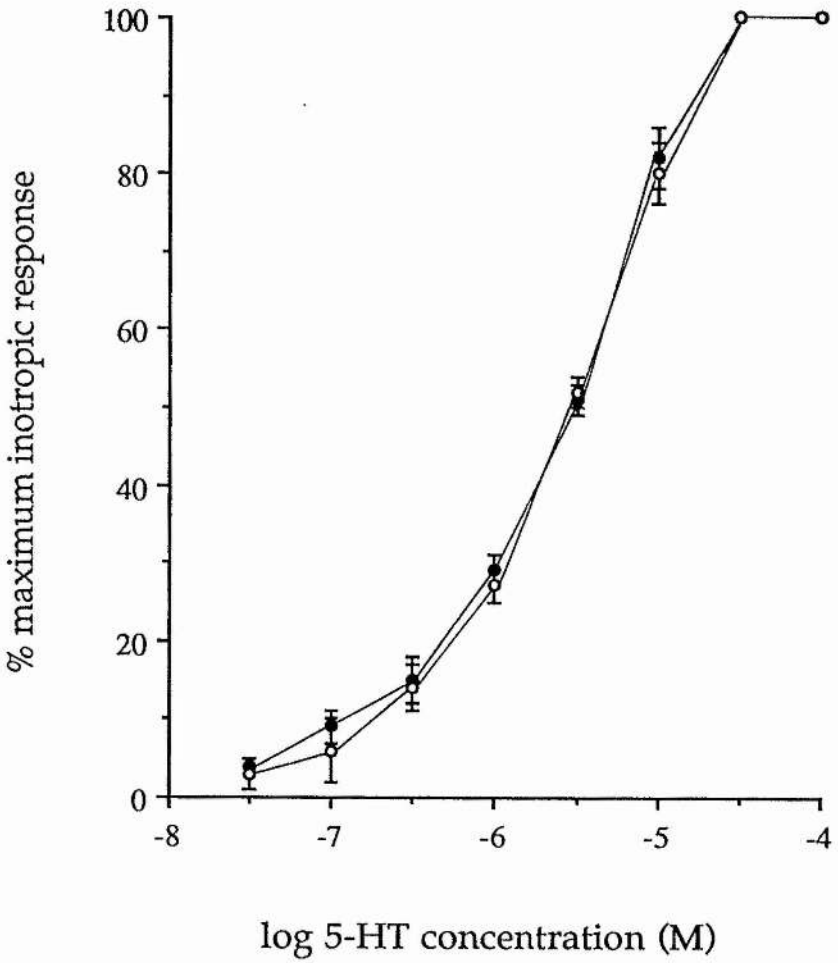


FIGURE 3.26

The concentration-effect curves to 5-HT on the isolated *Helix* heart in the absence (closed circles) and in the presence (open circles) of 1μM cocaine are shown in this figure. The x axis gives log of the 5-HT concentration while the y axis gives percentage maximum inotropic response. Vertical bars represent the arithmetic mean ± s.e. mean of n=8.

fact quite the opposite effect was apparent at 5-HT concentrations >10μM, because ondansetron caused a small potentiation of the 5-HT maximum inotropic response. This result was repeated with ICS 205-930 (Fig. 3.24). In the presence of 100μM ICS 205-930, and at 5-HT concentrations above 10μM, a small potentiation of the 5-HT maximum inotropic response was seen. At concentrations of 1μM and 10μM, ICS 205-930 has no antagonist action on the 5-HT inotropic response in *Helix* heart. Metoclopramide at concentrations of 1μM and 10μM would appear also to have no antagonistic action. In the presence of 1μM the 5-HT curves were superimposed, whereas in the presence of 10μM metoclopramide there was a small shift to the right, with a mean dose-ratio of 1.1 (Fig. 3.25). For 1μM cocaine (Fig. 3.26), once again, the two 5-HT curves were superimposed, indicating a lack of antagonist action on the 5-HT inotropic response in *Helix* heart These results are summarised in Table 3.4 below.

TABLE 3.4 Summary of the 5-HT receptor antagonists tested on *Helix* heart.

Antagonist	Concentration tested (μM)	n	Effect
Methiothepin	1	8	dose-ratio = 1.0
Ketanserin	1 and 10	8	dose-ratios of 0.8 ± 0.1 and 1.4 ± 0.4
Ritanserin	1 and 10	8	dose-dependent lowering of maximum response
Ondansetron	1	8	dose-ratio = 1.0
ICS 205-930	1, 10 and 100	8	dose-ratios = 1.0
Metoclopramide	1 and 10	8	dose-ratios of 1.0 and 1.1 ± 0.4
Cocaine	1	8	dose-ratio = 1.0

Effect of a MAO inhibitor on the inotropic response to 5-HT in the heart

Pargyline, a specific monoamine oxidase inhibitor, at a concentration of 10μM was seen to have little effect on the 5-HT inotropic response in *Helix* heart (Fig. 3.27). The 5-HT curves were superimposed except at high concentrations of 5-HT (>10μM) where a small potentiation was observed. The effect of 10μM pargyline was tested also on a tryptamine concentration-effect curve (Fig. 3.28). Pargyline showed no potentiation of the inotropic response to tryptamine in *Helix* heart.

Effects of two 5-HT uptake inhibitors on the 5-HT inotropic response in the heart

Two specific 5-HT uptake inhibitors were also tested also on the 5-HT response in *Helix* heart. They were fluvoxamine and zimelidine, both tested at a concentration of 10μM. Fluvoxamine (Fig. 3.29) and zimelidine (Fig. 3.30) both would appear to have very poor inhibitory action against the 5-HT uptake system within *Helix* heart tissue. This is indicated by the extent of overlap of the two 5-HT concentration-effect curves in each case.

Involvement of calcium in the 5-HT inotropic response in the heart

Verapamil, a calcium channel blocker, at a concentration of 1μM lowered the maximum inotropic response to 5-HT in *Helix* heart (Fig. 3.31). The fact that calcium ions contribute to the 5-HT inotropic response in *Helix* heart was demonstrated also by lowering the calcium concentration of the physiological saline (Fig. 3.32) When the calcium concentration was reduced from 7mM to 3mM, an increase in the sensitivity of *Helix* heart tissue to 5-HT was observed; this was noted as a shift of the 5-HT concentration-effect curve to the left. A potentiation of the 5-HT maximum inotropic response was observed also under these conditions.

Effects of the molluscan cardioactive peptides on the isolated heart

In *Helix* heart the cardioactive neuropeptide FMRMamide had no effect on the heart-beat amplitude or the heart rate when added to the bath as an agonist over the concentration range 10nM-100μM. The 5-HT concentration-effect curve obtained in the presence of 100μM FMRMamide (Fig.3.33) was

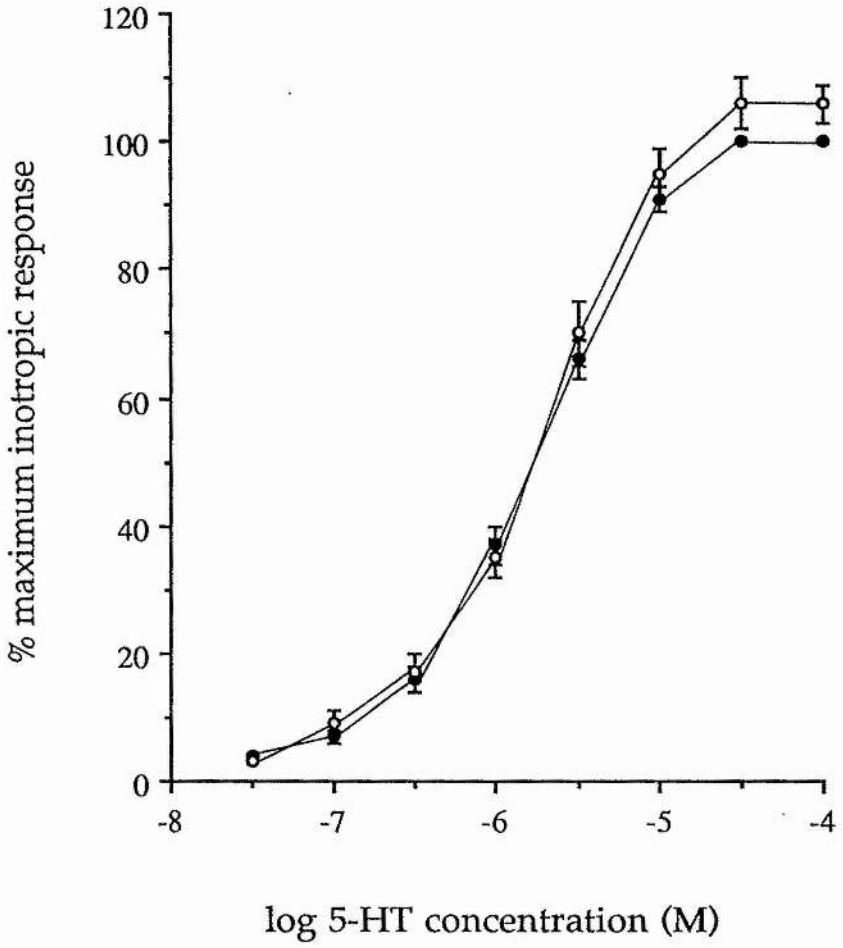


FIGURE 3.27

The concentration-effect curves to 5-HT on the isolated *Helix* heart in the absence (closed circles) and the presence (open circles) of 10μM pargyline are shown in this figure. The x axis gives log of the 5-HT concentration while the y axis gives percentage maximum inotropic response. Vertical bars represent the arithmetic mean ± s.e. mean of n=8.

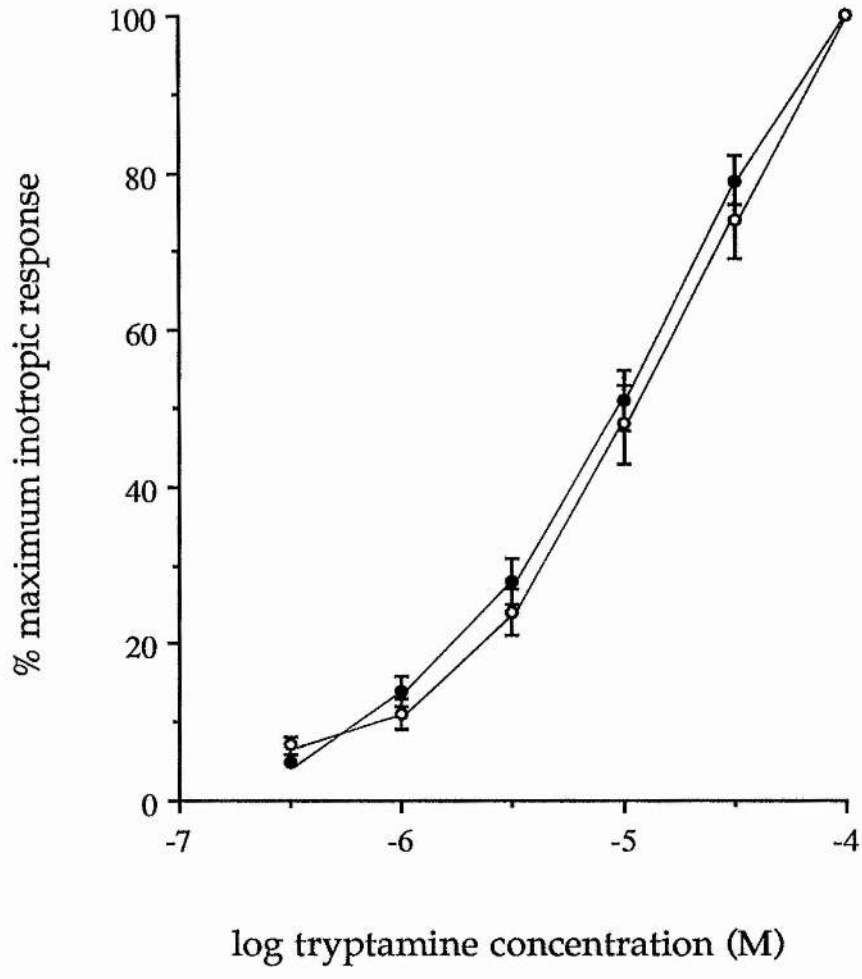


FIGURE 3.28

The concentration-effect curves to tryptamine on the isolated *Helix* heart in the absence (closed circles) and in the presence (open circles) of 10μM pargyline are shown in this figure. The x axis gives log tryptamine concentration while the y axis gives percentage maximum inotropic response. In this case the response obtained with 100μM tryptamine was taken as being 100% maximum response i.e. tryptamine was assumed to be a full agonist. Vertical bars represent the arithmetic mean ± s.e. mean of n=8.

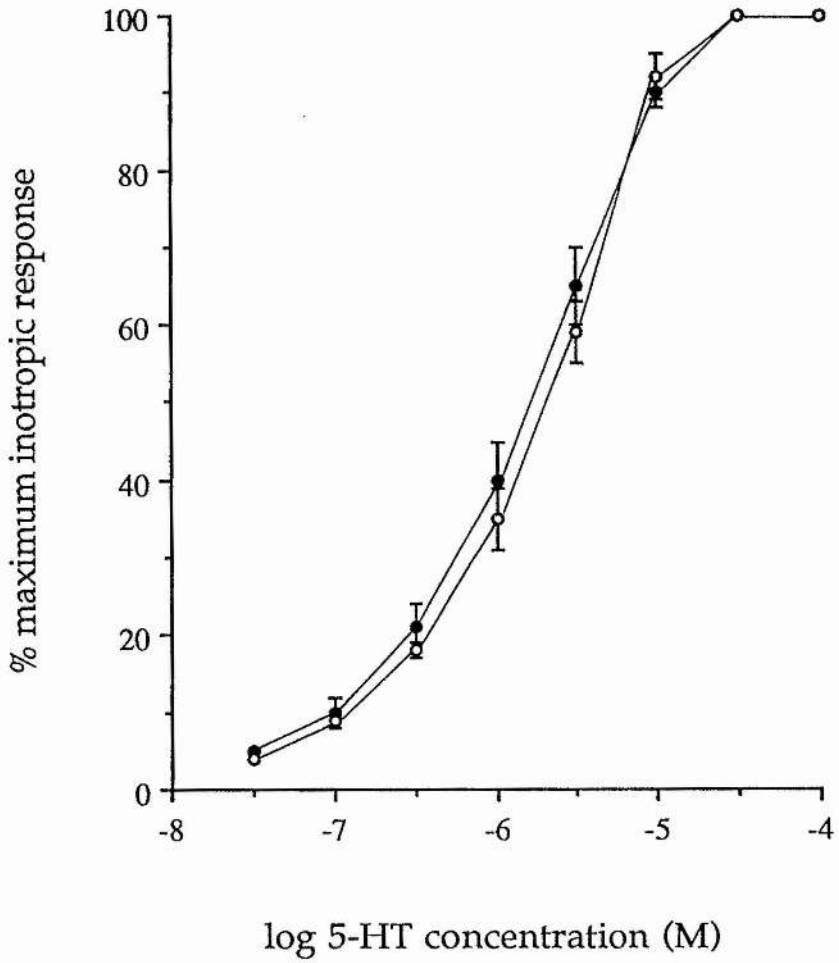


FIGURE 3.29

The concentration-effect curves to 5-HT on the isolated *Helix* heart in the absence (closed circles) and in the presence (open circles) of 10μM fluvoxamine are shown in this figure. The x axis gives log of the 5-HT concentration while the y axis shows percentage maximum inotropic response. Vertical bars represent the arithmetic mean ± s.e. mean of n=9.

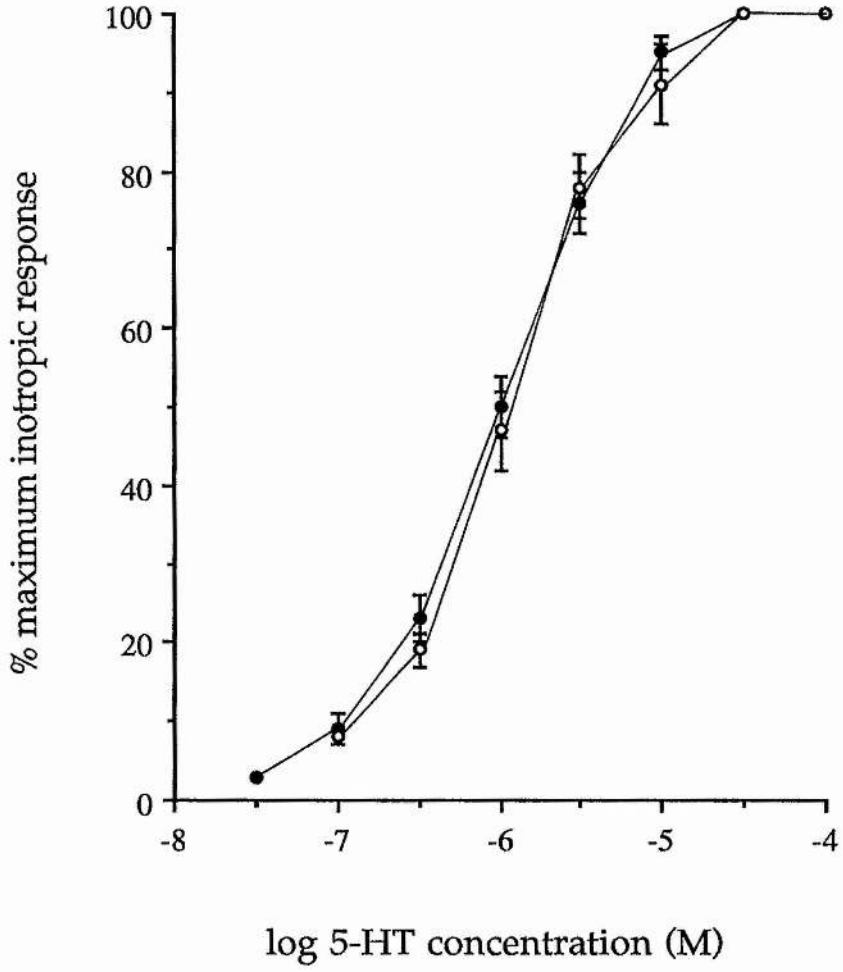


FIGURE 3.30

The concentration-effect curves to 5-HT on the isolated snail heart, in the absence (closed circles) and in the presence (open circles) of 10μM zimelidine are shown in this figure. The x axis gives log of the 5-HT concentration while the y axis gives percentage maximum inotropic response. Vertical bars represent the arithmetic mean ± s.e. mean of n=9.

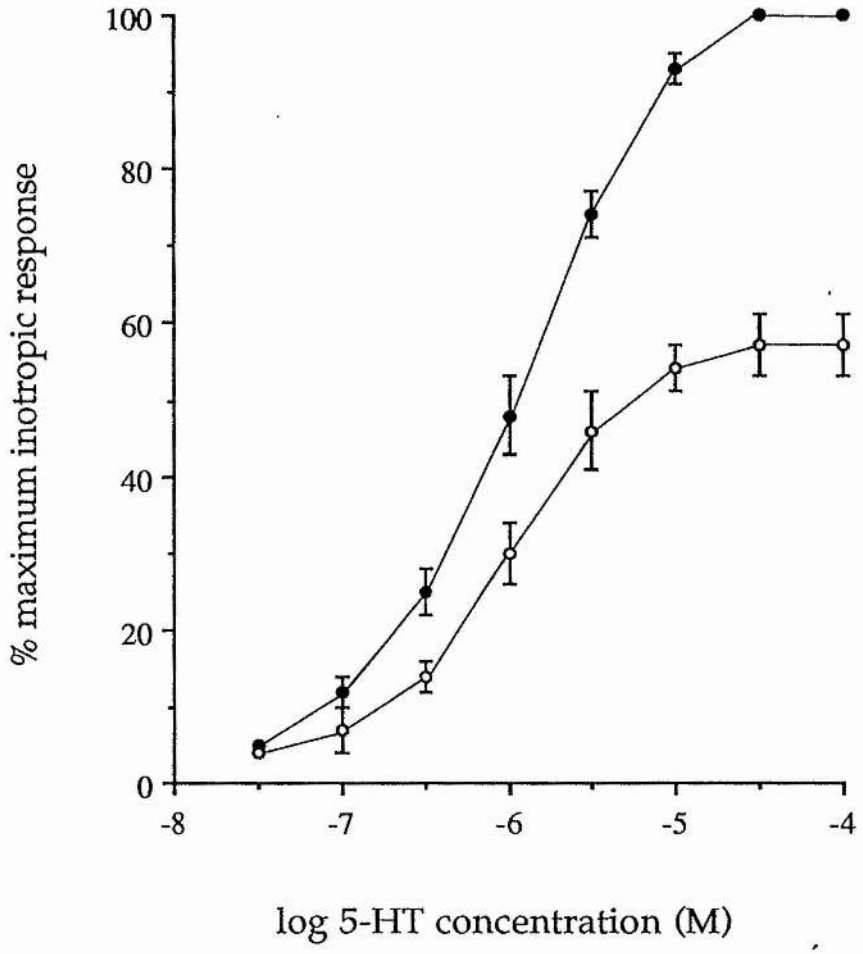


FIGURE 3.31

The concentration-effect curves for 5-HT on the isolated *Helix* heart in the absence (closed circles) and in the presence (open circles) of 1μM verapamil are shown in this figure. The x axis gives log of the 5-HT concentration while the y axis gives percentage maximum inotropic response. Vertical bars represent the arithmetic mean ± s.e. mean of n=9.

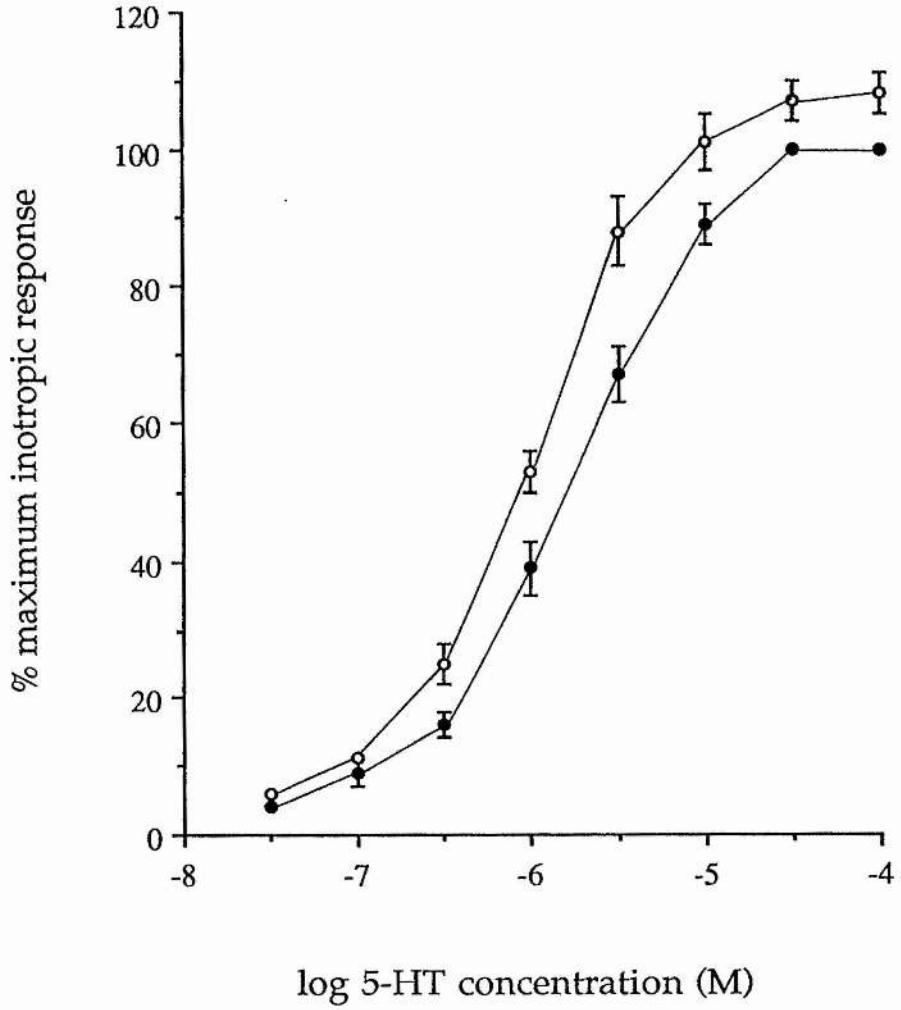


FIGURE 3.32

The concentration-effect curves to 5-HT on the isolated *Helix* heart in normal physiological saline (closed circles) and in physiological saline with a low calcium concentration (open circles) are shown in this figure. The x axis gives log of the 5-HT concentration while the y axis gives percentage maximum inotropic response. Normal physiological saline contains 7mM calcium whereas in the low calcium saline the calcium concentration was 3mM. The isotonicity of this saline was maintained by the addition of sucrose. Vertical bars represent the arithmetic mean ± s.e. mean of n=10.

superimposed upon the control curve, indicating that FMRMamide acted neither as agonist nor antagonist to the 5-HT inotropic response in *Helix* heart. However, the related extended FMRMamide-like peptide pQPFLRFamide was found, like 5-HT, to have a positive inotropic effect. The concentration-effect curve of the extended peptide was found to be to the left of the 5-HT control curve (Fig. 3.34). This indicates the greater potency of the extended peptide, which has a mean EC_{50} value of 6.7 compared to that of 5-HT which has a mean value of 5.7, a 10-fold difference. The threshold value for the extended peptide on *Helix* heart was approximately 5-10nM.

Effect of isoprenaline on the isolated heart

The potent β receptor agonist, isoprenaline, was tested also on *Helix* heart. It had no effect as an agonist on heart-beat amplitude when tested over the concentration range 10nM-100μM. The 5-HT concentration-effect curve obtained in the presence of 100μM isoprenaline was superimposed on the control curve (Fig. 3.35).

Effects of IBMX and forskolin on the 5-HT inotropic response in the heart

The phosphodiesterase inhibitor, IBMX tested at a concentration of 10μM, had no effect on the contractile response elicited by 5-HT in *Helix* heart. In the presence of IBMX (10μM) the 5-HT concentration-effect curve was unaffected, being superimposed on that of the control (Fig. 3.36).

Forskolin, a plant diterpene which is a strong activator of cAMP, had no effect on the 5-HT excitatory response in *Helix* heart. In the presence of 10μM forskolin, the 5-HT curve overlapped with that of the control (Fig. 3.37), indicating a complete lack of effect by forskolin on the 5-HT inotropic response seen in *Helix* heart.

Effect of 5-HT on cAMP levels in *Helix* heart tissue

5-HT caused a dose-dependent increase in cAMP levels within *Helix* heart (Fig. 3.38). From the results, the threshold 5-HT concentration for the stimulation of cAMP was between 50 and 100nM. Also indicated is the lack of effect of 100μM IBMX on the stimulation of cAMP levels in *Helix* heart tissue. Forskolin (10μM), in the absence of 5-HT, increased cAMP levels to 5 times greater than the control level (Table 3.5). Methysergide caused a small dose-

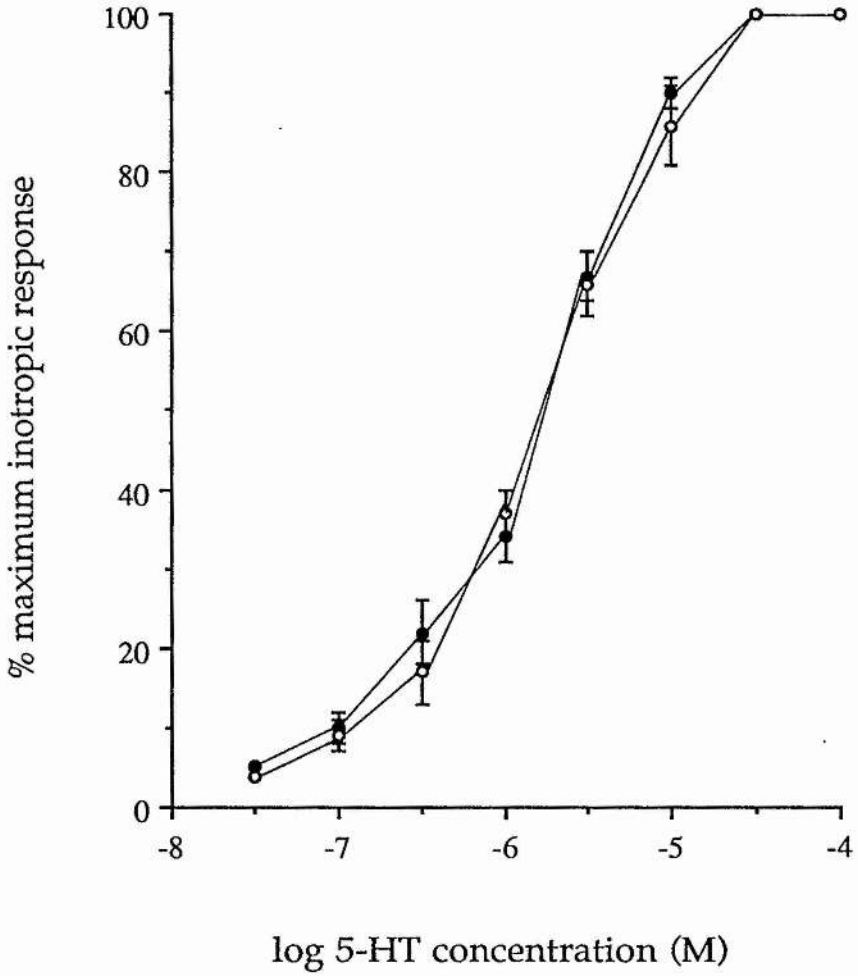


FIGURE 3.33

The concentration-effect curves to 5-HT on the isolated *Helix* heart before (closed circles) and after (open circles) FMRFamide over a concentration range of 10nM-100μM had been added to the bath, are shown in this figure. The x axis gives log of the 5-HT concentration while the y axis gives percentage maximum response. Vertical bars represent the arithmetic mean ± s.e. mean of n=9.

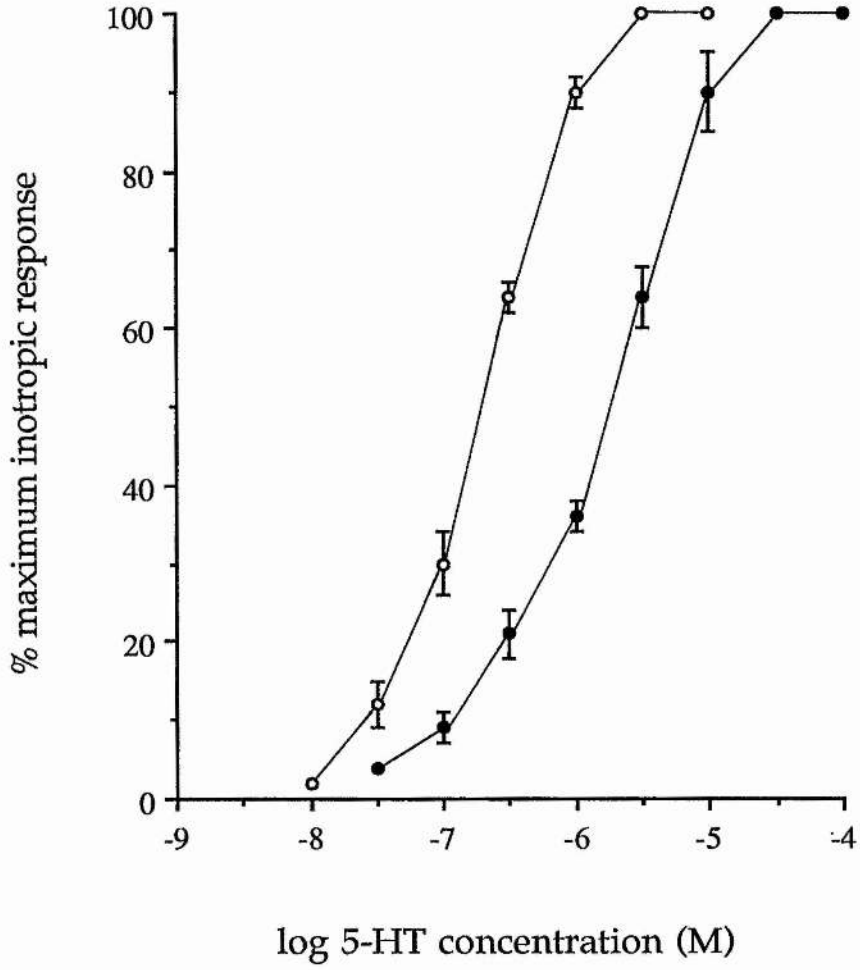


FIGURE 3.34

The concentration-effect curves for 5-HT (closed circles) and pQDPFLRFamide (open circles) on the isolated *Helix* heart are shown in this figure. The x axis gives log of the 5-HT concentration while the y axis gives percentage maximum inotropic response. Vertical bars represent the arithmetic mean ± s.e. mean of n=9.

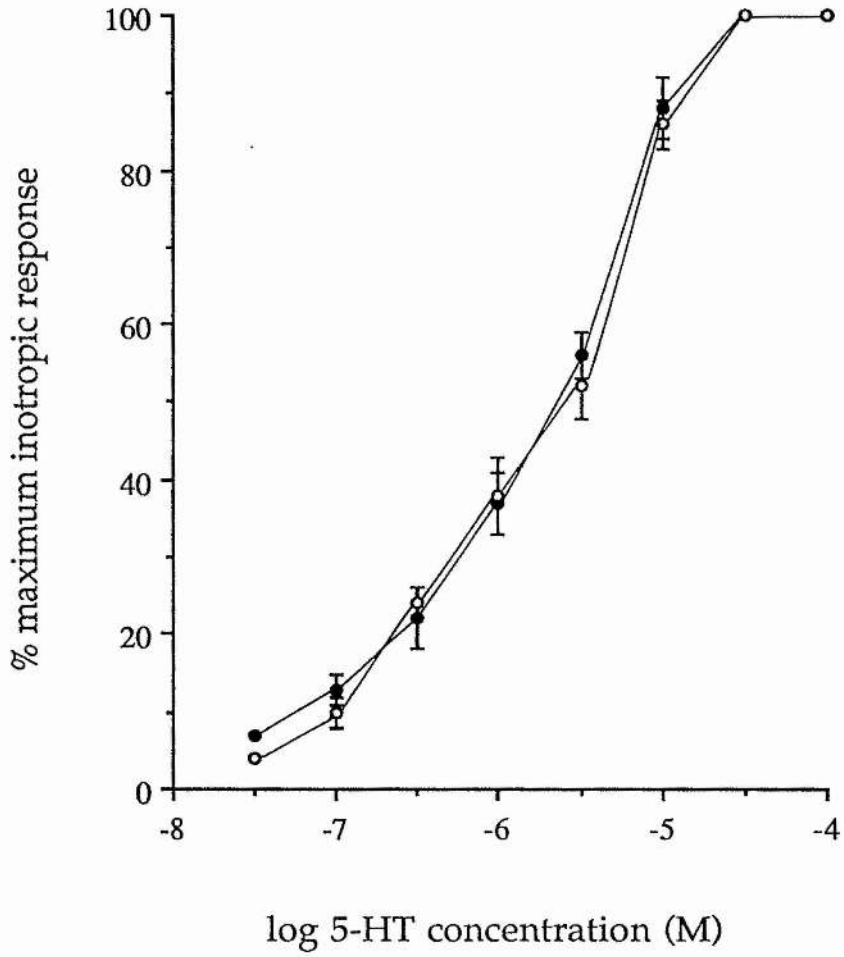


FIGURE 3.35

The concentration-effect curves to 5-HT on the isolated *Helix* heart in the absence (closed circles) and the presence (open circles) of 100μM isoprenaline are shown in this figure. The x axis gives log of the 5-HT concentration while the y axis gives percentage maximum inotropic response. Isoprenaline itself over a concentration range of 10nM-100μM had no effect on heart beat amplitude when added to the bath as an agonist. Verticals bars represent the arithmetic mean ± s.e. mean of n=9.

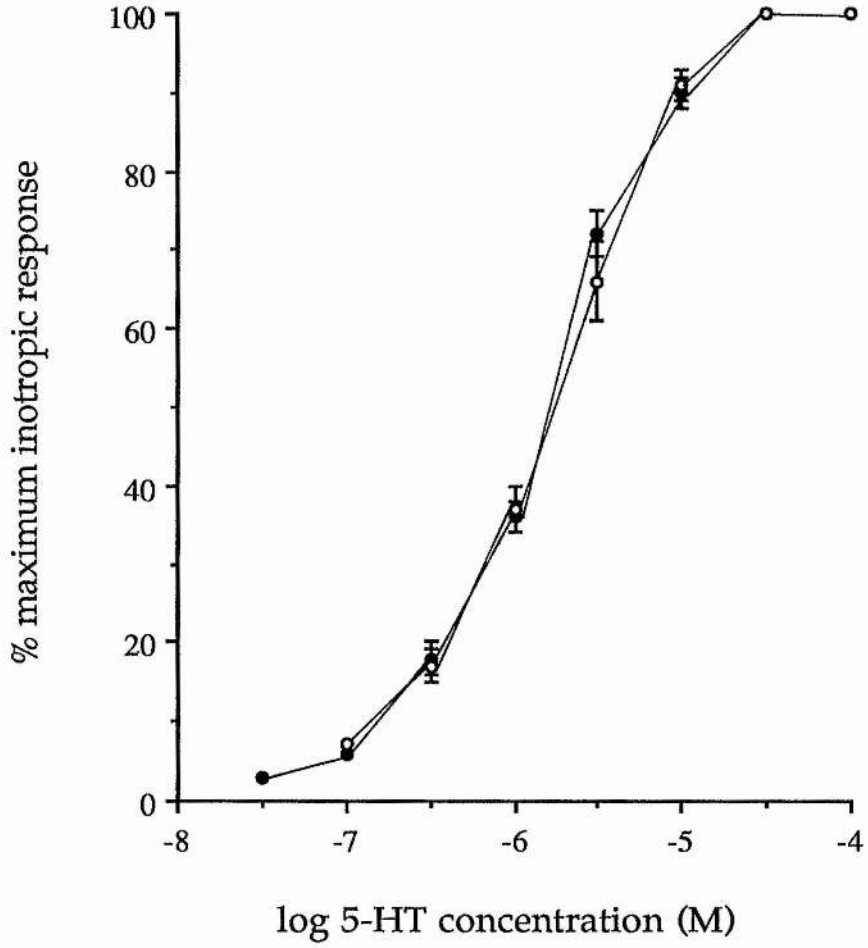


FIGURE 3.36

The concentration-effect curves to 5-HT on the isolated *Helix* heart in the absence (closed circles) and in the presence (open circles) of 10μM IBMX are shown in this figure. The x axis gives log of the 5-HT concentration while the y axis gives percentage maximum inotropic response. Vertical bars represent the arithmetic mean ± s.e. mean of n=9.

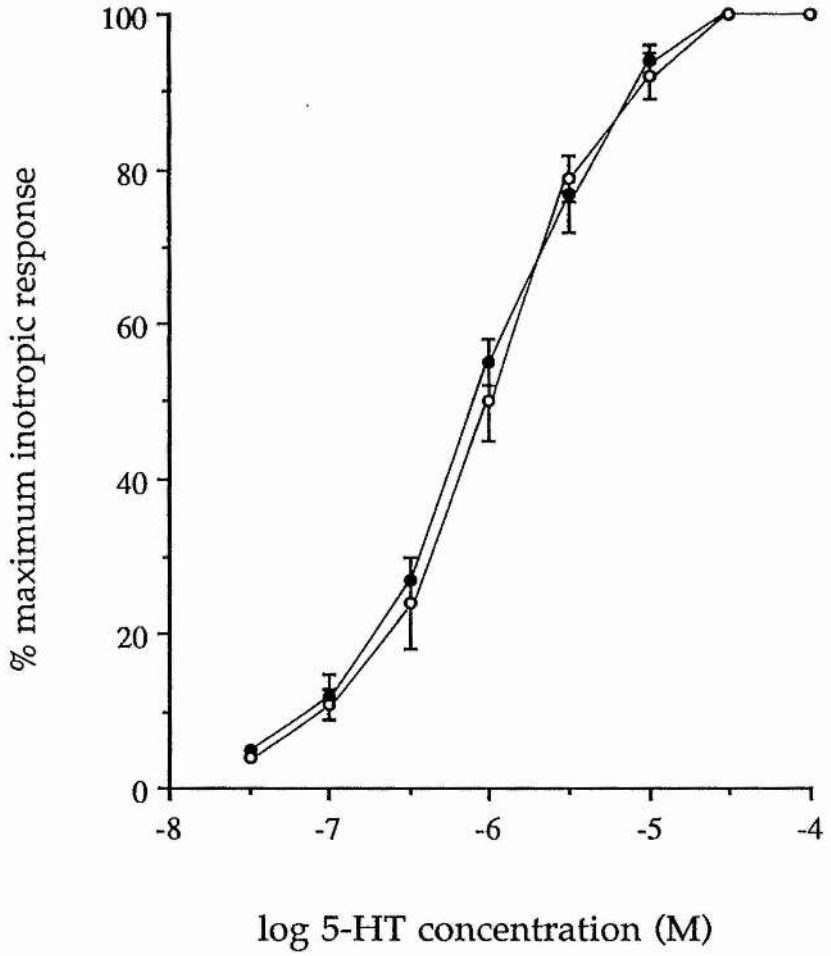


FIGURE 3.37

The concentration-effect curves to 5-HT on the isolated *Helix* heart in the absence (closed circles) and in the presence (open circles) of 10μM forskolin are shown in the figure. The x axis gives log of the 5-HT concentration while the y axis gives percentage maximum inotropic response. Vertical bars represent the arithmetic mean ± s.e. mean of n=9.

dependent increase in cAMP levels when pre-incubated with the hearts for 30 minutes in the absence of 5-HT (Table 3.5). This increase was only a fraction of that seen with 5-HT. When methysergide was pre-incubated with the hearts in the presence of 5-HT (10μM) the effect on cAMP levels was cumulative and the increase in cAMP levels then seen was larger than that seen for 10μM 5-HT in the absence of methysergide.

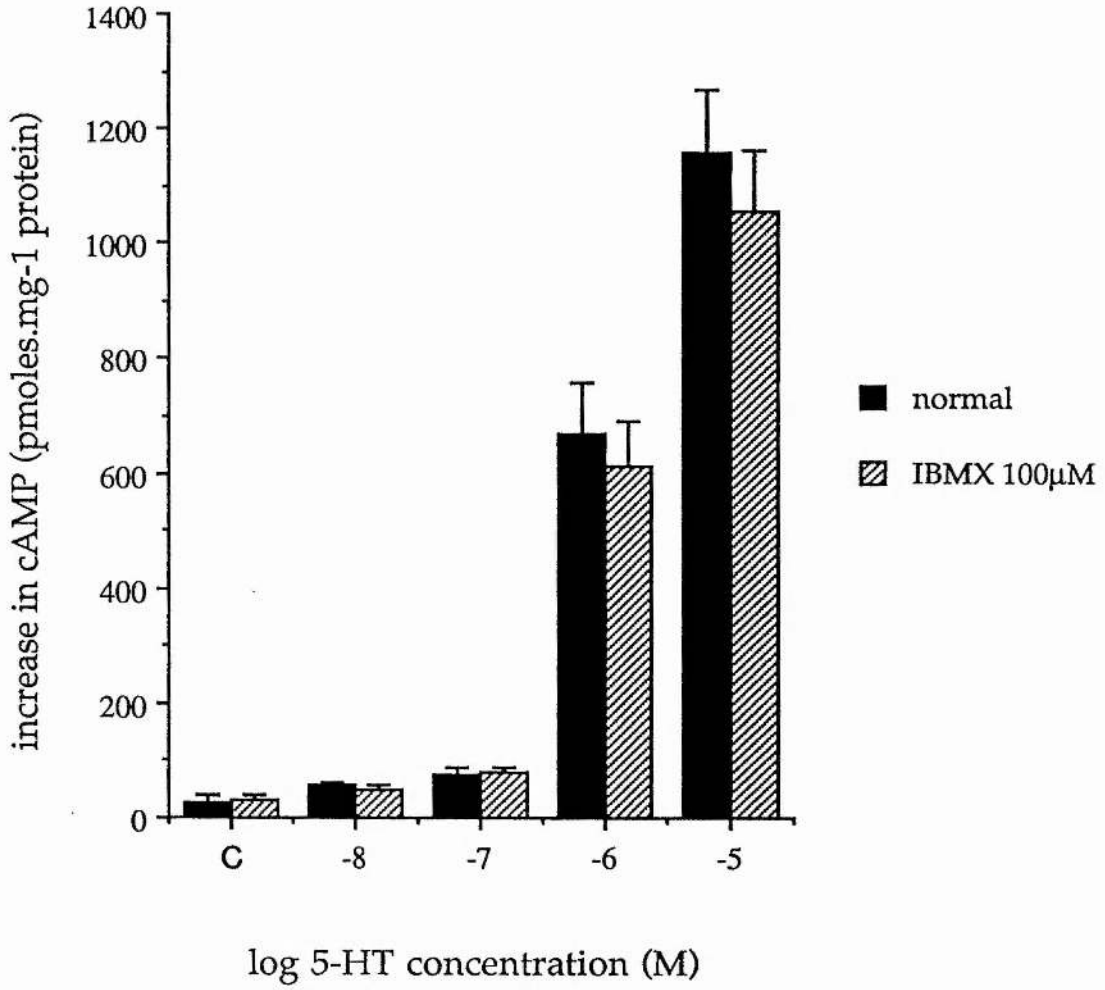


FIGURE 3.38

The increase seen in cAMP levels in *Helix* heart tissue with increasing concentrations of 5-HT are shown in this figure. The bar chart shows two sets of results. The first set of results (solid columns) were obtained under normal conditions and functioned as controls while the second set (dashed columns) were obtained in the presence of 100µM IBMX. The x axis gives log of the 5-HT concentration and shows a control value, marked by the letter C, obtained in the absence of 5-HT and the y axis gives increase in cAMP in pmoles.mg^{-1} protein. Vertical bars represent the arithmetic mean ± s.e. mean of n=5.

TABLE 3.5 Summary of the effects of 5-HT, forskolin, methysergide, isoprenaline and IBMX on cAMP levels in *Helix* heart tissue.

Treatment	n	pmoles cAMP.mg^{-1} protein normal	pmoles cAMP.mg^{-1} protein IBMX (100μM))
Control	5	28 ± 6	31 ± 6
5-HT (10nM)	5	56 ± 8	48 ± 9
5-HT (100nM)	5	77 ± 12	81 ± 9
5-HT (1μM)	5	688 ± 90	611 ± 106
5-HT (10μM)	5	1158 ± 110	955.± 106
Forskolin (10μM)	5	139 ± 11	132 ± 9
Methysergide (100nM)	3	99 ± 8	
Methysergide (1μM)	3	139 ± 8	
Methysergide (10μM)	3	153 ± 12	
Methysergide(100nM) + 5-HT (10μM)	3	1249 ± 137	
Methysergide (1μM) + 5-HT (10μM)	3	1329 ±137	
Methysergide (10μM) + 5-HT (10μM)	3	1417 ± 120	
Isoprenaline (10μM)	3	29 ± 5	

DISCUSSION

The difference in sensitivity of the *Helix* heart to 5-HT observed for the two methods

As the preceding results have shown, 5-HT has a marked inotropic and chronotropic effect on the isolated *Helix* heart. For experimental purposes the inotropic effect only of 5-HT was monitored because the chronotropic effect was found to be variable and occurred only at high concentrations of 5-HT. The two methods for monitoring the inotropic effect of 5-HT on the isolated heart differed only in threshold concentration for 5-HT. For the perfused heart method, the threshold concentration was between 1 and 10nM whereas for the non-perfused heart method the threshold concentration was 10-30nM, a 10-fold difference in sensitivity to 5-HT. One possible reason for the difference in sensitivity of the *Helix* heart to 5-HT could be the actual method employed. In the perfused heart method the heart was internally perfused exposing the interior of the heart to the stated concentration of 5-HT. By contrast, in the non-perfused heart method the ventricle was tied off and thereby closed to the surrounding medium. The means of entry of external medium, containing the experimental drugs, were limited in this method; through the small piece of aorta which was open to the external environment or by diffusion. How much of the medium penetrated inside of the heart was unknown, but was presumably dependent on the amplitude of heart beat of each individual preparation.

Ripplinger (1957) had shown that one branch of the extrinsic nerve supply to the *Helix* heart terminated in the AV junction. Later Cottrell and Osborne (1969) described a dense network of nerve fibres in the AV junction of *Helix*. They suggested that this network could have a neurosecretory function. Could it be possible that the majority of the 5-HT receptors in the *Helix* heart are located at or near the nerve terminals in the AV junction, with a greater distribution on the internal surface of the heart in comparison to the external surface of the heart ? Such a distribution of 5-HT receptors could then explain the greater sensitivity of the heart to 5-HT in the perfused method because this method inflicted the lesser damage of the two on the AV junction. The perfused heart method results in the internal surface of the heart being in

direct contact with the drug-containing medium; the non-perfused does not. However a combination of other events, such as sites of loss, presence of deactivating enzymes and uptake sites for 5-HT can not be overlooked when discussing the difference in sensitivity to 5-HT between the two methods.

Despite the between-method difference in sensitivity of *Helix* heart to 5-HT, the non-perfused heart method was still preferred over the perfused heart method. The endogenous molluscan cardioactive neuropeptides, FMRFamide and pQDPFLRFamide, were tested on the isolated *Helix* heart as a reference point to ascertain the reliability of the method used. In most studies with *Helix* hearts (e.g. Kerkut and Cottrell, 1963; Boyd *et al.*, 1985; Payza, 1987) the perfused heart method had been preferred. FMRFamide was found to be a weak agonist on the *Helix* heart by Greenberg and Price (1980), but in the present study it had no effect on the isolated heart. In contrast, pQDPFLRFamide was found to be more potent than 5-HT. This supports the finding by Price *et al.* (1985), who found that pQDPFLRFamide was about 100 times more potent than FMRFamide on this preparation. They suggested that an increased resistance to proteolytic degradation might explain, at least in part, the relatively high potency of pQDPFLRFamide compared to FMRFamide on the heart of *Helix*. This potent action of pQDPFLRFamide in stimulating the *Helix* heart served to demonstrate the fact that there was little difference between the perfused and non-perfused heart methods.

Another possible reason for the difference in sensitivity to 5-HT could be related to the state of the activity of the snails. Such changes in sensitivity to 5-HT in *Helix aspersa* heart were noted first by Kerkut and Laverack (1960). They observed that the heart of the winter-hibernating or aestivating snail had a threshold response to a 5-HT dilution of 10^{-12}g.l^{-1} compared to the heart of a non-hibernating or active snail which had a threshold response of 10^{-8}g.l^{-1} 5-HT. In *Helix aspersa* 5-HT levels appeared to show seasonal variation both in heart and nervous tissue (Cardot, 1971). Snails kept in dry conditions fell into an aestivating state and showed a rise in the 5-HT levels both in the brain and heart. When aroused from hibernation, the 5-HT level fell both in the heart and ganglia (Hiripi and Salánki, 1973). The snails used in the present perfused heart experiments had all been in a state of aestivation as a result of being kept in a dry environment for anything up to two months before use. However, all the animals used in the non-perfused heart method were in the

active state, having been kept in a humid environment at constant temperature and with regular feeding. Seasonal changes in the 5-HT level within the *Helix* hearts could therefore be attributable, at least in part, for the differences in sensitivity of *Helix* heart to 5-HT observed in the experiments.

5-HT as sole mediator for the observed inotropic effect on the isolated heart

That the inotropic effect observed with 5-HT on the isolated *Helix* heart was solely attributable to 5-HT has been investigated in this study. None of the different antagonists, at a concentration of 1μM, had any effect on the 5-HT concentration-effect curve or on the mechanical activity of the heart when they were added to the organ bath.

Certainly ACh played no role in the inotropic effect seen with 5-HT in *Helix* heart. This was hardly surprising because ACh has been shown to mimic the actions of endogenously released cardioinhibitory transmitter, with which it is considered identical (Prosser, 1940). The cardioactive effects seen by 5-HT-like agonists acting, not only on tryptamine receptors, but also on nicotinic receptors in the sympathetic cardiac nerves in the isolated rabbit heart (Fozard and Mobarok Ali, 1978) relates to a system probably confined to the vertebrates.

Neither of the catecholamine blocking compounds tested on the isolated heart had any effect. It was therefore assumed that catecholamines played no part in the excitatory action of 5-HT on *Helix* heart. 5-HT did not seem to be causing the release of noradrenaline from the isolated *Helix* heart, as has been shown in the isolated rabbit heart (Fozard and Mwaluko, 1976). With respect to 5-HT being an excitatory transmitter in the molluscan heart, it would appear that in this phylum 5-HT plays a role equivalent to that of the catecholamines, adrenaline and noradrenaline, in vertebrates. Despite the evidence for catecholamine excitation of the *Helix pomatia* heart (Osborne and Cottrell, 1970), from these results it was assumed that if these compounds acted at all they had only a very low potency compared to 5-HT. Clearly there were no β_1 and β_2 adrenoreceptors on *Helix* heart because isoprenaline had no effect on the heart beat amplitude or heart rate. Isoprenaline also had no effect on the level of cAMP in *Helix* heart cells. It is in the vertebrate heart that the β receptor is found and where it is responsible for the positive inotropic and

chronotrophic effects of catecholamines. The β receptor mediates these effects through adenylate cyclase and stimulation of cAMP levels.

5-HT agonist potency

Of all the specific 5-HT receptor agonists tested on *Helix* heart, none proved to be as potent as 5-HT itself. The full rank order of potency of the agonists, including the ergot derivatives because they had been observed to have agonist actions on the isolated heart, was 5-HT > methylergometrine = ergotamine = 5-CT > α-Me-5-HT = sumatriptan > methysergide = 2-Me-5-HT = tryptamine > 8-OH-DPAT. This rank order of potency is not comparable to others which have been elucidated for the different 5-HT receptor types in the vertebrates.

5-HT receptor antagonist potency

The inotropic effect of 5-HT was found to be resistant to antagonism by the 5-HT_1 receptor antagonist methiothepin, by the 5-HT_2 receptor antagonist ketanserin, by the 5-HT_3 receptor antagonists cocaine, ondansetron and ICS 205-930 and by the M receptor antagonist metoclopramide. Ritanserin showed dose-dependent lowering of the maximum inotropic response observed with 5-HT. Ritanserin has been shown in most vascular and tracheal preparations to act as an unsurmountable, apparently non-competitive, 5-HT_2 receptor antagonist. An exception is the rabbit femoral artery where the antagonism was competitive (Van Neuten *et al.*, 1986). This non-competitive action of ritanserin would therefore explain the dose-dependent lowering of the inotropic response to 5-HT seen in this study. The small potentiations seen with high concentrations of ICS 205-930 (>10μM) plus >10μM 5-HT and ondansetron (1μM) plus >10μM 5-HT cannot be explained in terms of a particular 5-HT receptor subtype or by the action of these drugs.

Some of the findings in this study paralleled those of Boyd *et al.* (1985). 5-HT produced excitation at concentrations >1-10nM and involved an increase in contraction size and amplitude of the heart beat. 8-OH-DPAT had direct excitatory action but it differed temporally from that produced by 5-HT. The 5-HT_3 receptor antagonist MDL 72222 produced excitation at high concentrations similar to that seen with ondansetron and ICS 205-930 in this study. Ketanserin was a weak antagonist only against 5-HT excitation of *Helix*

heart. The 5-HT receptor mediating cardioexcitation in the *Helix* heart could not be separated clearly into either 5-HT_1 or 5-HT_2 types of receptor by Boyd *et al.* (1985). From the present study the 5-HT receptor is clearly not either 5-HT_1 or 5-HT_2, due to the complete lack of antagonism either by methiothepin and ketanserin. Neither would the 5-HT receptor appear to be of the 5-HT_3 subtype, owing to the lack of effect of cocaine, ondansetron and ICS 205-930.

The novel 5-HT_4 receptor, and its possible involvement in molluscan hearts, was implicated by Kaumann *et al.* (1989). The 5-HT receptors of the human atrium were found to resemble the cerebral 5-HT receptors, designated 5-HT_4 by Dumuis *et al.* (1988). The 5-HT_4 receptor was found to be positively linked to cAMP and it was this increase in cAMP, as noted by Sawada *et al.* (1984) in molluscan hearts, which led Kaumann *et al.* (1989) to suggest that the 5-HT receptor on molluscan hearts might be of the 5-HT_4 subtype. Both Kaumann *et al.* (1989) and Dumuis *et al.* (1988) found that ICS 205-930 was a fully competitive antagonist at this receptor although at a much lower blocking potency than would be expected from its affinity with 5-HT_3 receptors (Bradley *et al.*, 1986). The possibility of the presence of a 5-HT_4 receptor on the isolated *Helix* heart could not be demonstrated by the results in this study. ICS 205-930, even at 100μM, had no antagonist effect on the 5-HT inotropic response within *Helix* heart. The lack of effect of metoclopramide provided evidence that the *Helix* heart 5-HT receptor was not of the M receptor type.

Recent experimental evidence indicates that central 5-HT_4 receptors which are positively coupled to adenylate cyclase, are stimulated by a family of 2-methoxy-4-amino-5-chloro substituted benzamide derivatives (Dumuis *et al.*, 1991). In their study the ability of three azabicycloalkyl benzimidazolone derivatives, BIMU 1, BIMU 8 and DAU 6215, to stimulate cAMP formation in colliculi neurones in primary culture was tested. Two of the compounds, BIMU 1 and BIMU 8, were found to be good agonists at the 5-HT_4 receptors which are present on these neurones. Unfortunately this information was not available at the time of this investigation and consequently these novel 5-HT_4 agonists were not tested on this isolated heart preparation.

Mediation by cAMP of the 5-HT inotropic response in the isolated heart

The evidence emerging from this investigation that the inotropic effect of 5-HT was mediated by cAMP was also significant. The cAMP results are in agreement with those obtained in earlier studies (Higgins and Greenberg, 1974; Higgins, 1977). 5-HT caused a dose-dependent increase in intracellular cAMP concentration which was not antagonized by methysergide. It is extremely unlikely that the 5-HT receptor is either 5-HT_{1A}, 5-HT_{1B} or 5-HT_{1D} because each of these has been shown to be negatively linked to adenylate cyclase, resulting in decreases in cAMP, not the inverse as observed with 5-HT in *Helix* heart. In vertebrates, the 5-HT_3 receptor was found to be coupled directly to a receptor-ion complex (see Bobker and Williams, 1990). This precludes the presence of a 5-HT_3 receptor in the isolated *Helix* heart. From the 5-HT_2 receptor antagonist potencies observed on *Helix* heart, the 5-HT receptor under investigation is unlikely to be a 5-HT_2 receptor; the 5-HT_2 receptor (along with the 5-HT_{1C} receptor) has been linked to phosphatidyl inositol turnover. Importantly Deaton and Gray (1990) have shown that phorbol esters increase both the beat frequency and amplitude of isolated *Mercenaria* ventricles. Phorbol esters have been demonstrated to directly stimulate protein kinase C, which is a crucial link in signal transduction for a variety of biologically active substances (Nishizuka, 1984). There is also increasing evidence of 5-HT receptors which are positively linked to cAMP and which do not appear to correspond to any of the other known subtypes. This situation is the case for NCB-20 cells (Cossery *et al.*, 1990) and for porcine vena cava (Trevethick *et al.*, 1986).

Corroborative pharmacological evidence for the hypothesis that cAMP is acting as the intracellular mediator for neurotransmitter-stimulated changes in molluscan heart contractility is lacking. Neither exogenous cAMP, nor its more lipid-soluble derivatives, had any effect on the contractility of the isolated molluscan ventricle. Similarly, the cyclic nucleotide phosphodiesterase inhibitor, theophylline, did not alter the rate or strength of contraction (Higgins, 1974). The results of the present study with the phosphodiesterase inhibitor, IBMX, supported the finding of Hess *et al.* (1981) that IBMX was a poor inhibitor of *Mercenaria mercenaria* phosphodiesterase enzyme. IBMX at a concentration of 100μM did not alter significantly the

strength or rate of contraction of the isolated *Helix* heart, nor did it have an appreciable effect on myocardial intracellular cAMP concentrations in the absence of 5-HT. Thus it could be seen that IBMX was a relatively poor inhibitor of *Helix aspersa* phosphodiesterase activity.

Although increasing the intracellular cAMP concentration may mediate some of the inotropic actions of 5-HT on *Helix* heart, other unknown mechanisms must be involved. Forskolin (10μM) in the absence of 5-HT increased the intracellular cAMP concentration without affecting the myocardial contractility. Forskolin had no effect on the inotropic or chronotropic effect of 5-HT when added directly to the bath. The increase in cAMP levels seen with forskolin were expected because forskolin was known to directly potentiate adenylate cyclase without neurotransmitter receptor activation (Seamon and Daly, 1982). 5-HT could conceivably have induced the membrane ionic events necessary to generate the myocardial response while cAMP could have mediated the other cellular events necessary to increase the magnitude of this response. It is unlikely that forskolin directly altered other intracellular enzyme activities (Seamon and Daly, 1982). Higgins and Greenberg (1974) demonstrated that cAMP stimulated phosphorylation and increased net calcium uptake by a microsomal fraction of *Mercenaria* ventricles. They proposed that cAMP stimulated the phosphorylation of proteins of the sarcoplasmic reticulum by a cAMP-dependent protein kinase. This is turn augmented the calcium sequestration by the sarcoplasmic reticulum and other intracellular calcium-sequestering membrane systems. Similarly, adrenergic agonists also increased mammalian ventricular cAMP levels, sarcoplasmic reticulum protein phosphorylation and calcium sequestration by isolated sarcoplasmic reticulum (Tada and Kirchberger, 1975). Such a mechanism could explain the positive inotropic response of the molluscan myocardium to 5-HT in that more calcium would be released per beat. This would increase actin-myosin cross-bridging and subsequently generate greater myocardial tension.

In the isolated *Helix* heart 5-HT caused inotropic and chronotropic effects with a threshold between 1 and 10nM. However, increases in cAMP were detectable only at 5-HT concentrations above 100nM. A similar discrepancy between thresholds for 5-HT-induced mechanical events and elevation of cAMP levels has been reported in bivalve catch muscle (Köhler

and Lindl, 1980) and in *Aplysia* heart (Mandelbaum *et al.*, 1979). The very large changes in cAMP levels induced by 5-HT suggest that there may be an hierarchy of cAMP-mediated processes in the muscles studied, different processes being initiated by different levels of cAMP. These observations suggest that changes in cytoplasmic levels of cAMP cannot fully explain the cardioactive effects of 5-HT in *Helix* heart, and there is, therefore, the possibility of the involvement of other intracellular second messengers.

The LSD derivatives and their effect on the isolated heart

The LSD derivatives have been demonstrated to have potent actions on molluscan hearts. Methysergide has been shown to antagonise the actions of 5-HT on *Mercenaria* heart (Welsh, 1954; Greenberg, 1960; Loveland, 1963) and *Patella* heart (Leake *et al.*, 1971) whereas LSD, along with other ergot compounds, has been shown to stimulate *Mercenaria* (Greenberg, 1960) and *Helix* heart (Gaddum and Paasonen, 1955). In *Aplysia* heart LSD, Bromo-LSD and methysergide all had direct effects on the heart, which made their degree of block on the 5-HT response variable (Liebeswar *et al.*, 1975).

Methysergide probably was the first specific antagonist of the contractile effects of 5-HT in a variety of smooth muscle preparations. However the nature of the interaction of methysergide with 5-HT differed between smooth muscle preparations. In some methysergide acted as a competitive antagonist, in others as a partial agonist and in yet others as an insurmountable antagonist (see Feniuk, 1984). Thus for both vertebrate and invertebrate tissues the actions of methysergide and other ergot derivatives are diverse and could be seen to range from agonist and antagonist.

In the present study all of methysergide, methylergometrine, ergotamine and LSD were shown to have potent agonist actions on the isolated *Helix* heart. The block of the 5-HT inotropic response, shown by methysergide, ergotamine and methylergometrine, at a concentration of 100μM, was only a very modest one. This was in contrast to Boyd *et al.* (1985) who found that methysergide not only strongly antagonised the 5-HT response in *Helix* heart but in itself produced weak excitation of the heart. With the antagonism of the 5-HT response in the *Helix* heart by methysergide, methylergometrine and ergotamine being only minimal, the data was difficult

to interpret. The high degree of agonism shown by the above three compounds made difficult the determination of their affinity for the 5-HT receptor in *Helix* heart.

The lack of high affinity shown by methysergide unfortunately fails to prove that the drug is acting through the same receptor as 5-HT. It may well be that all these agonist effects of 5-HT and methysergide are mediated through a common receptor, a possibility which remains unclear from the data presented here. A specific receptor-blocking drug, which failed to be forthcoming in this investigation, will be necessary in order to substantiate the hypothesis. Alternatively, the data with the ergot derivatives, in particular the potency of LSD as an agonist and the temporal difference exhibited by LSD, and to a lesser extent, methysergide, in eliciting a similar response to 5-HT in *Helix* heart, suggests the possibility of the presence of a separate receptor which, has a high affinity for the ergot compounds on *Helix* heart tissue. It could be speculated that this suggested separate receptor could be a peptide receptor.

The small dose-dependent increases in cAMP levels in *Helix* heart cells seen with methysergide did not provide any definitive evidence as to whether or not methysergide and 5-HT were acting at the same receptor. It served to prove that methysergide lacked antagonistic action towards the inotropic effects of 5-HT in *Helix* heart.

Inactivation of 5-HT in the isolated heart

For vertebrate brain tissue it is well known that the enzyme MAO is involved in the degradation of biogenic amines (Werman, 1966). Although small amounts of MAO have been shown to exist in *Helix* brain (Guthrie *et al.*, 1975), no investigations have been made into the presence of this enzyme within *Helix* heart. From the lack of effect of pargyline, a specific MAO inhibitor, it would appear that *Helix* heart either lacked this enzyme or that the enzyme itself was not sensitive to the inhibitors that were tested in the present study. This lack of effect of pargyline assisted in accounting for the low potency of tryptamine. It was thought that the low potency exhibited by tryptamine on the isolated *Helix* heart could be due to its accessibility to enzymatic breakdown by MAO within the heart. This has been observed in

other systems, such as the rat fundic strip, where it occurs because the cell membrane acts as a selective diffusion barrier excluding 5-HT but allowing the less polar tryptamine molecules to gain access to intracellular MAO enzymes with subsequent breakdown (Vane, 1959).

In the rat caudal artery preparation, both 5-HT and tryptamine had contractile actions although tryptamine was much less potent than 5-HT (Bradley *et al.*, 1985). This suggested that tryptamine was stimulating receptors distinct from those of 5-HT. However, in the presence of pargyline, the 5-HT antagonists were more potent against tryptamine, such that the pA_2 values were not significantly different from those against 5-HT. This indicated that tryptamine was indeed acting at the same receptor as 5-HT and that the differential inactivation of tryptamine by MAO largely accounted for the different susceptibilities of 5-HT and tryptamine to the antagonists examined. Pargyline also had no effect on the inotropic response to tryptamine in the isolated *Helix* heart. This indicated that tryptamine was only a weak agonist at the 5-HT receptor in *Helix* heart and its low potency could not be accounted for by its differential inactivation by MAO.

There have been few specific studies on the characteristics of the 5-HT uptake mechanism for invertebrates and those that have been undertaken are confined to the 5-HT uptake into neurones. It is known that *Helix* central ganglia have specific high affinity mechanisms for the uptake of 5-HT, which were dependent on temperature and sodium ions (Osborne *et al.*, 1975). These specific uptake mechanisms were imipramine-sensitive (Cottrell, 1971). The lack of action of the 5-HT uptake blockers fluvoxamine and zimelidine on the 5-HT response in *Helix* heart provided evidence that either 5-HT within *Helix* heart is deactivated by a novel method, which is highly unlikely or that the 5-HT uptake system present in the heart tissue was unsusceptible to blockade by the specific drugs used. From the above evidence this latter hypothesis would seem the more feasible. There could perhaps be some difference in structure of the 5-HT uptake mechanism in the heart of the molluscs, compared to that of the vertebrates, which would account for the possible difference in susceptibility to the specific uptake inhibitors that were used.

Involvement of calcium in the inotropic response to 5-HT in the isolated heart

The action of verapamil on the excitatory 5-HT response in the isolated *Helix* heart indicated a possible involvement of calcium channels with associated movement of calcium ions in the inotropic response to 5-HT in the isolated heart. Verapamil, a slow calcium channel blocker, more properly described as a calcium entry blocker (Sperelakis, 1984), not only decreased the maximum inotropic response to 5-HT in the isolated heart but also caused a small decrease in sensitivity to 5-HT in the heart cells.

Molluscan cardiac muscle cells possess an elaborate sarcoplasmic reticulum which could act as a site for intracellular calcium storage and release, although this molluscan cardiac muscle lacks a typical T-system (Sanger, 1979). Extracellular calcium influx, as well as release of calcium from extracellular stores, could both have a role in contraction of *Helix* heart. The influx of calcium through a calcium channel whose activity is regulated by 5-HT may be necessary to mediate the excitation-contraction coupling within *Helix* heart muscle cells. 5-HT, by increasing not only the plateau height of the cardiac action potential, but also prolonging its plateau phase (Hill, 1974), serves to increase the time available for calcium entry into myocardial cells. This in turn would allow for increased force of contraction as observed with 5-HT. Filippov *et al.* (1985) suggested that a slow calcium channel (but not a fast sodium channel) was responsible for the generation of action potentials in the heart of *Spisula sachalinensis*. Their experiments with 5-HT demonstrated that the regulation of molluscan heart tension by 5-HT was not related to the membrane potential-dependent ionic channels; rather the effect of 5-HT was mediated through chemosensitive calcium channels.

Of interest is the study by Huddart (1986) on the influence of, amongst other compounds, 5-HT on calcium efflux from locust foregut muscle. 5-HT stimulated calcium entry to the fibres, which was monitored as the subsequent calcium efflux in the experiments. This calcium entry was abolished in calcium-free media. Verapamil was also shown to block field stimulation-induced responses of the foregut. Huddart concluded that the effects of 5-HT on the contractile activity of the foregut muscle resulted from a rise in cellular

free calcium. The mechanism for elevating this free cellular, or activator, calcium appeared to be an agonist-induced influx of calcium from the external medium via voltage-dependent slow calcium channels. This inward calcium signal was secondarily amplified by the triggering of a calcium response from cellular storage sites. It is possible, from the evidence discussed here, that this pathway might be common in invertebrates and an important agonist-induced mechanism to increase intracellular calcium; possibly via cAMP, by opening voltage-dependent slow calcium channels.

There is a significant modulatory effect exerted by divalent cations such as calcium upon the excitatory effect of 5-HT on the isolated heart. By lowering the external calcium concentration, the response of the isolated heart to 5-HT was potentiated. This was consistent across several cell types and tissues. Early intracellular recordings from N1E-115 cells demonstrated that depolarising responses to 5-HT were augmented by the omission of calcium from the recording solution (Peters and Usherwood, 1983). More recent patch-clamp studies on N1E-115 cells have shown that calcium, magnesium and other divalent cations modulate the amplitude and duration of the 5-HT-induced currents: the effect occurred in the absence of any change in the reversal potential of 5-HT and showed no obvious voltage dependency (Peters *et al.*, 1988).

Precisely how a reduction in extracellular calcium facilitates the response to 5-HT in *Helix* heart remains unclear. In the *Helix* heart, the influx of extracellular calcium, as well as release of calcium from intracellular stores, could both play a role in contraction of the heart. The results with verapamil support this hypothesis because verapamil failed to completely block the inotropic 5-HT response in the isolated heart. This appears to indicate that the release of calcium from intracellular stores is significant in the inotropic response to 5-HT in the isolated heart. Peters *et al.* (1988) suggested that calcium could affect the affinity of the 5-HT receptor by modulation of receptor-ion channel or receptor-enzyme coupling via changes in surface potential. This hypothesis remains to be verified.

The possibility of the presence of more than one 5-HT receptor

Investigations of recent years have proved that 5-HT possessed both depolarizing (excitatory) and hyperpolarizing (inhibitory) effects on molluscan heart muscle cell membranes (Hill, 1974). It was suggested by Kiss and S-Rózsa (1978) that the different 5-HT responses of *Helix* heart muscle cells involved different specific 5-HT receptors, similar to the molluscan neurones (Gerschenfeld and Paupardin-Tritsch, 1974). Kiss and S-Rózsa (1978) measured the effects of 5-HT on the membrane potential of *Helix pomatia* heart which had been pretreated with a wide range of drugs. Two types of 5-HT receptor were distinguished, one causing depolarization the other hyperpolarization. Concentrations of between 10^{-8} to 10^{-6} excited while 10^{-6} to 10^{-4} inhibited. However no specific antagonist was found for either of the suggested 5-HT receptors.

Further evidence of the presence of more than one 5-HT receptor in molluscan hearts was put forward by Akagawa *et al.* (1988). They found that 5-HT had dual effects on the isolated ventricle of *Achatina fulica*. 5-HT at concentrations above 1nM enhanced frequency and amplitude of heart beat while at high concentrations ≈ 10μM 5-HT produced a periodic arrest of beating after potentiation. Methysergide inhibited the potentiation of heart beat and cinanserin strongly antagonized the periodic arrest of beating which suggested that the dual effect of 5-HT was mediated through separate receptors. In the present study 5-HT was observed both to have a positive inotropic and chronotropic effect. However only the inotropic effect was monitored in these experiments as the chronotropic effect was variable and only noted at high 5-HT concentrations. Unlike that seen in *Achatina* heart no periodic arrest of beating was observed at 5-HT concentrations over 10μM in *Helix* heart. Evidence from this investigation therefore, gives no indication of the presence of more than one 5-HT receptor on the heart muscle cells of *Helix aspersa*.

Conclusion

From the evidence presented it would appear that the 5-HT receptor which mediates the inotropic response of 5-HT on *Helix* heart is unique and does not fall into any of the 5-HT receptor types which have been described in vertebrate tissues. This is supported by the results that 5-HT was mediating its

inotropic action by activation of cAMP levels. Very few, the 5-HT_4 and 5-HT_1-like receptor, of the fully characterized and classified types has been positively linked to cAMP. The presence of a 5-HT_4 receptor has been discounted, due to the failure of ICS 205-930 at high concentrations to antagonise the 5-HT inotropic response of *Helix* heart. The presence of a 5-HT_1-like receptor has been discounted also because of the lack of effect of methiothepin. However it has become apparent in this investigation that the inotropic effect of 5-HT on the isolated heart could not be attributed entirely to cAMP activation. Paciotti and Higgins (1985) argued that if cAMP was the sole mediator of the effect of 5-HT on molluscan hearts, 5-HT would theoretically be unable to elicit a response if the 5-HT receptor and associated adenylate cyclase were uncoupled: an interesting concept and one still to be evaluated.

The activation of *Mercenaria* heart by phorbol esters, reported by Deaton and Gray (1990), suggested the involvement of protein kinase C and associated inositol phospholipids. Is the action of 5-HT on molluscan hearts similar to that in the salivary gland of the blowfly, *Calliphora erythrocephala*? There, 5-HT increased the rate of secretion and both calcium and cAMP were second messangers for 5-HT. The first indication of a link between calcium mobilization and phosphatidylinositol breakdown in blowfly salivary glands was the demonstration that 5-HT increased phosphatidylinositol breakdown through a mechanism that was not secondary to elevated cAMP levels (Fain and Berridge, 1979). Since that time it has been demonstrated that separate 5-HT receptors are involved in activation of adenylate cyclase and phosphatidylinositol breakdown. Could it be that there is more than one 5-HT receptor present in *Helix* heart? This possibility requires further investigation because none of the data presented in this study gives any indication of the presence of more than one 5-HT receptor.

CHAPTER FOUR

THE 5-HT RECEPTOR ON *HELIX* PRM

INTRODUCTION

In addition to the potent actions of 5-HT on molluscan neurones and cardiac muscle, as described in the preceding chapters, 5-HT has been shown to have an effect on other molluscan tissues. Since the 1950s there has been an accumulation of evidence suggesting a transmitter role for 5-HT in molluscan somatic and visceral muscles. Most of the investigations on the action of 5-HT have been undertaken on the anterior byssus retractor muscle (ABRM) of *Mytilus*. In contrast, there have been few studies on the action of 5-HT on molluscan visceral muscle.

Action of 5-HT on molluscan muscle

The ABRM of *Mytilus edulis* is a non-striated paramyosin-containing catch muscle which maintains the shell of these lamellibranchs closed for long periods. This muscle was found to be capable of two types of contraction; a brief contraction which persists only during stimulation and a prolonged contraction which continues long after stimulation has ceased. This sustained contraction was called catch. In this catch state there was an absence of electrical potentials and membrane depolarization (Twarog, 1960a). In the absence of the catch state in Mytilus nerve-muscle preparation, 5-HT had no effect on resting membrane potential, junction potentials, spikes or contraction (Hikada *et al.*, 1967). 5-HT had been found to cause relaxation of catch contraction in the ABRM. The evidence for a transmitter role for 5-HT in the muscle has already been discussed.

The PRM of *Helix* is another molluscan somatic muscle found to be sensitive to 5-HT. It is a large discrete muscle which runs from the columellar region of the shell to the buccal mass. Each half of the muscle is innervated by a short nerve running from the suboesophageal ganglia. The PRM is innervated diffusely and there is no evidence for the presence of neuronal cell bodies in the muscle itself (Ramsay, 1940). The PRM was used first by Bozler (1930), who investigated heat production of smooth muscle. The mechanical properties both of resting and active PRM have been described (Abbott and Lowy, 1958a,b). Two of the major findings of these studies were that: i) the

PRM exerted no significant rest tension until it had been stretched past the largest length that it reached in the animal, and ii) that the contraction of the PRM far outlasted the active state in the muscle. The relationship between the mechanical and electrical properties of the PRM were studied by Sato *et al.* (1960). They recorded both neuromuscular junction potentials and muscle action potentials.

In addition the PRM has been shown to be sensitive to 5-HT. Lloyd (1978) found that the PRM contained large quantities of 5-HT. Application to the muscle of fractions obtained by gel filtration of a PRM extract demonstrated that 5-HT was the only relaxing factor present in the muscle. Lloyd (1980) subsequently provided further evidence for the mediation of 5-HT in the post-tetanic increase in relaxation rate by showing the effect of reserpine on the PRM. Reserpine markedly reduced the 5-HT content of the PRM. Furthermore, the PRM of reserpine-treated animals showed no significant post tetanic increase in relaxation rate. He observed also that, on the PRM, the commonly used 5-HT antagonist methysergide was unsuccesful in blocking the action of 5-HT. It proved to be an effective agonist but an ineffective antagonist for the increase in relaxation rate in the PRM. The actions of 5-HT on the PRM were similar to its effect on the ABRM of *Mytilus*. When stimulated to contract in certain ways, the ABRM entered catch, a prolonged contraction (Twarog, 1954). Exogenous 5-HT caused a relaxation of this catch contraction. Lehman and Greenberg (1987), while investigating the effects of not only molluscan peptides, but also 5-HT and ACh, on visceral and somatic muscles of *Helix aspersa*, noted that the relaxing effect of 5-HT on the PRM was best observed after the muscle had been contracted with ACh.

Mediation of the effect of 5-HT by cAMP in molluscan muscle

There has been considerable interest in cAMP and the action of 5-HT in molluscs. Higgins (1974), Higgins and Greenberg (1974) and Achazi *et al.* (1974) have provided evidence that there is a correlation between 5-HT action and cAMP levels within molluscan tissues. In the ABRM, cAMP had been implicated as the second messenger in the relaxant action of 5-HT. Interest in this hypothesis led Cole and Twarog (1972) to survey the pharmacology of cAMP, dibutyryl cAMP and certain compounds said to act on adenylate cyclase. Although application of cAMP had no effect, dibutyryl cAMP produced relaxation similar to that evoked by 5-HT, but only at 5°C and not in all

preparations. Theophylline, a phosphodiesterase inhibitor, potentiated the relaxation in the ABRM. Achazi *et al.* (1974) confirmed some of the above pharmacological results and extended the study. Marchand-Dumont and Baguet (1975) reported that cAMP induced relaxation in chemically treated, permeable muscle fibre bundles in *Mytilus*. They proposed that the relaxing effect of 5-HT was due to an increase in the rate of calcium release from the contractile proteins and that this was mediated through cAMP.

In addition $^{45}Ca^{2+}$ flux studies on the whole muscle (Bloomquist and Curtis, 1972, 1975a), and measurements of net Ca^{2+} flux with an extracellularly placed calcium electrode (Bloomquist and Curtis, 1975b), have shown that 5-HT caused a transient increase in Ca^{2+} efflux. This suggested that changes in calcium concentration or calcium permeability of the muscle surface membrane were involved also in the relaxation of catch contraction. Ishii *et al.* (1989) investigated the effects of 5-HT and forskolin on intracellular free calcium in isolated and Fura-2 loaded smooth muscle cells from the ABRM of *Mytilus*. Their results suggested that 5-HT had multiple effects on the intracellular free calcium concentration in the ABRM and that cAMP was implicated in this effect. One of the mechanisms which underlay these responses was the inhibition of an agonist-induced release of stored calcium. From these results it appeared that the relaxant effect of 5-HT on the *Mytilus* muscle was linked intimately both with cAMP levels and intracellular free calcium concentration though it still remained for the entire mechanism of catch to be elucidated.

Action of 5-HT on molluscan visceral muscles

Studies of the action of 5-HT have not been limited solely to the somatic muscles but have also focused on the molluscan visceral muscles. Greenberg and Jegla (1963) investigated the actions of 5-HT on the rectum of *Mercenaria*. 5-HT excited the rectum, inducing rhythmical activity at lower concentrations, and at high concentrations 5-HT increased the tone. The actions of 5-HT were mimicked by tryptamine and LSD and antagonised by methysergide. Benzoquinonium, tubocurarine and morphine antagonised the tone increase while augmenting the beat induced by 5-HT. From their study they suggested two sites of action for 5-HT on the rectum: one which could be antagonised by methysergide, directly on the muscle and one presynaptically onto cholinergic nerve terminals which could be modified by ACh antagonists. They implied

that the situation was somewhat similar to the hypothesis that had been proposed by Gaddum and Picarelli (1957) to explain the actions of 5-HT on the guinea pig ileum.

Lehman and Greenberg (1987) investigated the actions not only of FMRFamide peptides, but also of ACh and 5-HT, on several visceral and somatic muscles in *Helix aspersa*. Their muscle preparations included the ventricle, epiphallus, crop, tentacle retractor muscle and PRM. 5-HT induced phasic contractions of the epiphallus which were not reduced by methysergide or 2-bromo-LSD. The latter two drugs acted as weak agonists. On the crop, 5-HT reduced the tone while increasing the frequency of spontaneous contractions. The action of 5-HT was not blocked by methysergide or 2-bromo-LSD. Both on the tentacle retractor muscle and PRM 5-HT had a relaxant effect which could not be distinguished by 5-HT antagonists.

An attempt to define the properties of the 5-HT receptor in the ABRM led to a structure-activity study by Twarog and Cole (1972). Dopamine and bufotenine were approximately equipotent with 5-HT, but 6-HT was approximately 100 times less potent than 5-HT. Tryptamine, noradrenaline and adrenaline all showed equally low potency with 5-HTP, N-acetyl-5-HT, α-Me-dopa and melatonin having no effect at all.

Aims

The aim of the present study was to attempt to characterize the 5-HT receptor mediating relaxation in the PRM of *Helix aspersa* as one of the vertebrate 5-HT receptor types. Using organ bath methodology, the relaxant effect mediated by 5-HT in the PRM has been investigated. This was achieved by utilising 5-HT's ability to inhibit ACh-induced contraction in the muscle, which proved to be dose-dependent and was quantifiable. In an attempt to characterize the receptor, specific 5-HT receptor agonists and antagonists have been tested on the muscle preparation. Because the evidence relating to smooth muscle preparations amongst molluscs has suggested that 5-HT might be mediating its relaxant effect through elevated cAMP levels, measurements of cAMP concentrations in the muscle, under appropriate conditions, were obtained in order to provide further information with which to characterize this 5-HT receptor.

Before the PRM was finally selected, not only for its consistent response to 5-HT, but also for the overwhelming evidence that 5-HT was indeed the relaxant neurotransmitter in this muscle, a number of other muscle preparations from *Helix* were investigated with reference to the action of 5-HT. The search for a suitable preparation as a bioassay included muscles of the visceral type, because previous evidence had shown that both molluscan rectal and oesophageal preparations could be used in pharmacological assays and were responsive to 5-HT. The preparations used from the *Helix* therefore included the rectum and the oesophagus. The same experimental protocol was used as that utilized for the PRM and the effects of 5-HT on these muscle preparations were monitored. The results both with the rectum and the oesophagus are shown and discussed later in further detail.

METHODS AND MATERIALS

Animals

Common garden snails, *Helix aspersa* were obtained from Blades Biological. They were maintained in humid tanks enclosed with wire mesh, at room temperature and fed at regular intervals. All snails used in this study were kept in the active state for at least one week prior to use.

Dissection

The snail shell was cut away carefully with the aid of a pair of sharp scissors and removed entirely. The snail was then pinned in a wax-bottomed dissecting dish. The body wall was cut dorsally from near the top of the head and the genital organs were removed. A diagram of the internal organs is given in Fig. 4.1. In the case of the PRM, the position of which is shown in Fig. 4.2, the visceral organs were lifted with forceps and cut away from the buccal mass. The nerve collar which comprises a pair of cerebral ganglia and which cover the oesophagus, was slipped forward over the buccal mass and then cut away. This left visible the buccal mass and the PRM attached to its ventral surface. The muscle was then ligated at both ends with short pieces of silk thread. The ligated preparation with the two small thread loops could then be cut away from the rest of the body, at one end close to the buccal mass and at the other end close to the columella of the shell. In the case of the oesophagus, once the snail was opened the crop was lifted, thus making visible the oesophagus. The oesophagus was again ligated with short pieces of silk thread which were knotted to form small loops at each end. The preparation was then cut away from the rest of the body. The rectum was rather difficult to find and required that the snail be completely opened up. The easiest way was to trace the gut from the oesophagus through the digestive gland past the nephridium and out towards the anus. Once the rectum had been identified it, like the other preparations, was ligated also with silk thread leaving small thread loops. The rectum was then cut away from the rest of the snail body.

Experimental protocol

Once all these preparations had been cut away from the animal they were mounted vertically and secured, by means of the small thread loops, in

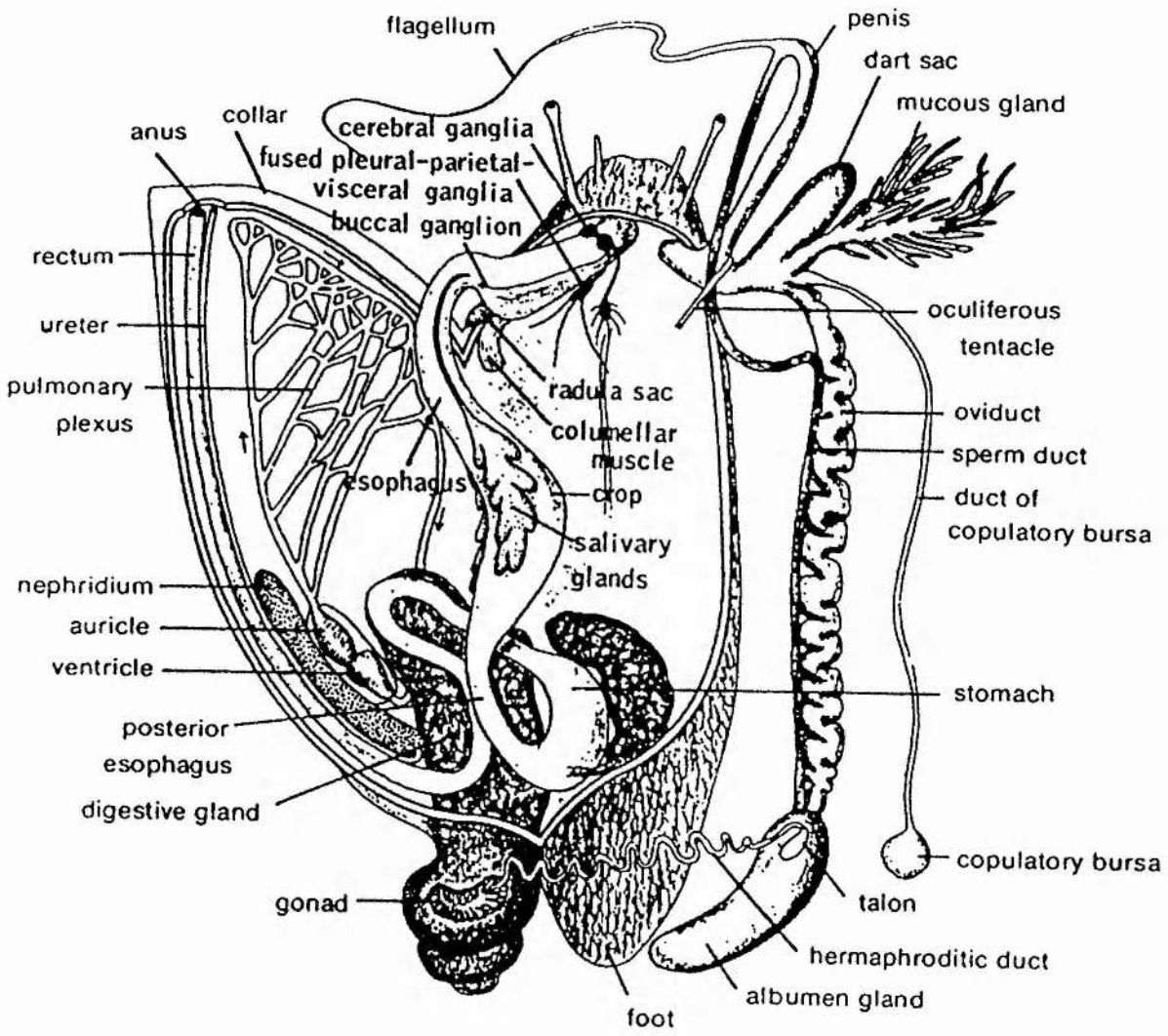


FIGURE 4.1

Diagram of the internal organs of the snail, *Helix aspersa*. This diagram shows the intestinal tract with reference to the oesophagus, anterior and posterior parts, and the rectum which were tested for their responses to 5-HT in this study. This figure was modified from Invertebrate Zoology ed. R.D. Barnes, Sanders College (1980).

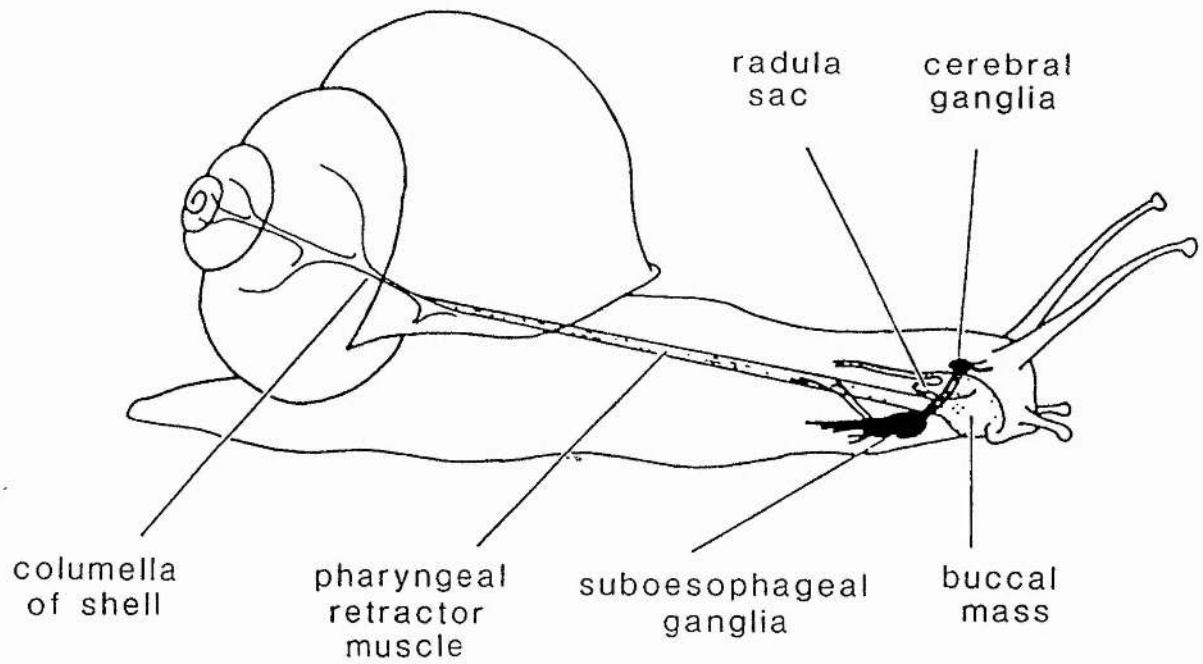


FIGURE 4.2

Diagram to show the position of the PRM in *Helix aspersa* and its innervation. As can be seen from the diagram, the PRM runs from the buccal mass to the columella of the shell where it is attached. In this diagram the PRM is shown in the elongated form. In a non-active snail inside its shell, the muscle would be considerably shortened. The main innervation of the muscle comes from connections to the suboeosophageal ganglia. The drawing was modified from Leake and Walker (1980).

10ml organ baths containing physiological saline (NaCl 80mM, KCl 5mM, $CaCl_2$ 7mM, $MgCl_2$ 5mM, Hepes 20mM; pH 7.5). Fig. 4.3 shows diagrammatically the experimental apparatus. The upper end of the preparation was connected via a small hook to a Dynamometer (UFI) isometric transducer, which in turn was connected to an 8-channel Lectromed recorder. The lower end of the preparation was attached to an L-shaped stainless steel wire support which was clamped above the bath. In this way any muscle movement could be detected by the transducer. This type of transduction measured the shortening of the muscle/gut preparation. All the experiments were undertaken at room temperature (18-20^{o}C) and were gassed by a 95% 0_2, 5% CO_2 mixture.

In the case both of the oesophagus and rectum preparations, these were used only as trials in an attempt to find a preparation which responded well to 5-HT. Here 5-HT was added to the bath in appropriate concentrations in a Gilson pipette. The drugs were always added to the airstream caused by the gassing mixture so that this ensured rapid and even dispersion. The sensitivity of the tissue to the 5-HT was then observed on the chart recorder, examples of which are shown later. No other drugs were tested on these two preparations.

In the case of the PRM it was found that the effect of 5-HT on the muscle was to cause relaxation. This response was adequately observed only if the muscle had previously been contracted. ACh was used to contract the muscle. Full concentration-effect curves to ACh were obtained because ACh caused dose-dependent increases in the size of contraction. These curves were obtained both in the presence and absence of eserine (1μM), a naturally occurring tertiary amine which acts effectively as an acetylcholinesterase inhibitor. Once a full concentration-effect curve for ACh had been established a concentration resulting in approximately 50-70% maximum contractile response was chosen. This concentration of ACh, usually 3μM, was then used to induce contraction in the PRM. The appropriate ACh concentration was added to the bath every 20 minutes, the muscle allowed to contract and the drug washed out with fresh physiological saline. In this way the muscle gave reproducible responses to ACh which were consistent in size and the muscle tissue did not appear to exhibit desensitization.

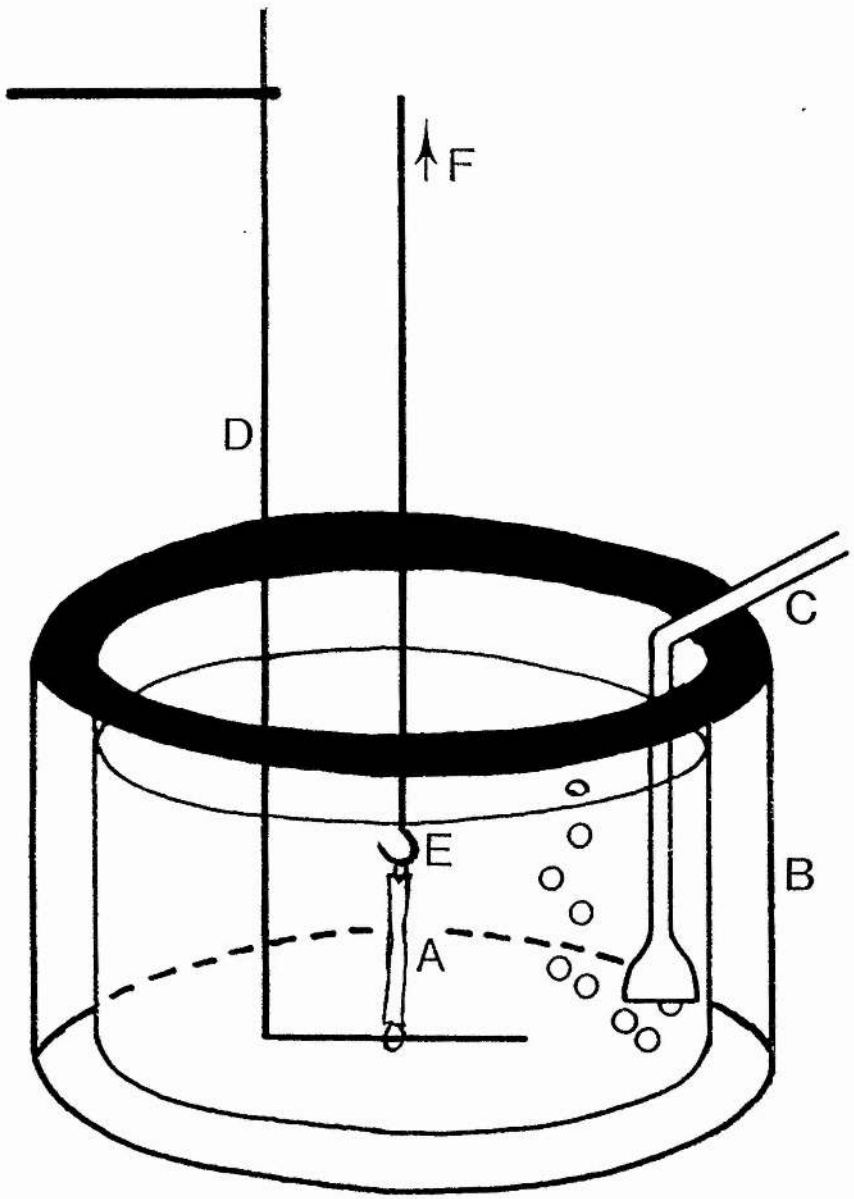


FIGURE 4.3

Diagram of the experimental apparatus used in the muscle experiments. The ligated PRM (**A**) was suspended in a 10ml jacketed organ bath (**B**) in physiological saline at room temperature. The muscle was held in position between a fixed L-shaped steel wire support (**D**) which was kept rigid in a clampstand above the bath, and a wire hook (**E**) which was attached to a force transducer (**F**). In this way any movement of the muscle could be monitored. The physiological saline was gassed with a 95% 0_2, 5% CO_2 mixture (**C**).

Effects of agonists

For the experimental protocol involving 5-HT and the testing of the relevant agonists and antagonists, eight muscle preparations were set up in individual organ baths. ACh at a concentration of 3μM was then added to each of the eight organ baths once every 20 minutes. This allowed the PRM to contract and the ACh to be washed out properly before the next administration. Of the eight muscle preparations, two would act as control with ACh added every 20 minutes for the whole period of the experiment. No other drugs were added to the control preparations. This could then be used to verify the sensitivity of the PRM to ACh and to demonstrate any deterioration of the contractile response to ACh.

Of the remaining preparations, once the ACh contractile responses were consistent, a concentration of 5-HT was added to the baths three minutes before the next administration of ACh. These drugs were then washed out of the bath once a contractile response had been obtained. 5-HT caused an inhibition of the ACh-induced contractile response so that this response had to return to its original size before the next concentration of 5-HT could be added. ACh was repetitively administered every 20 minutes with three washes of fresh physiological saline being used to remove the drug from the bath. After the contractile response to ACh had returned to its original size, the next concentration of 5-HT could be added and the same procedure followed.

The results are expressed as a percentage of inhibition of the ACh-induced contraction that was measured from the experiments and these values were plotted against log of the molarity of the 5-HT concentration to give the log concentration-effect curves. The range of concentrations over which 5-HT and other test agonists were tested was 0.1μM to 100μM. From the concentration-effect curves, the EC_{50} (molar concentration of each compound to produce 50% inhibition) value could be obtained. This was used to provide an indication of the potency because the EC_{50} value for the test agonist was divided by that of 5-HT to give the relative potency value (EC_R). Where quoted, the EC_{50} value is usually the pEC_{50} value (negative $\log arithm_{10}$ of the EC_{50} value). The maximum percentage inhibitions elicited by the agonists tested were compared to that obtained with 5-HT. All values shown in the results are the arithmetic mean ± s.e. mean from n experiments. The values

for n are given in the results section. All concentrations stated are the final bath concentrations to which the PRM was exposed. The agonists tested were 5-CT and sumatriptan (both 5-HT_1 receptor agonists), α-Me-5-HT (a 5-HT_2 receptor agonist) and 2-Me-5-HT (a 5-HT_3 receptor agonist). The LSD derivative, methysergide and the ergot derivative ergotamine were also tested as agonists in this preparation.

Effects of antagonists

For antagonists, their effect was tested by the addition to the bath of the antagonist over a period of at least 30 minutes both before, during, and after ACh administration, to test if the antagonist affected the contractile response to ACh in any way. The same procedure as for the agonist was followed, the only difference being the presence of the said antagonist. If antagonist action was established, the agonist concentration-ratio could then be calculated for each antagonist concentration (Apperley *et al.*, 1976). The estimates of concentration-ratio could then be used to calculate the PA_2 values (Arunlakshana and Schild, 1959). The antagonists tested were methiothepin (a 5-HT_1 receptor antagonist), ketanserin (a 5-HT_2 receptor antagonist), ondansetron and metoclopramide (both 5-HT_3 receptor antagonists).

Drugs used

The full list of the drugs used in the experiments and from where they were obtained is given: 5-HT creatinine sulphate (Sigma), ACh chloride (Sigma), eserine/physostigmine salicylate (Sigma), ergotamine tartrate (Sigma), methysergide hydrogen maleate (Sandoz), metoclopramide hydrochloride (Sigma), methiothepin maleate (Hoffman La Roche) and ketanserin (Janssen). 5-CT maleate, α-Me-5-HT hydrochloride, sumatriptan and ondansetron were all synthesized by the Chemistry Reseach Department at Glaxo. All stock solutions of the drugs were made up fresh on the day of use in physiological saline and kept on ice, with the exception of ketanserin and ondansetron both of which were first dissolved in 0.1M tartaric acid with further dilutions in physiological saline. Where appropriate, vehicle controls were made to see if the solvent had any effect.

Measurement of cAMP

The same experimental protocol as in the previous chapter was followed for measurement of cAMP. There were, however, two differences in the apparatus. The muscle tissue was homogenized using a teflon-glass motor-driven homogenizer (Jencons Scientific) and the optical density of each protein assay sample was determined at 750nm on a Phillips PU8620 UV spectrophotometer. The results were expressed as pmoles cAMP.mg^{-1} tissue protein. These values were the arithmetic mean ± s.e. mean of n, n being the number of experiments.

RESULTS

Results relating to the effect of 5-HT on the oesophagus and rectum of *Helix*

The degree of torsion within the shell of *Helix aspersa* resulted in the dissection of the rectum being more difficult than that of the oesophagus. A simplified version of the internal organs of *Helix* is shown in Fig. 4.1. As can be seen from this figure, when the snail is cut open the intestinal tract from the buccal mass to the rectum was easy to identify, once the genital organs had been removed, and could be followed throughout the animal. In this way small pieces of the oesophagus and the rectum could be ligated and mounted in an organ bath.

The spontaneous activity of the freshly isolated oesophagus was highly irregular despite frequent washes and periods of approximately 30-45 minutes, in order to allow the muscle to settle. Different parts of the oesophagus were also used in the various preparations. It was found that the anterior oesophagus, the region between the buccal mass and the crop, was the most robust preparation, having a high degree of spontaneous activity and giving a good response to the application of 5-HT. Examples of the activity of the anterior oesophagus, and its response to 5-HT, are shown in Figs. 4.4 and 4.5. The less spontaneously active posterior oesophagus, and its different response to 5-HT application, is demonstrated in Fig. 4.6. In most preparations 5-HT reduced the tone of the oesophagus and decreased the frequency of contractions in a dose-dependent manner (Figs. 4.4 and 4.5). However, in a small minority of the preparations, those from the posterior region of the oesophagus, 5-HT was observed to have a contractile effect and to increase the frequency of contractions (Fig. 4.6). The observed spontaneous activity of the oesophagus, in particular the anterior region, made quantification of the response to 5-HT extremely difficult. Eventually the PRM was used in preference to the oesophagus because the relaxant response to 5-HT seen in the PRM was easier to quantify and allowed for the testing of specific agonists and antagonists. The response to 5-HT in the PRM was also consistent as compared to the varying responses seen with different parts of the intestinal tract.

The rectum was also used in several preparations. This tissue was difficult to dissect from the animal and much more fragile than the

FIGURE 4.4

Three examples of the response shown by an isolated *Helix* oesophagus to increasing concentrations of 5-HT (**A**, **B** and **C**). The addition of 5-HT is indicated by a closed circle. The stated concentration of 5-HT was the final bath concentration to which the oesophagus was exposed. Washes with fresh physiological saline are indicated by the letter w.

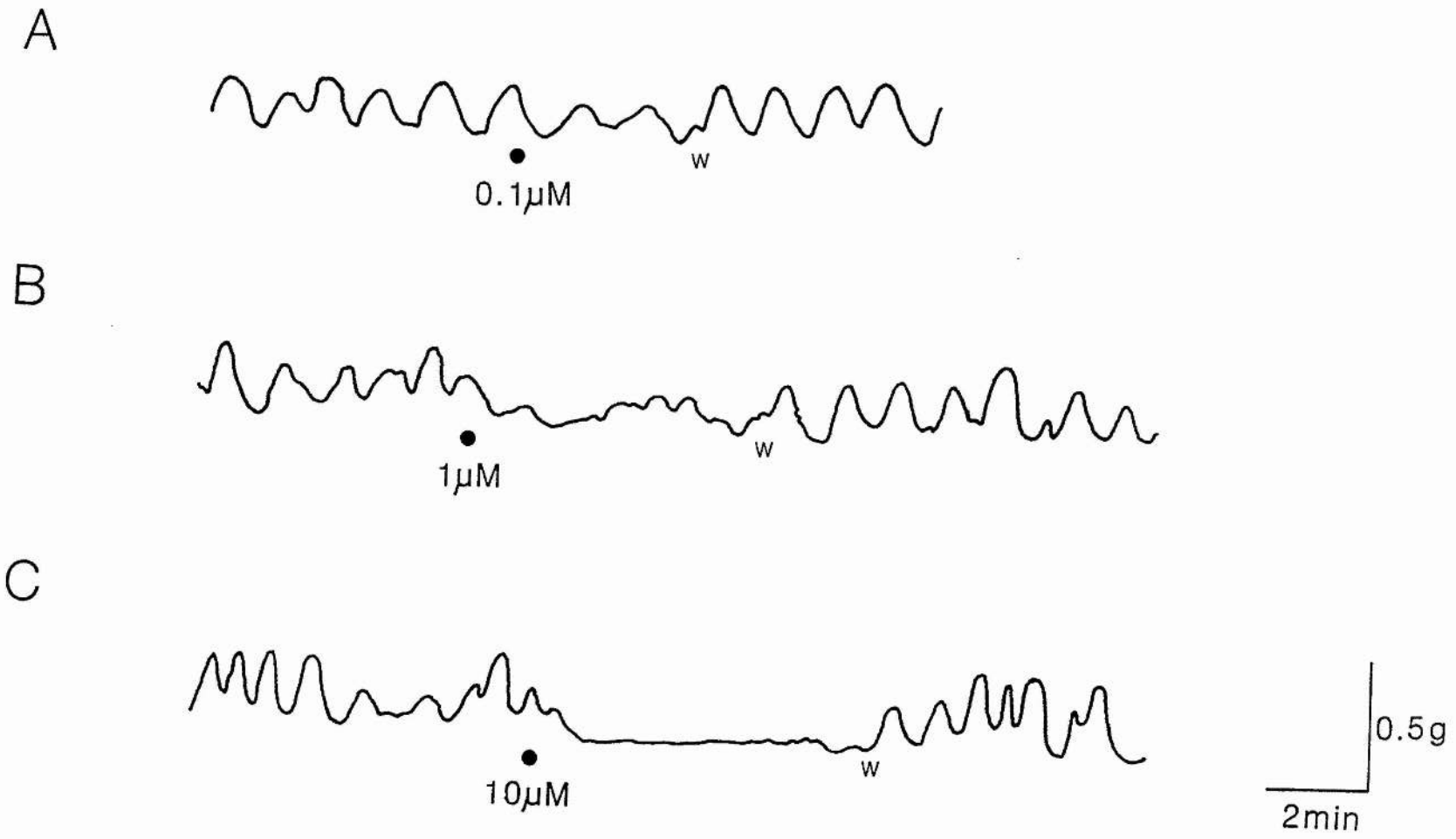

A
0.1μM
W
B
1μM
W
C
10μM
W
0.5g
2min

FIGURE 4.5

Three examples of the response shown by an isolated *Helix* oesophagus to increasing concentrations of 5-HT (**A**, **B** and **C**). The addition of 5-HT is indicated by a closed circle. The stated concentration of 5-HT was the final bath concentration to which the oesophagus was exposed. Washes with fresh physiological saline are indicated by the letter w.

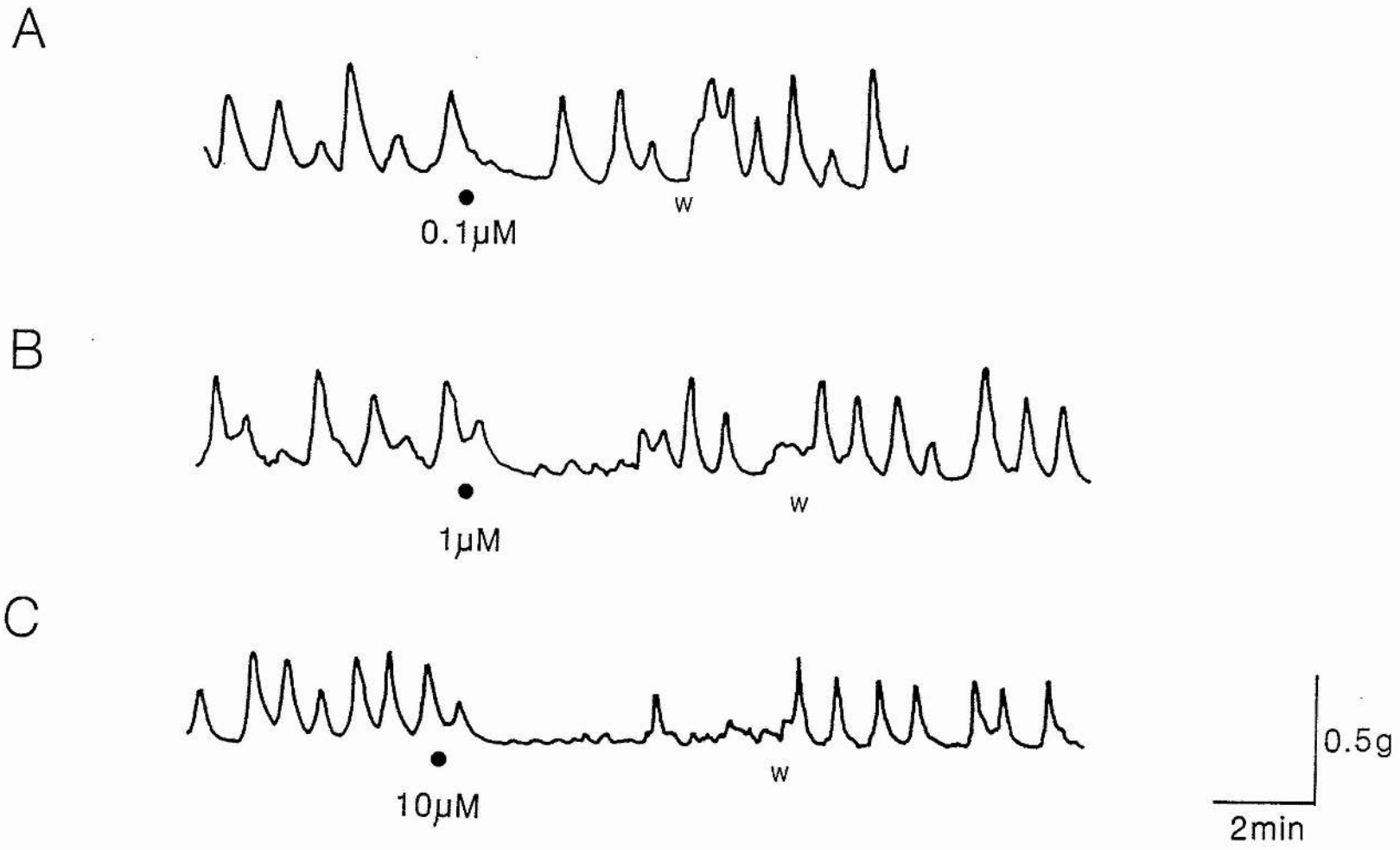
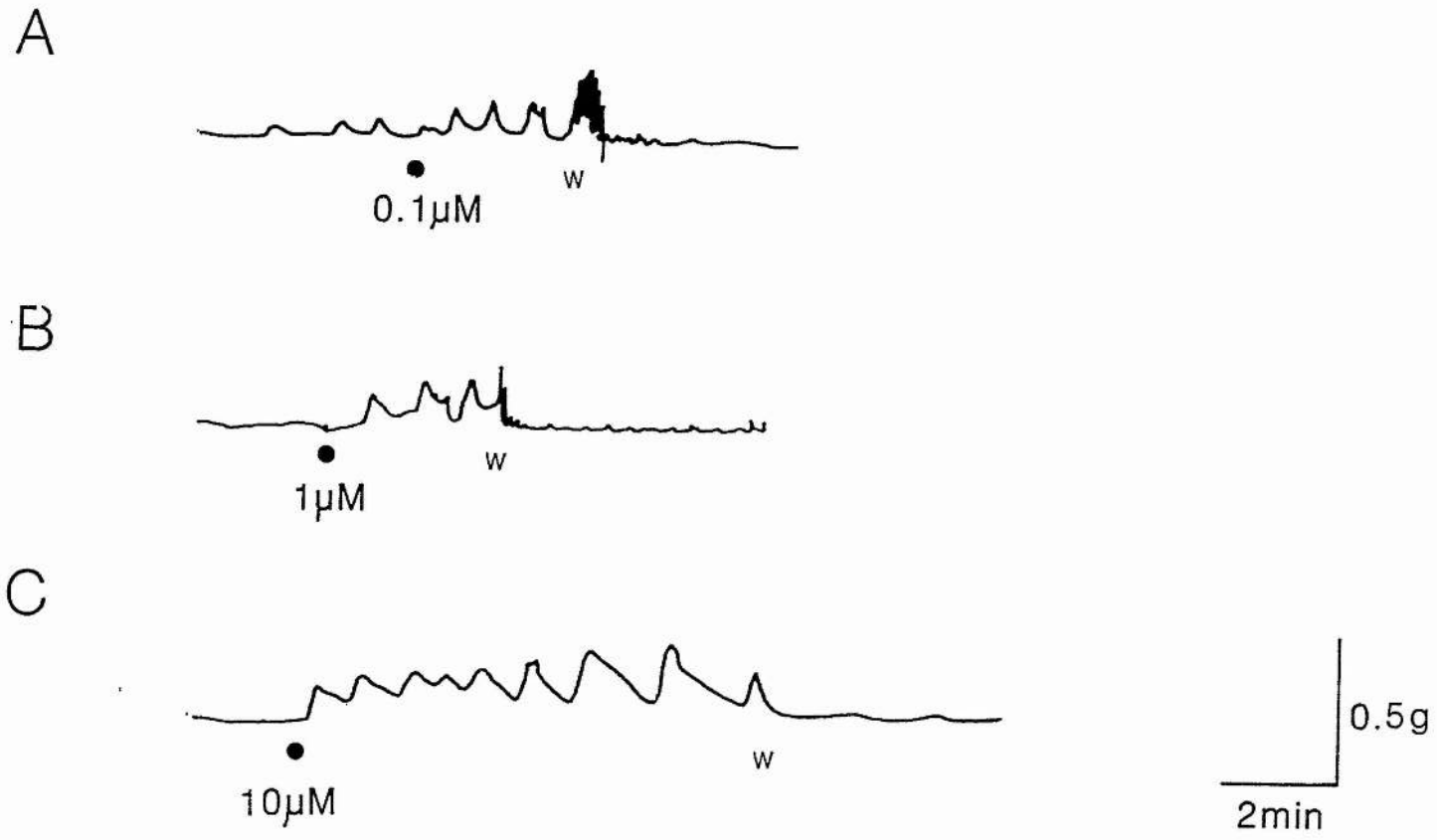

A
0.1μM
W
B
1μM
W
C
10μM
W
0.5g
2min

FIGURE 4.6

Three examples of the response shown by an isolated *Helix* oesophagus to increasing concentrations of 5-HT (**A**, **B** and **C**). The addition of 5-HT is indicated by a closed circle. The stated concentration of 5-HT was the final bath concentration to which the oesophagus was exposed. Washes with fresh physiological saline are indicated by the letter w.


A
0.1μM
W
B
1μM
W
C
10μM
W
0.5g
2min

oesophagus, and therefore easily damaged. The rectum, once isolated in the organ bath, was quiescent and showed little or no response to 5-HT. The results are not shown here because only two experiments were undertaken before the rectum preparation was discarded in preference for the PRM.

Effect of ACh on the PRM

The PRM was usually quiescent when isolated in the organ bath and only infrequently showed spontaneous activity. It was a robust preparation and easily dissected from the animal. The position of the PRM in the snail is shown in Fig. 4.2. After mounting the PRM in the organ bath with an initial tension the PRM relaxed back to its resting state. Once the relaxation was complete and the baseline steady, ACh was applied to the organ bath.

The contractile response of the PRM to applications of increasing concentrations of ACh was dose-dependent (Fig. 4.7A). The PRM tissue was desensitized not only to higher concentrations of ACh (Fig. 4.7B), but also if the ACh was incompletely removed from the organ bath leaving enough time for complete recovery. The experimental protocol, therefore, demanded that the ACh applications be made at 20 minute intervals, with three washes of fresh physiological saline between each application. In the presence of eserine (1μM) the PRM became much more sensitive to applications of ACh (Fig. 4.8). The PRM also showed more rapid desensitization and this was apparent at lower concentrations of ACh in the presence of eserine. In the presence of eserine, the PRM required more frequent washes and a longer time interval between ACh applications in order to avoid desensitization. The concentration-effect curve for ACh, in the absence of eserine, had a threshold concentration of approximately 50nM reaching a maximum at 50μM. In the presence of eserine, the threshold was lowered to 10nM, whereas the maximum response was achieved at a lower ACh concentration (Fig. 4.9). Eserine was seen also to potentiate the effect of ACh, increasing the maximum response seen and shifting the concentration-effect curve to the left.

Due to the problems with desensitization of the ACh responses in the PRM in the presence of eserine, and the subsequent longer time course of the experiment to avoid this problem, a decision was taken not to use eserine in the following experiments. A response of approximately 50-80% of the maximum was taken from the concentration-effect curve (Fig. 4.9) and that concentration of ACh (3μM) was then used to contract the PRM in the

FIGURE 4.7

A. The contractile responses of *Helix* PRM to increasing concentrations of ACh are shown in the figure. The additions of ACh to the bath are indicated by arrows. The stated concentration was the final bath concentration to which the PRM was exposed. Washes with fresh physiological saline are indicated by the letter w.

B. The desensitization shown by *Helix* PRM to higher concentrations of ACh. The additions of ACh to the bath are indicated by arrows. The stated concentration was the final bath concentration to which the muscle was exposed. The letter w indicates washes with fresh physiological saline.

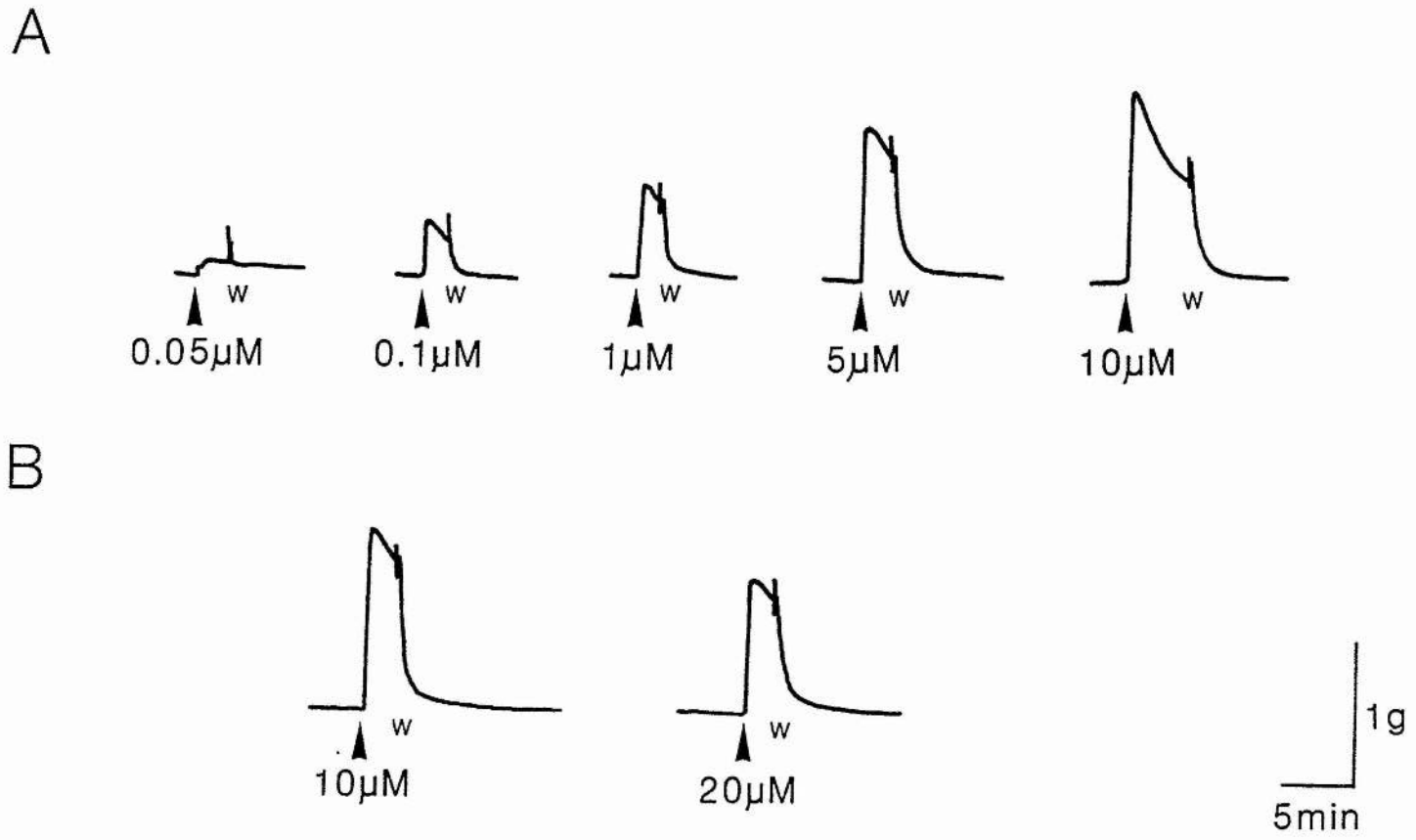
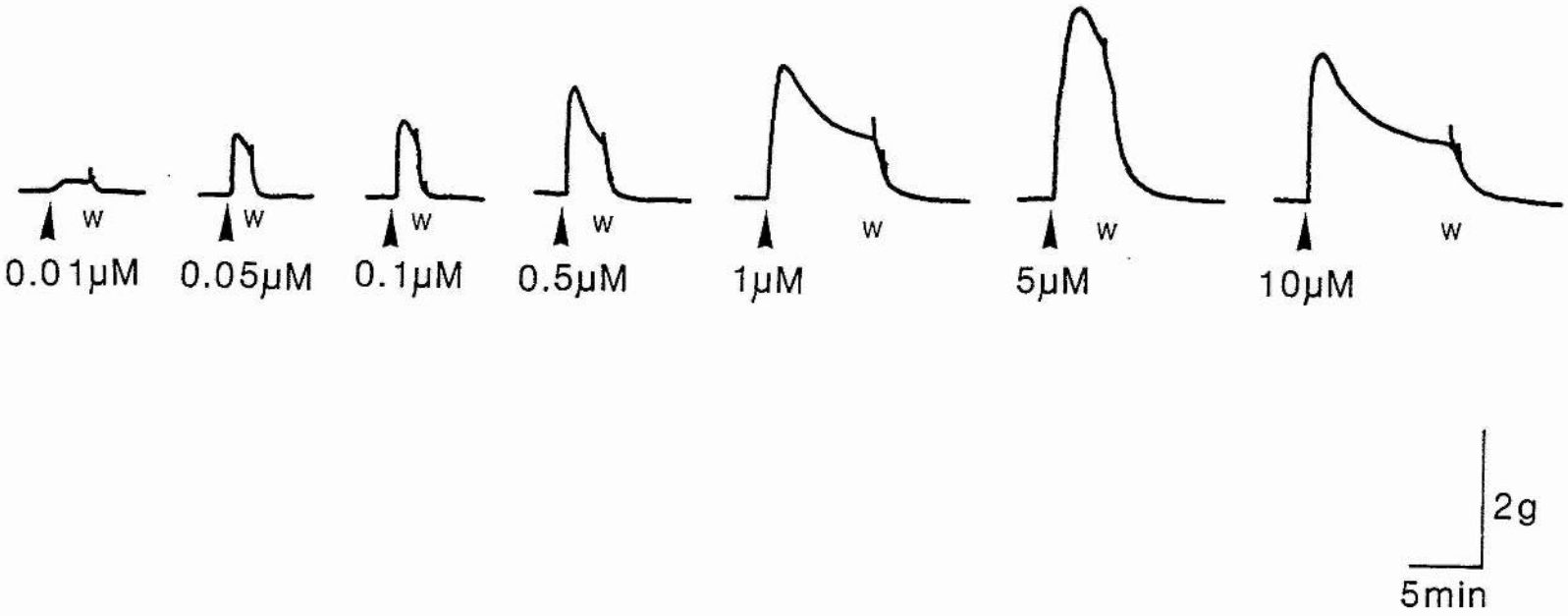

A
W
0.05μM
W
0.1μM
W
1μM
W
5μM
W
10μM
B
W
10μM
W
20μM
1g
5min

FIGURE 4.8

The contractile responses of *Helix* PRM to increasing concentrations of ACh in the presence of 1μM eserine are shown in this figure. The ACh was added to the bath as indicated by the arrows and the concentration stated was the final bath concentration to which the PRM was exposed. The letter w indicates washes with fresh physiological saline. Note the desensitization of the PRM to ACh concentrations >5μM in the presence of 1μM eserine.


W
W
W
W
W
W
W
0.01μM
0.05μM
0.1μM
0.5μM
1μM
5μM
10μM
2g
5min

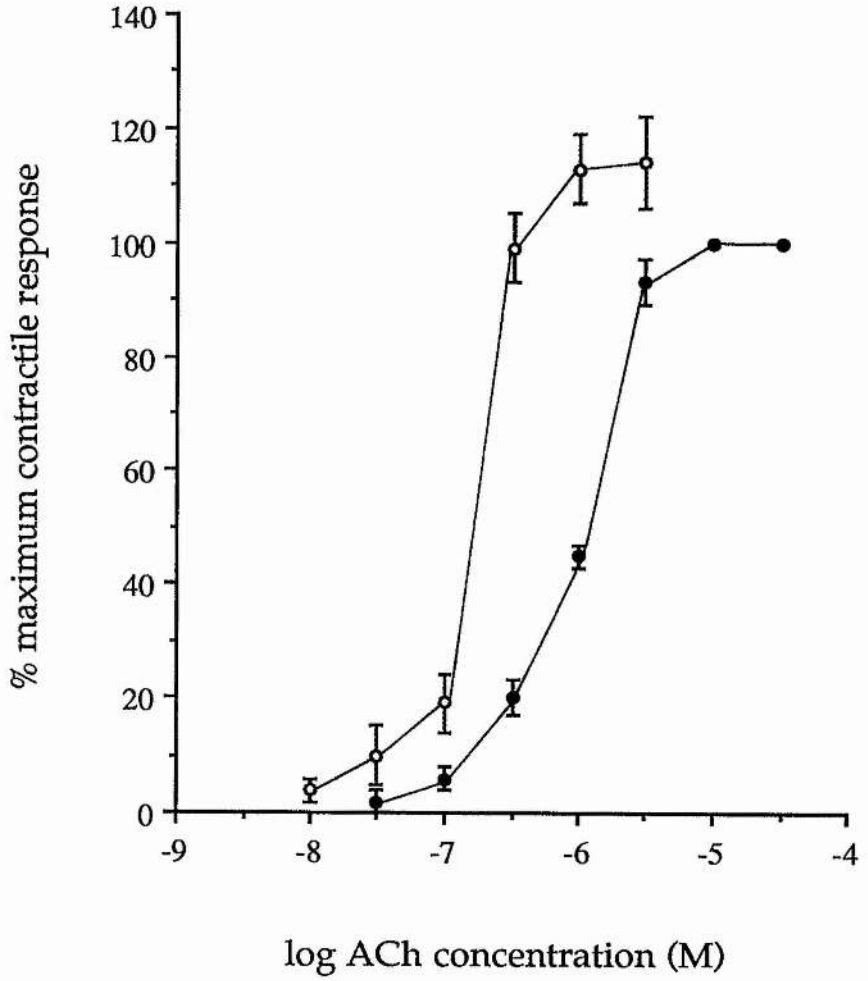


FIGURE 4.9

The concentration-effect curves for ACh-induced contraction in the PRM in the absence (closed circles) and the presence (open circles) of 1μM eserine are shown in this figure. The x axis gives the log of the ACh concentration while the y axis gives the percentage maximum contractile response. Vertical bars represent the arithmetic mean ± s.e. mean of n=6.

following experiments. This value was just above the EC_{50} value (approximately 1μM) that was obtained for the contractile effect of ACh on the PRM. The response of the muscle to repetitive additions of ACh was consistent and showed less than 5% deterioration over the time course of an experiment which could last for anything up to 7 hours. This small deterioration in the ACh response is not shown in the results because it did not interfere with the results and was, therefore, considered insignificant.

Effect of 5-HT on ACh-induced contraction in the PRM

Because 5-HT was known to have a relaxant effect on the *Helix* PRM, the response to 5-HT could be adequately seen only if the muscle had previously been contracted. ACh (3μM) was used to induce the contraction in the PRM. 5-HT relaxed the ACh-induced contraction in the PRM and increased the rate of relaxation in a dose-dependent manner (Fig. 4.10). The PRM was in the process of relaxing after the contraction due to ACh (3μM) when 5-HT was added and a rapid response was seen. However, this relaxant response in the PRM to 5-HT was difficult to quantify because the ACh contractions were not well maintained and an alternative approach to observing the effect of 5-HT was investigated.

Finally it was found that if 5-HT was added to the bath three minutes before the addition of ACh, 5-HT caused a subsequent inhibition of the ACh-induced contraction and that this was dose-dependent (Fig. 4.11). This 5-HT response could easily be quantified as the percentage inhibition of ACh-induced contraction in the PRM. When plotted against the log of the 5-HT concentration, a typical sigmoid concentration-effect curve for 5-HT was obtained (Fig. 4.12). 5-HT had a thresold range of 5-10nM, reaching a maximum at a concentration of 100μM. The threshold for inhibition of ACh-induced contraction by 5-HT was the same for that seen for the relaxant response. An increased rate of relaxation was seen also and was comparable once again to that seen with the relaxant response of 5-HT on the PRM. The time course of the experiment was limited by the fact that over time the ACh contractile responses were themselves very slowly desensitized. Thus 5-HT along with the specific 5-HT receptor agonists and antagonists were tested over a concentration range of only 0.1μM-100μM. The EC_{50} value for 5-HT was between 0.3-0.5μM.

FIGURE 4.10

The relaxant effect of increasing concentrations of 5-HT (**A-C**) on *Helix* PRM are shown after the muscle has been contracted with 3μM ACh. Application of ACh is indicated by arrows. The addition of 5-HT is indicated by closed circles. The stated concentration of 5-HT was the final bath concentration to which the PRM was exposed.

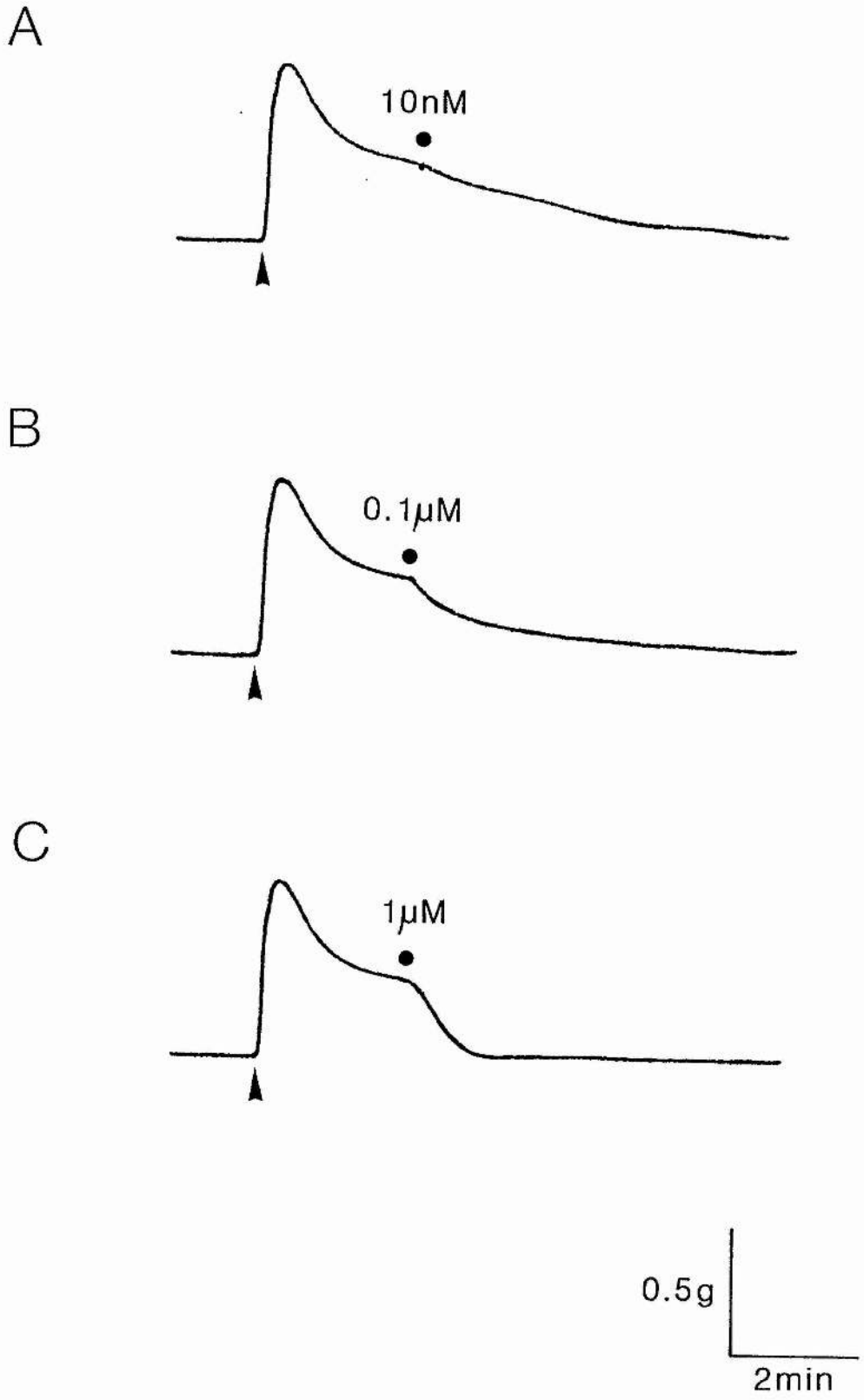
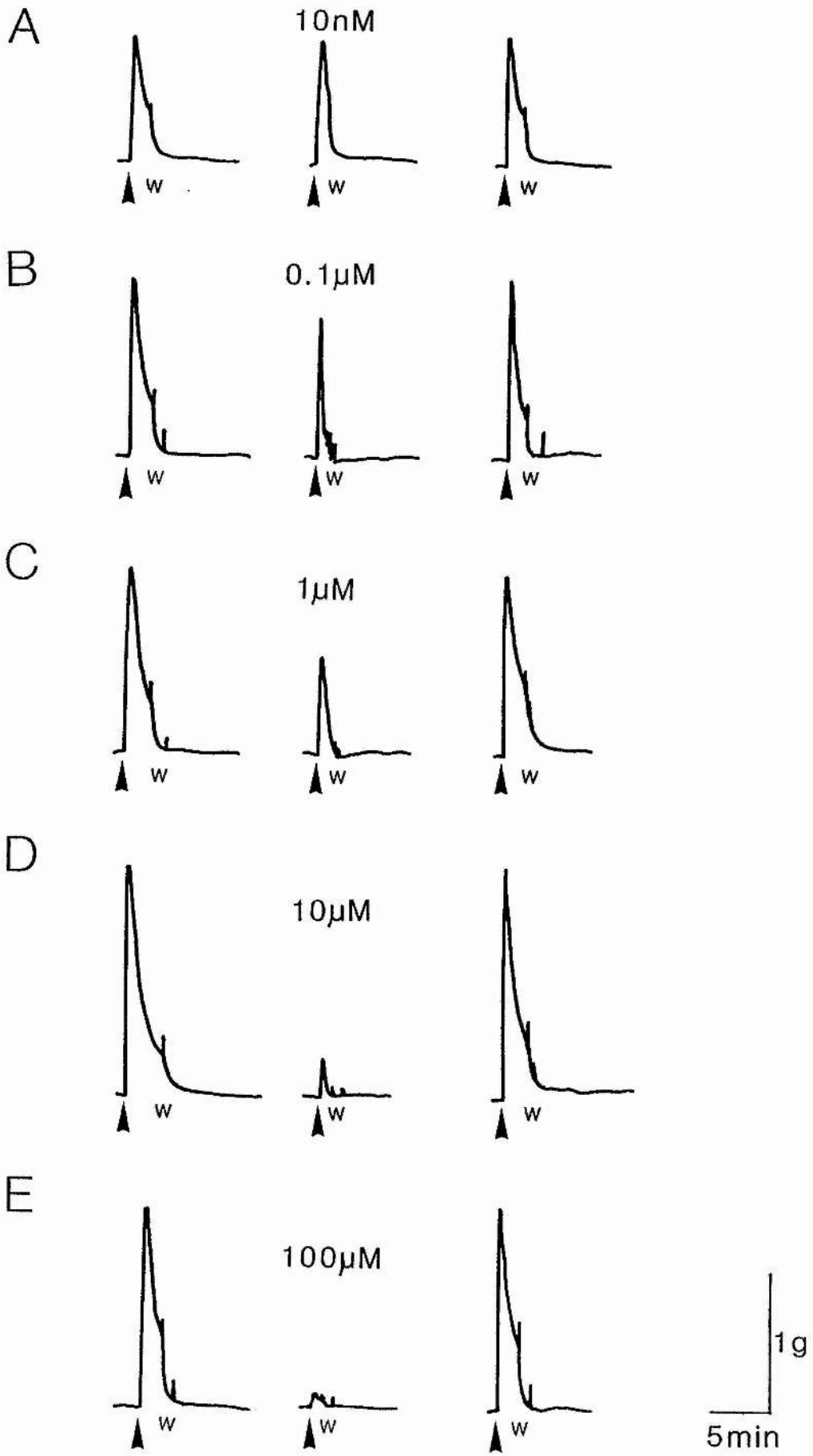

A
10nM
B
0.1μM
C
1μM
0.5g
2min

FIGURE 4.11

The contractile responses of *Helix* PRM to a given concentration of ACh and inhibition of this ACh-induced contraction of the PRM by 5-HT. Five examples (**A-E**) are given in this figure to illustrate the ability of 5-HT to inhibit the ACh-induced contractions in the PRM. Each example shows a control response to ACh, a response to ACh in the presence of 5-HT and a response to ACh after 5-HT has been thoroughly washed out. ACh (3μM) was applied at the arrows. The concentration of 5-HT was increased from **A-E**. The stated concentration of 5-HT was the final bath concentration to which the PRM was exposed. The letter w indicates washes with fresh physiological saline.


A
10nM
W
B
0.1μM
W
C
1μM
W
D
10μM
W
E
100μM
W
1g
5min

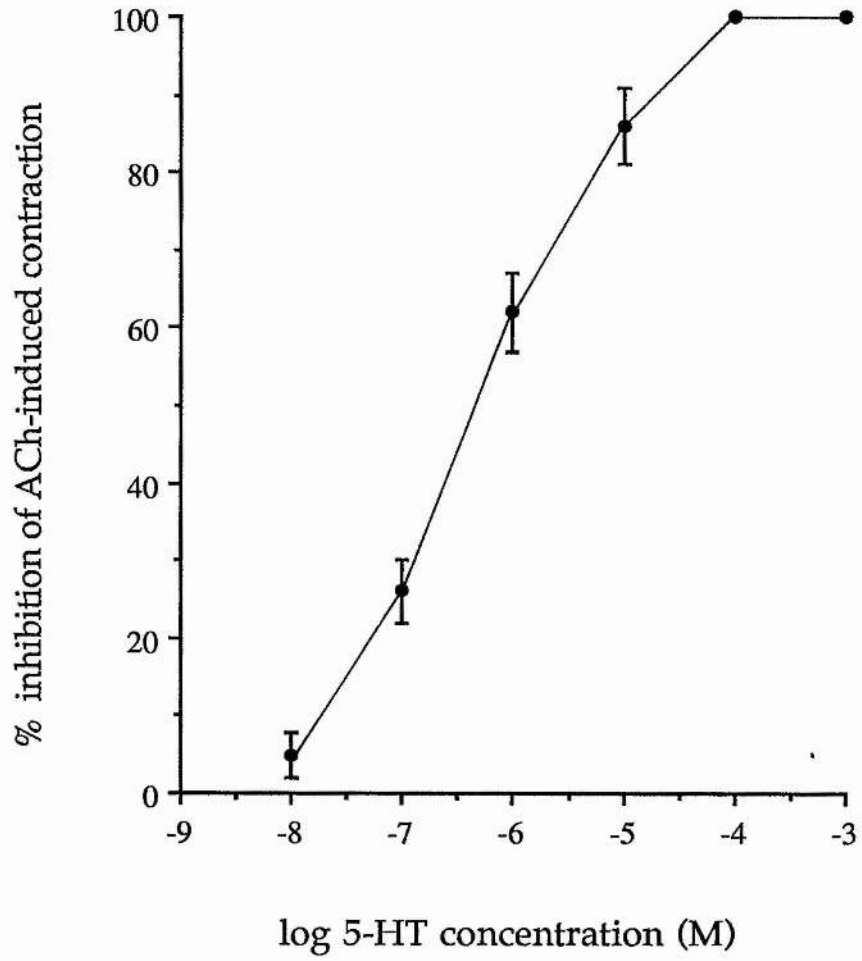


FIGURE 4.12

The concentration-effect curve for the inhibiton of ACh-induced contraction by 5-HT in the PRM is shown in this figure. The x axis gives log of the 5-HT concentration while the y axis gives the percentage inhibition of ACh-induced contraction. The ACh concentration used to induce contraction in the PRM was 3μM. Vertical bars represent the arithmetic mean ± s.e. mean of n=6.

Effects of 5-HT agonists

The 5-HT receptor agonists were tested for their ability to induce inhibition of ACh-induced contraction in the PRM. These were 5-CT, sumatriptan, α-Me-5-HT and 2-Me-5-HT (Figs. 4.13 and 4.14). Also tested as agonists on this system were ergotamine and methysergide. Of the agonists tested, 5-CT was the most potent. 5-CT caused a 75% inhibition at a concentration of 10μM (Fig. 4.15). Sumatriptan caused a similar percentage inhibition (73%) to 5-CT at 10μM. Ergotamine and methysergide shared similar potency, causing a 50-60% inhibition of ACh-induced contraction at 10μM (Figs. 4.15 and 4.16 respectively). α-Me-5-HT and 2-Me-5-HT were much less effective showing only a 5-10% inhibition of ACh-induced contraction at 10μM (Fig. 4.17). From the examples of inhibition caused by these agonists in the figures quoted above, log concentration-effect curves could be obtained. EC_{50} values for each agonist could then be obtained and these were used to estimate the relative potency values (EC_R).

Ergotamine and methysergide were potent as agonists on this preparation in showing no antagonist effect (Fig. 4.16). Sumatriptan showed similar agonist effects to the two latter compounds but was more effective (Fig. 4.15) whereas 5-CT was clearly the most potent agonist mimicking closely the inhibition of ACh-induced contraction effects by 5-HT (Fig. 4.15). Both α-Me-5-HT and 2-Me-5-HT showed little agonist effect producing only maximum inhibitions of only 27% and 23% respectively (Fig. 4.17). 5-CT was only found to be 3 times less potent whereas sumatriptan was 16 times less potent than 5-HT. Ergotamine and methysergide were approximately similar, being 40-45 times less potent than 5-HT. No EC_R values for α-Me-5-HT or 2-Me-5-HT were obtained because they failed to even elicit a 50% inhibition value. The full rank order of potency for inhibition of ACh-induced contraction in the PRM was 5-HT > 5-CT > sumatriptan > ergotamine = methysergide >>> α-Me-5-HT = 2-Me-5-HT. The agonist results are summarized in Table 4.1 overpage.

FIGURE 4.13

The contractile responses of *Helix* PRM to 3μM ACh and inhibition of this ACh-induced contraction by the specific 5-HT receptor agonists tested. An example of the ability of the stated agonist to inhibit the ACh-induced contraction in the PRM, at a concentration of 10μM, is shown in this figure. The agonists are 5-CT (**A**), sumatriptan (**B**) and ergotamine (**C**). Each example shows the control response to ACh, the response to ACh in the presence of agonist (10μM) and the response to ACh once the agonist had been thoroughly washed out. ACh (3μM) was applied at the arrows. The letter w indicates washes with fresh physiological saline. The stated agonist concentration was the final bath concentration to which the PRM was exposed.

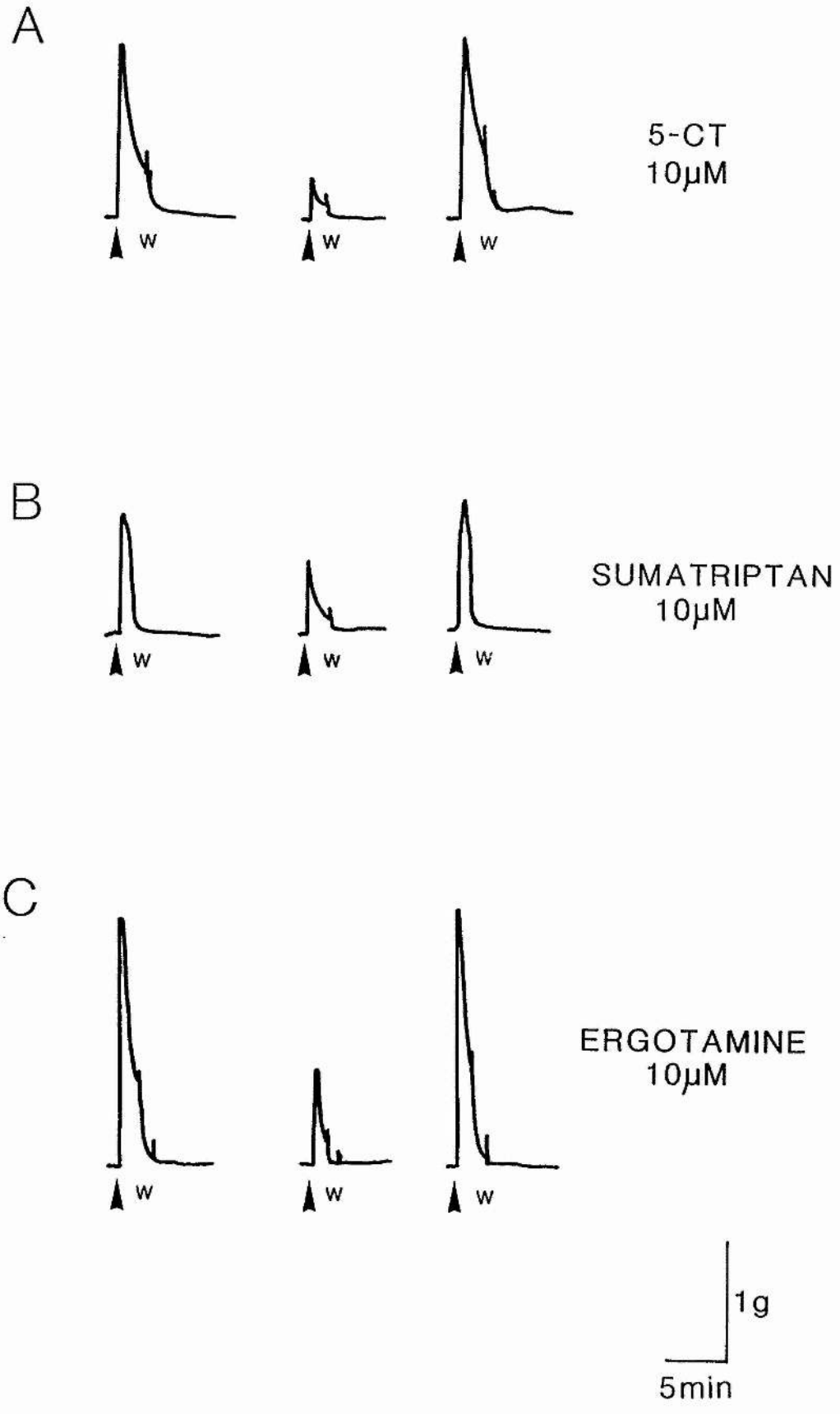
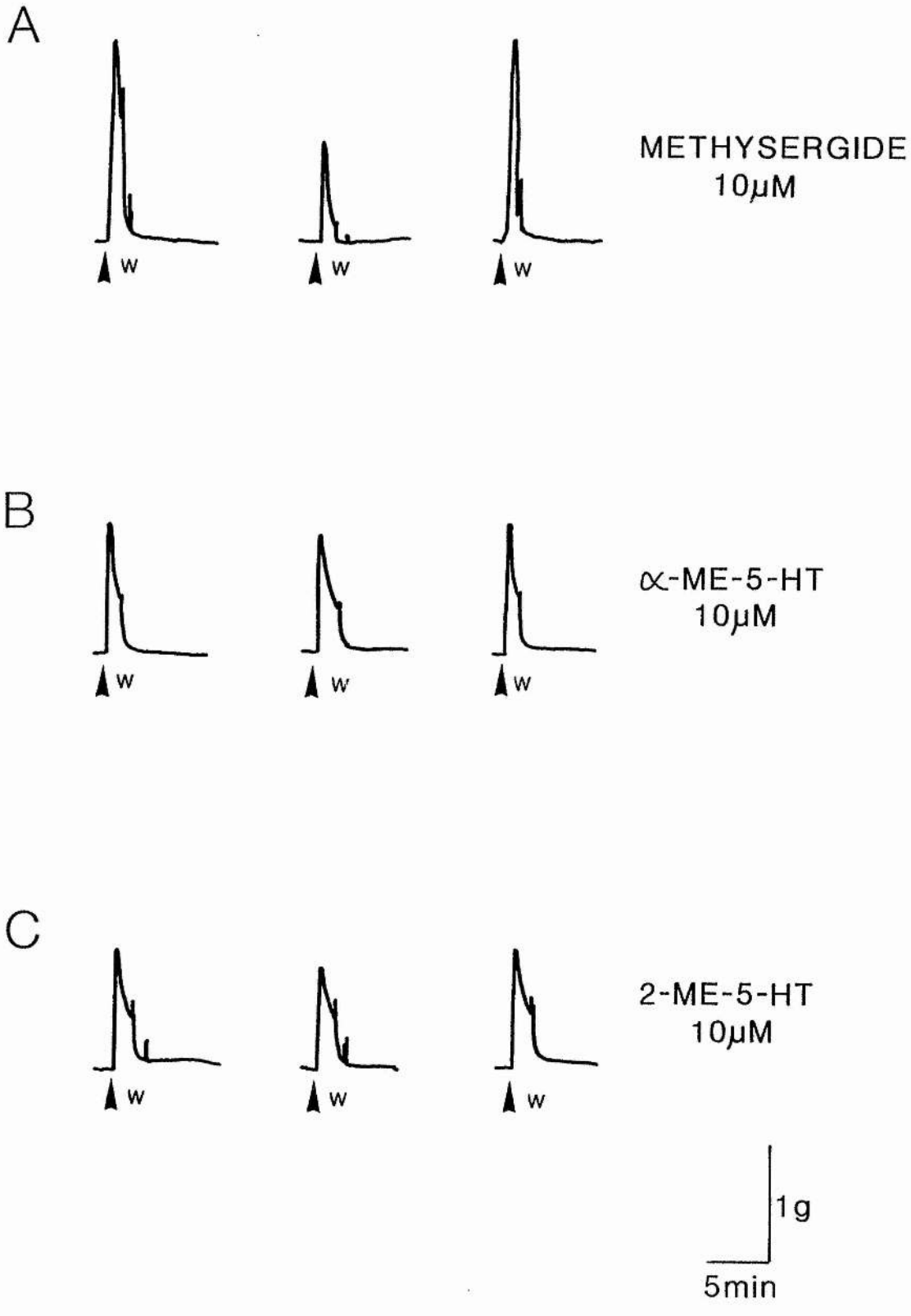

A
W
W
W
5-CT
10μM
B
W
W
W
SUMATRIPTAN
10μM
C
W
W
W
ERGOTAMINE
10μM
1g
5min

FIGURE 4.14

The contractile responses of *Helix* PRM to 3μM ACh and inhibition of this ACh-induced contraction by the specific 5-HT receptor agonists tested. An example of the ability of the stated agonist to inhibit the ACh-induced contraction in the PRM, at a concentration of 10μM, is shown in this figure. The agonists are methysergide (**A**), α-Me-5-HT (**B**) and 2-Me-5-HT (**C**). Each example shows, the control response to ACh, the response to ACh in the presence of agonist (10μM) and the response to ACh once the agonist had been thoroughly washed out. ACh (3μM) was applied at the arrows. The letter w indicates washes with fresh physiological saline. The stated agonist concentration was the final bath concentration to which the PRM was exposed.


A
w
w
w
METHYSERGIDE
10μM
B
w
w
w
α-ME-5-HT
10μM
C
w
w
w
2-ME-5-HT
10μM
1g
5min

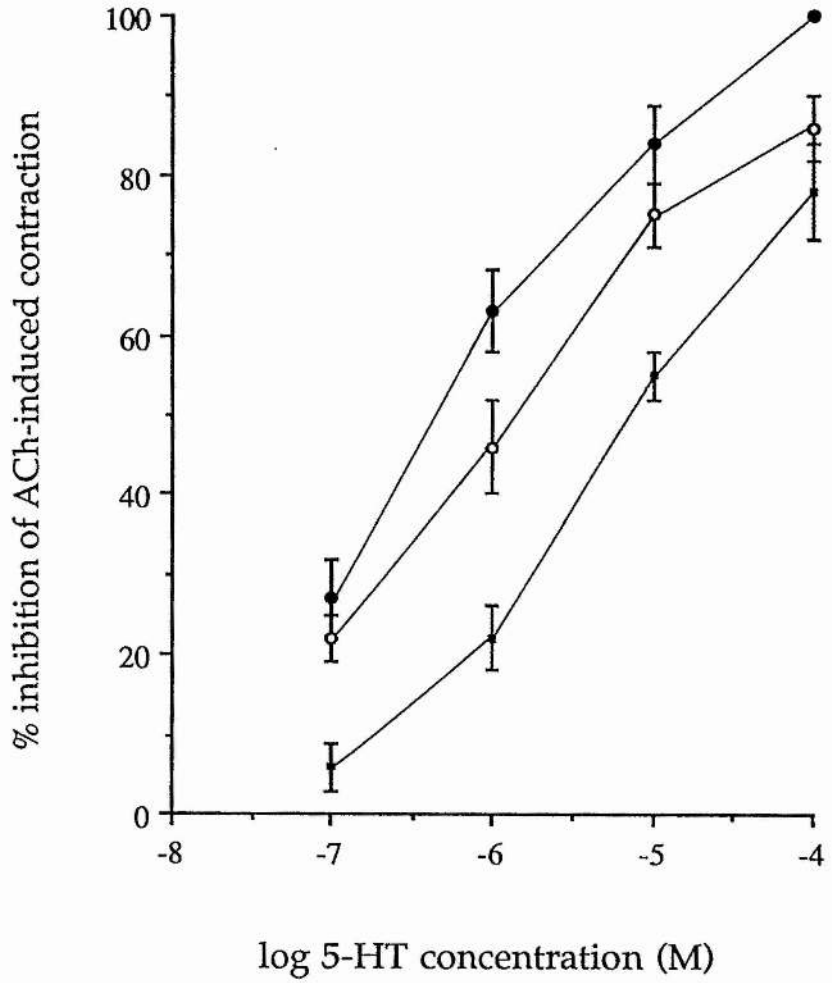


FIGURE 4.15

The concentration-effect curves for inhibition of ACh-induced contraction in *Helix* PRM for 5-HT (closed circles), for 5-CT (open circles) and for sumatriptan (squares) are shown in this figure. The x axis gives log of the 5-HT concentration while the y axis gives percentage inhibition of ACh-induced contraction. The ACh concentration used to induce contraction in the PRM was 3μM. Vertical bars represent the arithmetic mean ± s.e. mean of n=8 for 5-HT and n=5 for 5-CT and sumatriptan.

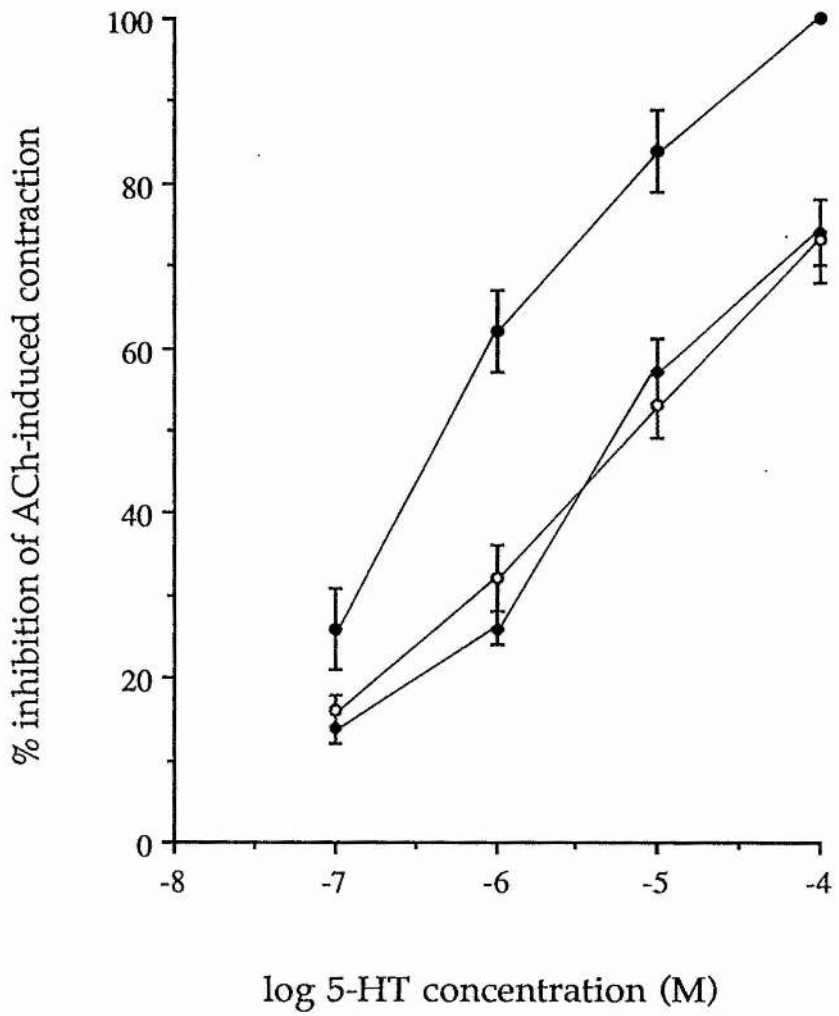


FIGURE 4.16.

The concentration-effect curves for inhibition of ACh-induced contraction in *Helix* PRM for 5-HT (closed circles), for ergotamine (diamonds) and for methysergide (open circles) are shown in this figure. The x axis gives log of the 5-HT concentration while the y axis gives percentage inhibition of ACh induced contraction. The ACh concentration used to induce contraction in the PRM was 3μM. Vertical bars represent the arithmetic mean ± s.e. mean of n=8 for 5-HT and n=5 for ergotamine and methysergide.

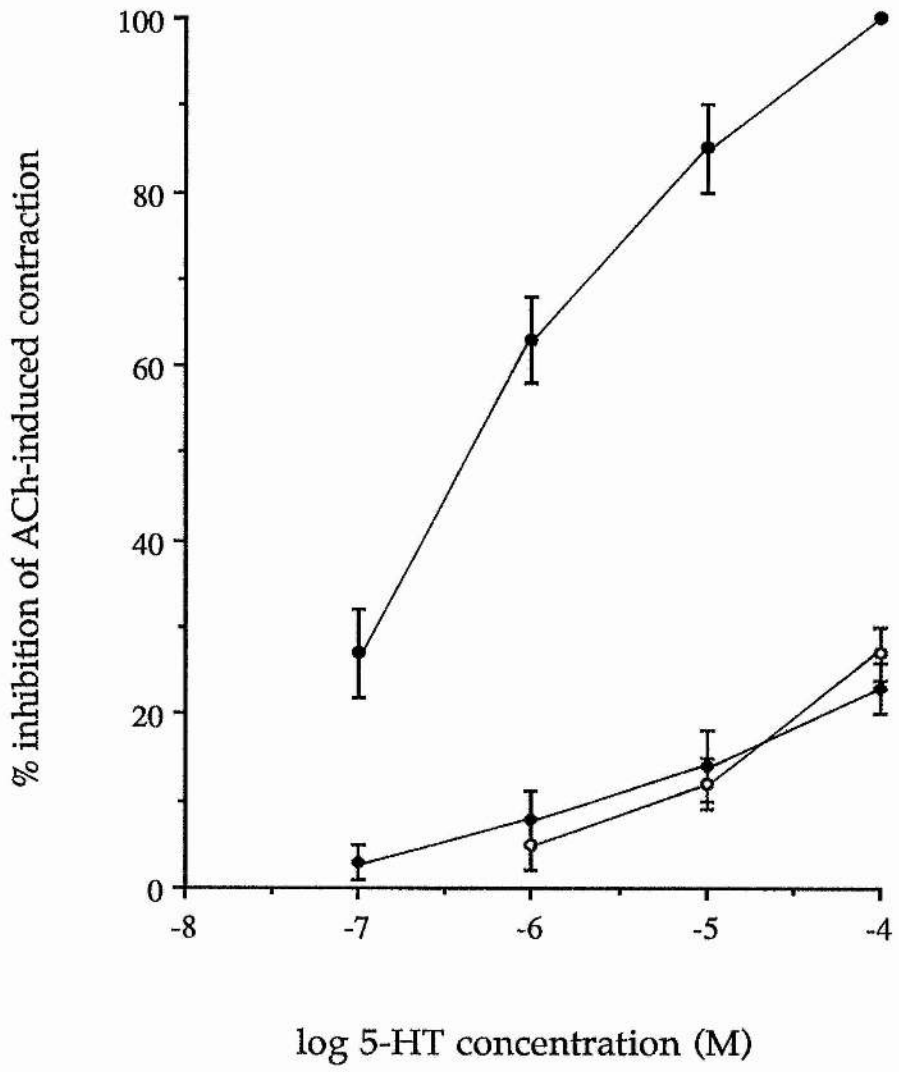


FIGURE 4.17

The concentration-effect curves for inhibition of ACh-induced contraction in *Helix* PRM for 5-HT (closed circles), for α-Me-5-HT (open circles) and for 2-Me-5-HT (diamonds) are shown in this figure. The x axis gives log of the 5-HT concentration while the y axis gives percentage inhibition of ACh-induced contraction. The concentration of ACh used to induce contraction in the PRM was 3μM. Vertical bars represent the arithmetic mean ± s.e. mean of n=8 for 5-HT and n=5 for α-Me-5-HT and 2-Me-5-HT.

TABLE 4.1 Summary of the 5-HT agonists tested in *Helix* PRM. The EC_R and EC_{50} values are molar values.

Agonist	n	EC_{50}	EC_R	% inhibition
5-HT	20	6.5 ± 0.6	1	100
5-CT	5	6.0 ± 0.5	3.0 ± 0.4	86 ± 5
Sumatriptan	5	5.3 ± 0.3	16.0 ± 4.0	78 ± 6
Ergotamine	5	4.9 ± 0.3	40.0 ± 6.0	74 ± 4
Methysergide	5	4.8 ± 0.6	44.0 ± 4.0	73 ± 5
α-Me-5-HT				27 ± 4
2-Me-5-HT				23 ± 3

Effects of 5-HT antagonists

The specific antagonists tested against the ability of 5-HT to inhibit ACh-induced contraction in the PRM were methiothepin, ketanserin, ondansetron and metoclopramide. Methiothepin lacked antagonistic effect on the ability of 5-HT to inhibit ACh-induced contraction (Fig. 4.18). The 5-HT concentration-effect curve in the presence of 1μM methiothepin, is superimposed upon the 5-HT concentration-effect curve in the absence of the antagonist. Ketanserin also failed to have antagonist action on the inhibition of ACh-induced contraction seen with 5-HT Ondansetron also demonstrated no antagonistic properties, as once again, the two 5-HT curves, one in the presence and one in the absence of 1μM ondansetron showed little difference. The lack of effect of both ketanserin and ondansetron have not been shown as figures because both antagonists gave similar results to that of methiothepin which has been shown as an example. On the other hand metoclopramide would appear to have had an effect. The 5-HT concentration-effect curve in the presence of 1μM metoclopramide was shifted to the right indicating that metoclopramide had an antagonist action (Fig. 4.19). Metoclopramide acted as a muscarinic antagonist in this preparation. The results are summarized in Table 4.2 overpage.

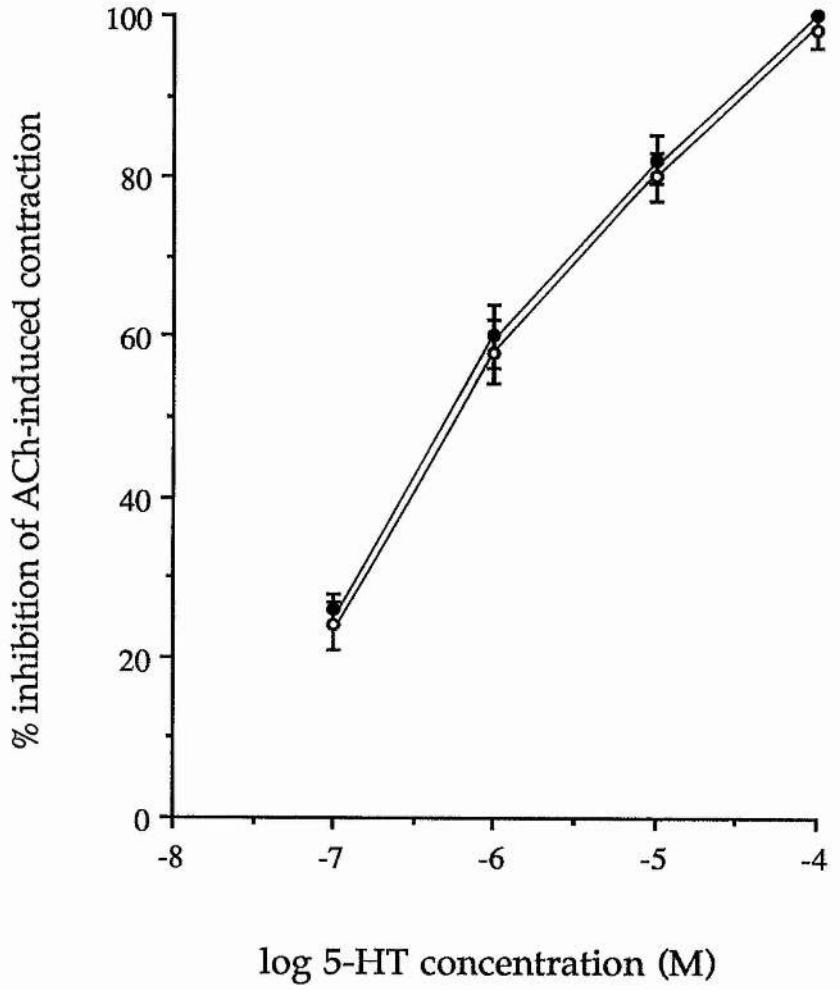


FIGURE 4.18

The concentration-effect curves for inhibition of ACh-induced contraction in *Helix* PRM for 5-HT in the absence (closed circles) and in the presence (open circles) of 1μM methiothepin are shown in this figure. The x axis gives log of the 5-HT concentration while the y axis gives percentage inhibition of ACh-induced contraction. The ACh concentration used to induce contraction in the PRM was 3μM. Vertical bars represent the arithmetic mean ± s.e. mean of n=8.

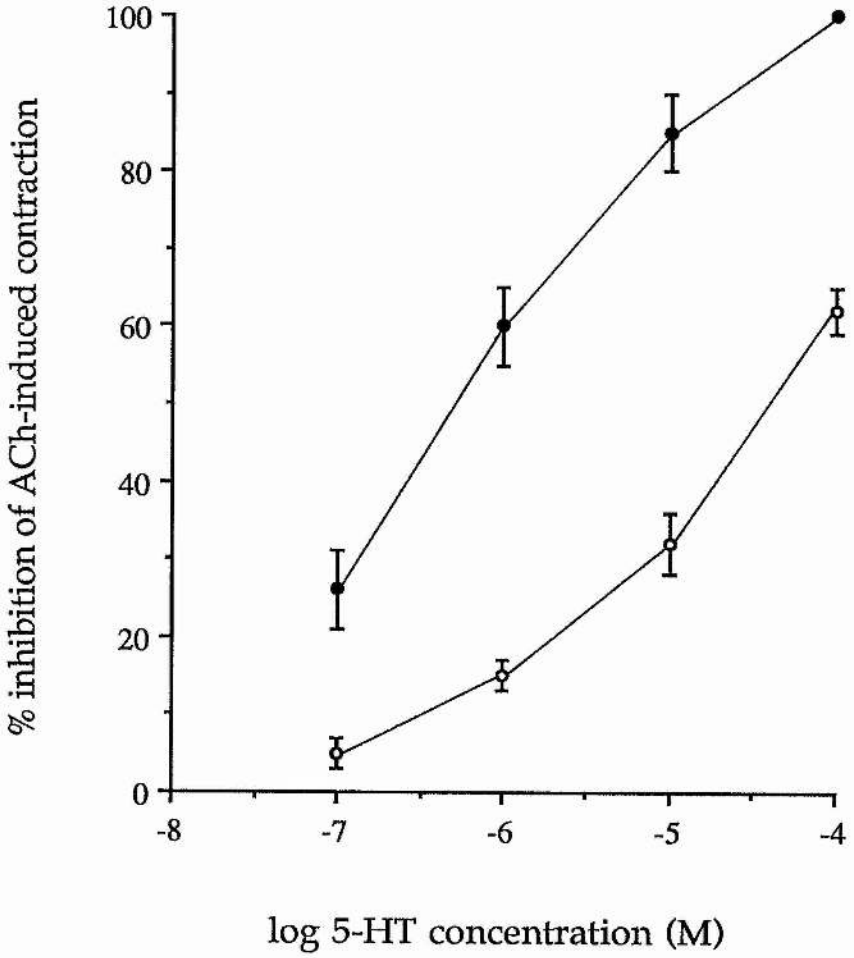


FIGURE 4.19

The concentration-effect curves for inhibition of ACh-induced contraction in *Helix* PRM for 5-HT in the absence (closed circles) and in the presence (open circles) of 1μM metoclopramide are shown in this figure. The x axis gives log of the 5-HT concentration while the y axis gives percentage inhibition of ACh-induced contraction. The ACh concentration used to induce contraction in the PRM was 3μM. Vertical bars represent the arithmetic mean ± s.e. mean of n=8.

TABLE 4.2 Summary of the 5-HT antagonists tested on *Helix* PRM.

Antagonist	Concentration tested (μM)	n	Effect
Methiothepin	1	8	No effect seen
Ketanserin	1	8	No effect seen
Ondansetron	1	8	No effect seen
Metoclopramide	1	8	Shift of 5-HT curve to right and lowering of maximum response

Cyclic AMP measurement

5-HT caused a dose-dependent increase in cAMP levels within the muscle cells in the PRM (Fig. 4.20). The presence of IBMX (100μM), a phosphodiesterase inhibitor, had no effect on the cAMP levels and indicated that IBMX is a relatively poor inhibitor of *Helix* cAMP phosphodiesterase enzyme. Forskolin, a potent cAMP activator, (10μM) increased the cAMP levels in the absence of 5-HT to 5 times greater than that obtained in the controls.

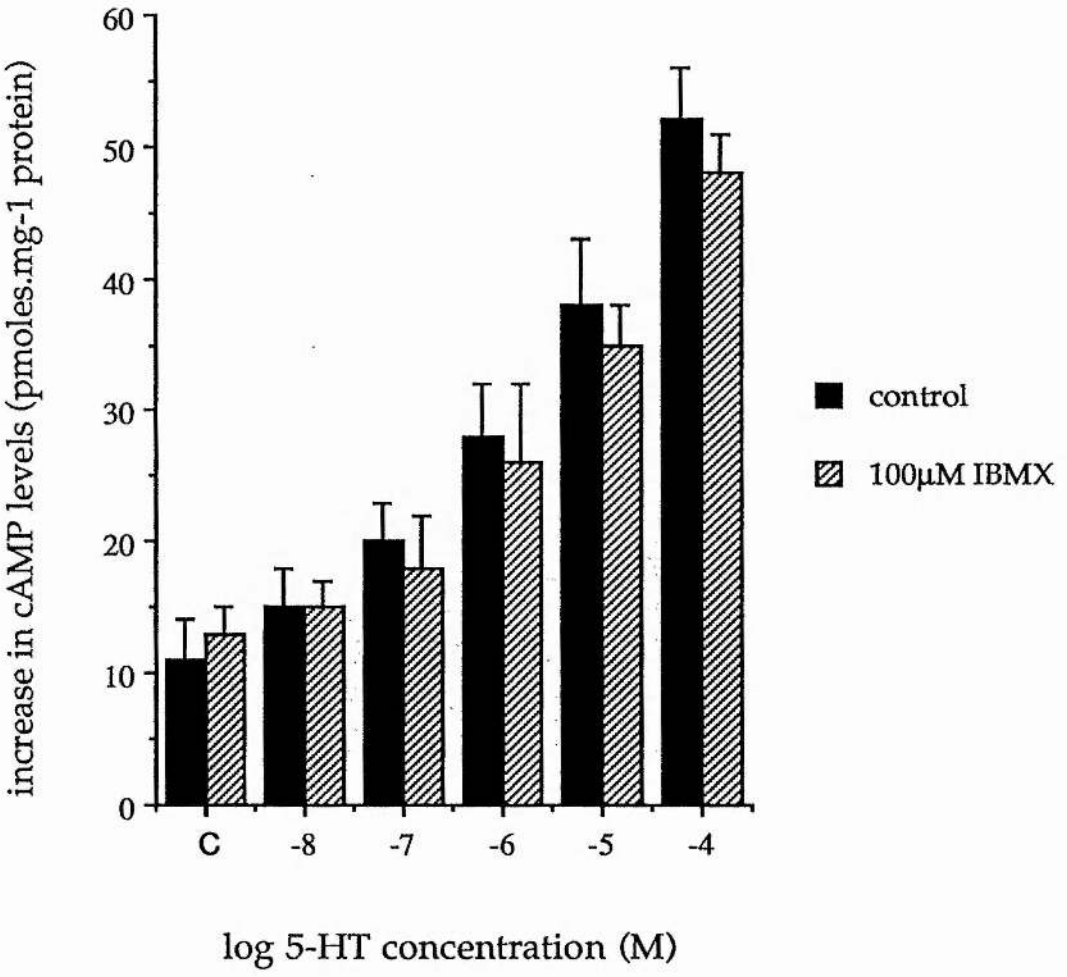


FIGURE 4.20

The increase seen in cAMP levels in the PRM tissue with increasing concentration of 5-HT are shown in this figure. The bar chart shows two sets of results. The first set of results (solid columns) were obtained under normal conditions and functioned as controls while the second set of results (dashed columns) were obtained in the presence of 100µM IBMX. The x axis gives log of the 5-HT concentration which includes a control, marked by the letter C, obtained in the absence of 5-HT and the y axis gives increase in cAMP in pmoles.mg^{-1} protein. Vertical bars represent the arithmetic mean ± s.e. mean of n=5

TABLE 4.3 Summary of the effects of 5-HT, forskolin and IBMX on cAMP levels in *Helix* PRM tissue.

Treatment	n	pmoles cAMP.mg^{-1} protein normal	pmoles cAMP.mg^{-1} protein IBMX (100μM)
Control	5	11 ± 3	13 ± 3
5-HT (10nM)	5	15 ± 3	15 ± 2
5-HT (100nM)	5	20 ± 3	18 ± 4
5-HT (1μM)	5	28 ± 4	26 ± 6
5-HT (10μM)	5	38 ± 5	35 ± 3
5-HT (100μM)	5	52 ± 4	48 ± 3
Forskolin (10μM)	5	63 ± 7	

DISCUSSION

Effects of 5-HT on the visceral muscles of *Helix*

The two visceral preparations from *Helix* that were tested for their response to 5-HT were the oesophagus and the rectum. The level of spontaneous activity, particularly in the oesophagus, made difficult the quantification of the response to 5-HT. The two different regions of the oesophagus, the anterior and the posterior region, showed different responses to 5-HT. The response of the posterior region to 5-HT was excitatory, whereas the response of the anterior region to 5-HT was inhibitory. The increase in frequency of contractions seen with 5-HT in the posterior region of the oesophagus agreed with results presented by Lloyd (1980). These results also mirrored those of Craveiro and Mendes (1990). Their results on the effect of 5-HT on the isolated oesophagus of an amphibious snail, *Pomacea lineata*, were similar in showing an increase in contractile activity. In contrast, the inhibitory effects seen here with 5-HT on the anterior region of the oesophagus were similar to those obtained by Ajimal and Ram (1981), who investigated the spontaneous activity and pharmacological sensitivity of *Aplysia* gastrointestinal tract. In their study 5-HT blocked spontaneous contractions. This inhibitory effect on spontaneous contractions, seen with 5-HT, was observed at concentrations as low as 9nM. This is in agreement with the present results where the threshold concentration for inhibitory effects was found to be approximately 10nM. 5-HT was seen also to inhibit ACh-induced responses in the isolated *Aplysia* oesophagus depending on the concentration of 5-HT used.

The rectum of *Helix aspersa* proved to be a difficult preparation, both to dissect without damage, and from which to elicit a response to 5-HT. Of the two preparations that were successful, little spontaneous activity was observed and the addition to the bath of increasing concentrations of 5-HT were notable by the absence of any response in the preparation. This contrasted with results observed by Phillis (1966), Greenberg and Jegla (1963) and by Ajimal and Ram (1981) on the rectum of *Tapes watlingi, Mercenaria mercenaria* and *Aplysia californica* respectively: there 5-HT excited rhythmic contractions and also caused an increase in tone.

Both excitatory and inhibitory effects have been observed previously in neuronal and various muscle responses to 5-HT in *Helix*. Gerschenfeld and Paupardin-Tritsch (1974a) reported that neurones both in *Helix* and *Aplysia* could respond to 5-HT in six different ways. In *Helix* heart, as seen in the preceding chapter, there is good evidence that 5-HT is the excitatory transmitter. In addition from the present chapter 5-HT is seen to have aa inhibitory or relaxant effect on *Helix* PRM. Thus the variety of responses to 5-HT in different regions of the gastrointestinal tract, in particular the oesophagus, conformed with the variety of effects of 5-HT observed elsewhere in the CNS and other muscles of *Helix*.

Lloyd (1980) demonstrated the presence of 5-HT both in the oesophagus and the rectum of *Helix*. His findings were mirrored by Nemcsok *et al.* (1986) who investigated the localization and quanititative distribution of biogenic amines in the intestinal tract, not only of the snail, but also the locust and the carp. They found 1.14μg 5-HT.g^{-1}wet weight in the intestinal tract of the snail which included the oesophagus and rectum. On the basis of their morphological and biochemical results, they suggested that the biogenic monoamines (such as 5-HT) were involved in the regulation of gut muscle functioning both in the form of transmitters as well as neurohormones.

Effects of ACh and 5-HT on *Helix* PRM

The PRM proved to be a robust preparation in the prolonged assays which were designed to attempt the characterization of the 5-HT receptor mediating relaxation of the PRM. Potentiation of the ACh contractile responses in the PRM in the presence of eserine matched those of Twarog (1954) who utilized the result to provide evidence that ACh was the probable excitatory transmitter in the ABRM.

The threshold concentration of 5-HT for causing relaxation of ACh-induced contractions was found to be 10nM. This is in agreement with the investigation by Lehman and Greenberg (1987). 5-HT had a threshold concentration for relaxation of the *Helix* PRM of 10-50nM. The prevention of ACh-induced contraction by 5-HT in the PRM of *Helix pomatia* was noted also by Sorokin (1986). Against a background of 5-HT, ACh-induced phasic contractions were smaller than those seen in the absence of 5-HT. These contractions had a greater rate of relaxation in the presence of 5-HT in

comparison to those seen in the absence of 5-HT. In the present investigation a quantifiable response for the relaxation caused by 5-HT was sought. The contraction to ACh was not well maintained, unlike that caused by contractile agonists in isolated porcine vena cava which has allowed the relaxant effects of 5-HT and its associated agonists to be studied (Sumner *et al.*, 1989). In this way the 5-HT receptor in porcine vena cava had been characterized as a 5-HT_1-like receptor. That method of characterization could not be used in the isolated *Helix* PRM. Therefore, the quantifiable response of inhibition of ACh-induced contraction in the *Helix* PRM, mediated by 5-HT was utilised in this study.

Effect of the 5-HT agonists on ACh-induced contraction in the PRM

5-HT proved to be the most potent agonist although 5-CT was only 3 times less potent. Sumatriptan was 16 times less potent than 5-HT whereas ergotamine and methysergide (which were found to have agonist activity in the PRM) shared the same potency of approximately 40-44 times less than that of 5-HT. α-Me-5-HT and 2-Me-5-HT failed to elicit a response to provide an EC_{50} value. Consequently they were considered to be only weak agonists at this 5-HT receptor. The full rank order of potency of the agonists was 5-HT > 5-CT > sumatriptan > ergotamine = methysergide >> α-Me-5-HT = 2-Me-5-HT; but this failed to provide an indication of what the 5-HT receptor type might be, being unlike any other rank orders of potency which have been elucidated for the vertebrate 5-HT receptors.

Effects of 5-HT antagonists on the inhibition of ACh-induced contraction by 5-HT

Murakami *et al.* (1986) suggested that the 5-HT receptor of the ABRM subserving the relaxant response appeared to be similar to those of the 5-HT_1 type of vertebrate receptors. Methysergide, cyproheptadine and 5-methoxygramine all acted as competitive antagonists of the 5-HT induced relaxation of catch contraction. Because both methysergide and cyproheptadine had been observed to act as antagonists of both 5-HT_1 and 5-HT_2 receptors in the brain (Leysen *et al.*, 1981), the antagonistic effects of mianserin, the specific 5-HT_2 antagonist, were investigated (Murakami *et al.*, 1986).

Mianserin had no significant effect on the 5-HT-induced relaxation of catch contraction. In the present study ketanserin, a specific 5-HT_2 receptor antagonist, had no antagonistic action on the inhibition of ACh-induced contractions by 5-HT on the PRM. This result concurs with the above finding with mianserin and therefore precludes the 5-HT receptor being of the 5-HT_2 type. Increasing evidence for non-involvement of the 5-HT_2 receptor type also comes from the lack of agonist action of α-Me-5-HT in inhibiting ACh-induced contraction in the PRM.

In contrast to Murakami *et al.* (1986), methysergide here acted as an agonist in the present study and mimicked the effect of 5-HT and its ability to elicit inhibition of ACh-induced contraction in the PRM. Methysergide had already been noted as a potent antagonist of the relaxation induced by 5-HT in ABRM (Northrop, 1964). Of the effects of the ergot alkaloids on the ABRM studied by Twarog *et al.* (1977), most imitated 5-HT and proved to be effective as relaxants of catch contraction. This was reflected in the results seen here with ergotamine, which mimicked the effects of 5-HT and caused inhibition of ACh-induced contraction in the PRM. Both methysergide and ergotamine proved to be equipotent on the PRM, being approximately 40-44 times less potent than 5-HT.

Twarog *et al.* (1977) suggested that both methysergide and bromo-LSD possessed blocking action against 5-HT in the ABRM, due to the fact that they both were unique in that they were substituted at or near the indole nitrogen. This substitution presumably decreased the effectiveness with which these ergots bound at the indole nitrogen and hindered combination at the relaxing site. In this way they were not as effective relaxants as the unsubstituted ergot derivatives.

A more recent study by Murakami *et al.* (1988) attempted to characterize the type of 5-HT_1-like receptor that they had previously reported in the ABRM. The ability of indole and non-indole 5-HT agonists to produce relaxation of catch contraction in the ABRM was investigated. Changes in cAMP levels in the indole and non-indole agonist-treated ABRM were studied also. Spiperone, propranolol and pindolol did not have any effect on the 5-HT-induced relaxation of catch contraction. They inferred that these presumably specific $5HT_{1A}$ and $5HT_{1B}$ antagonists (Pedigo *et al.*, 1981; Hoyer *et al.*, 1985) failed to recognise the 5-HT_1-like recognition sites in the ABRM.

5-Me-OT and 5-MeODMT induced dose-dependent relaxation of the catch contraction. Because both of these agonists were selective for $5HT_{1A}$ receptors (Sills *et al.*, 1984), the results suggested the presence of a 5-HT_{1A}-like receptor in the ABRM. 8-OH-DPAT also induced relaxation of catch contraction, adding more evidence that the 5-HT_1-like receptor was of the 5-HT_{1A} type. 8-OH-DPAT was a selective 5-HT_{1A} receptor agonist(Middlemiss and Fozard, 1983). The results with the 5-HT non-indole agonist were varied: TFMPP, quipazine and mCPP were selective for 5-HT_{1B} whereas PAPP was more selective for 5-HT_{1A} subtypes (Sills *et al.*, 1984). TFMPP, PAPP and mCPP induced dose-dependent relaxation of the catch contraction but quipazine did not. This led them to believe that the 5-HT_1-like receptor was of the $5HT_{1A}$ subtype, despite some inconclusive evidence with the non-indole agonists. This would appear not to be the case in the *Helix* PRM because methiothepin, a non-specific 5-HT_1-like receptor antagonist, failed to have an antagonistic effect on the inhibition of ACh-induced contraction by 5-HT.

The results seen with metoclopramide were of interest because this drug appeared to antagonize the action of 5-HT. However in the present study, it is unlikely that metoclopramide was acting as a 5-HT receptor antagonist because ondansetron, the more specific 5-HT_3 receptor antagonist, failed to have any effect on the inhibition of ACh-induced contraction by 5-HT. If it had been a 5-HT_3 receptor mediating the relaxant response of 5-HT in the PRM, both ondansetron and metoclopramide would have behaved as antagonists. Since this was not the case metoclopramide must be acting, not as a 5-HT_3 antagonist but rather as a muscarinic antagonist. Fosbraey and Johnson (1980) found that metoclopramide had selective antagonist properties on pre-junctional ACh receptors in the guinea pig illeum. Metoclopramide (>20μM) antagonized the post-junctional responses to ACh in a non-competitive manner. This was consistent with the findings of Bury and Mashford (1976) who had suggested that the antagonistic actions of metoclopramide might be due to a local anaesthetic action based on the structural similarity of metoclopramide to the tertiary amine local anaesthetics.

Kerkut and Leake (1966) found that the PRM of *Helix* was stimulated by ACh. These ACh-induced contractions were blocked by atropine, nicotine and muscarine, which indicated the presence both of nicotinic and muscarinic receptors in *Helix* PRM. It is likely, therefore, that metoclopramide acts on the

muscarinic receptors in the PRM in a competitive manner. By blocking the ACh receptors the contractile response to ACh can be seen to decrease in size with corresponding increases in metoclopramide concentration. Metoclopramide acting as a muscarinic antagonist would be seen to have the same effect as if it was acting as a 5-HT receptor antagonist. The most probable explanation is that metoclopramide is acting as a muscarinic antagonist.

Possible mediation of the action of 5-HT by cAMP

5-HT elevated cAMP levels significantly in the PRM, as had already been demonstrated in other molluscan muscles (Achazi *et al.*, 1974; Köhler and Lindl, 1980; Ishikawa *et al.*, 1981). However, although the 5-HT-induced relaxation of catch contraction in the ABRM has been coupled to elevated cAMP levels, several experimental results were inconsistent with the hypothesis that cAMP was the intracellular mediator for relaxation induced by 5-HT in the ABRM. 8-bromo-cGMP relaxed catch tension in the ABRM without any increase in cAMP levels (Painter, 1982), despite the fact that this drug seemed to act on relaxing nerve terminals to increase release of relaxing transmitter. Whether cGMP was involved in relaxation remained inconclusive because 5-HT had failed to increase cGMP levels in the ABRM (Köhler and Lindl, 1980; Ishikawa *et al.*, 1981). Neural stimulation with repetitive pulses did not increase cAMP levels, although it produced contraction followed by rapid relaxation (Köhler and Lindl 1980). Ishikawa *et al.* (1981) reported that dopamine could relax catch without any increase in cAMP levels. Köhler and Lindl (1980) reported that although 10^{-5}M dopamine increased cAMP levels, the increased amount was only 10% of that induced by 10^{-5}M 5-HT. Because dopamine was thought to relax catch tension by acting on its receptors on the muscle fibre membrane (Twarog *et al.*, 1977) this could not be explained by the cAMP hypothesis.

However, Köhler and Lindl (1980) have pointed out some discrepancies between relaxing events and intracellular levels of cAMP. Catch tension, the prolonged contraction exhibited by certain muscles in lamellibranchs, was relaxed in the ABRM by 5-HT at concentrations as low as 1nM, whereas an increase in cAMP could be observed only at a concentration of 0.5μM or higher. When 10μM 5-HT was applied the maximum cAMP concentration was reached in 2-3 minutes whereas complete relaxation required only 30-60

seconds. From these results they postulated that cAMP might not be the physiological second messenger for relaxation of catch tension.

In the investigation reported by Murakami *et al.* (1988) 5-MeOT and 5-MeODMT significantly reduced cAMP levels at a concentration sufficient to relax catch contraction. Of the other agonists tested TFMPP, QUIP and 8-OH-DPAT reduced cAMP levels whereas PAPP did not. Murakami *et al.* (1988) concluded from these results that changes in cAMP levels induced by the 5-HT agonists were unlikely to be directly coupled to the relaxation induced by them.

Such discrepancies are also shown by the results presented here. 5-HT, at a concentration of 1μM, fully relaxed ACh-induced contraction in the PRM in 20-40 seconds whereas increases in cAMP were seen after 2 minutes. The levels of cAMP showed only a marked increase at a concentration of 0.1μM whereas the relaxation is observed between 10-50nM. Further investigation is needed to prove whether cAMP is the intracellular second messenger for relaxation of ACh-induced contraction in the PRM, and that it is linked to the 5-HT receptor mediating relaxation in the PRM.

Such discrepanies between the threshold action of 5-HT and the associated increases in cAMP levels also have been noted in the *Helix* heart (see previous chapter). In heart muscle tissue, it is hypothesised that cAMP might not be the only intracellular signal through which 5-HT mediates its excitatory effects. As with the *Helix* heart, the action of 5-HT on molluscan muscle is associated also with calcium mobilization (Bloomquist and Curtis, 1975a,b; Ishii *et al.*, 1989). In the *Mercenaria* heart, recent findings have implicated the possible involvement of protein kinase C and inositol phospholipids (Deaton and Gray, 1990). Unfortunately phosphatidylinositol hydrolysis, which results in calcium mobilization cannot be implicated in the action of 5-HT on the PRM; it is muscle contraction that is associated with phosphatidylinositol turnover rather than muscle relaxation.

Conclusion

The 5-HT receptor mediating relaxation in *Helix* PRM could not be characterized as any of the already existing 5-HT receptor types present in the vertebrates. No specific 5-HT receptor antagonist was found to inhibit the action of 5-HT on *Helix* PRM. The rank order of potency of the 5-HT receptor

agonists was unlike any of those that had been elucidated for the different 5-HT receptor types in vertebrates. Presently it appears, as does *Helix* heart 5-HT receptor, to be unique and quite unlike the 5-HT receptors in vertebrates. As in the heart, the lysergic acid derivative methysergide and ergots like ergotamine are agonists, but it is not clear whether they act at the same receptor as 5-HT because no specific blocker was found. That the receptor mediates its effect through cAMP is still unclear. 5-HT does undoubtedly cause an increase in cAMP levels within the muscle tissue, but the complete pathway requires to be elucidated. Certainly the discrepancies with the cAMP levels, as already mentioned seem to question the involvement of cAMP.

CHAPTER FIVE

GENERAL DISCUSSION

Involvement with cAMP and possible linkage to a G-protein receptor family

The action of 5-HT has been implicated in the activation of cAMP in many invertebrate tissues. In the CNS of *Helix* and *Aplysia* 5-HT stimulated adenylate cyclase (Drummond *et al.*, 1980b) and these workers found a good correlation between cyclase activation and 5-HT-sensitive [^{3}H]LSD binding. From the present, and previous, evidence 5-HT appears to be linked closely to adenylyl cyclase and cAMP is implicated in the action of 5-HT both in *Helix* heart and PRM tissue. One could suggest that the 5-HT receptors within *Helix* may be members of the G-protein super family, as has been shown for 5-HT$_{1C}$, 5-HT$_2$ and 5-HT$_{1A}$. This receptor family has been characterized by the presence of seven transmembrane domains in each receptor and by the ability to activate G-protein dependent processes (see Hartig, 1989). The 5-HT$_2$ receptor shared overall sequence identity of 49% with the 5-HT$_{1C}$ receptor but the amino acid identity within the putative transmembrane domains was over 80% (Julius *et al.*, 1990). Julius *et al.* concluded that the stringent sequence conservation was likely to reflect the fact that those regions of the molecule were required for the maintenance of two primary functions shared by the 5-HT$_{1C}$ and 5-HT$_2$ receptor—ligand binding and G-protein-mediated activation of phospholipase C. The close structural and functional relationship between those two 5-HT receptors suggested that they should be grouped by nomenclature (Pritchett *et al.*, 1988).

The similarities between the neuronal 5-HT response in *Helix* and the 5-HT response mediated by the 5-HT$_3$ receptor in vertebrate tissues and cell lines suggested that the *Helix* neuronal receptor, under study, may be an ion channel; the same as the 5-HT$_3$ receptor has been shown to be (Derkach *et al.*, 1989). However it is more probable that the neuronal 5-HT receptor in *Helix* regulates ion channels through a specific G protein. It has been argued that specific G proteins, in response to a variety of receptors, can regulate one of several different channels and that it may be able to achieve this through multiple pathways, which include classical second messangers and potential direct interaction with the channel complex (see Sternweis and Pang, 1990). A

good example providing evidence for this hypothesis has been shown already in invertebrates; behavioural sensitization in *Aplysia*. Klein and Kandel (1978) found that 5-HT and cAMP closed single K^+ channels in *Aplysia* sensory neurones. They identified a 5-HT-sensitive K^+ channel which was active at the resting potential and was not dependent on the activity of intracellular calcium. Application of 5-HT to the cell body, or intracellular injection of cAMP, caused prolonged and complete closure of the channel, thereby reducing the effective number of active channels in the membrane. The depression of outward current, bought about by closure of these channels, delayed repolarization of the action potential and extended the duration of calcium influx into the presynaptic terminals, thereby contributing to the facilitation of transmitter release that is thought to underly behavioural sensitization in *Aplysia*.

Families of G-protein-coupled receptors have also been identified for cholinergic and adrenergic transmitters. The muscarinic acetylcholine receptors defined a family of receptors that were highly homologous to one another (Bonner *et al.*, 1987). The 5-HT_{1A} receptor gene was obtained by hybridization to a β_2-adrenoreceptor clone (Fargin *et al.*, 1988). A strong nucleotide sequence homology was found to exist between the two receptors. In several amino acid stretches the 5-HT_{1A} more closely resembled the β_2-adrenoreceptor than it did the other cloned 5-HT receptors. This evidence has raised intriguing questions about the functional and evolutionary relationships among these different receptors.

In the past there has been a strong tendency to base receptor characterization and subsequent classification on the characteristics of the binding sites for competitive antagonists, largely because the affinities of such antagonists could be determined reliably by the Schild method. Colquhoun *et al.* (1987), in their review of nicotinic ACh receptors, stated that if this had still been the case there would have been a major problem in defining nicotinic receptors. This was because antagonists which were thought primarily to be competitive on some receptors were not competitive on others but exerted effects on the ion channels. They concluded that it was not sensible to define nicotinic receptors solely in terms of their agonist and antagonist binding sites but that they must ultimately be defined, by means of recombinant DNA

methods, in terms of the structure of the entire receptor ion channel molecule.

It would be of interest to discover if the molluscan 5-HT receptors shared any sequence identity with those of the vertebrate 5-HT receptors which already have been cloned, particularly within the transmembrane regions where 80% of the amino acid sequence was shared by 5-HT_{1C} and 5-HT_2 (Julius *et al.*, 1990). 5-HT has been found in the tissues of coelenterates, flatworms and the nervous tissue of annelids (Welsh, 1968): these are some of the most primitive of the invertebrate phyla. It would appear, therefore, that 5-HT and its associated receptors arose at an early stage in the evolution of the nervous system of animals.

The application of modern techniques—such as radioligand binding, monoclonal antibodies and gene cloning technology—has enabled marked advances in the quantity and quality of information concerning receptor structure. In a review of the evolution of neurotransmitter receptor systems, concerning, in particular the adrenergic and cholinergic systems, Venter *et al.* (1988) proposed that the large family of receptors which mediate their effects via the cyclic nucleotide regulated systems (such as adenylate cyclase and phosphatidylinositol turnover) will be shown to have structural homology and a common ancestry. This proposal was based on the fact that tryptic-digest maps both of adrenergic and muscarinic cholinergic receptors, together with monoclonal antibody cross reactivity data, had indicated that adrenergic and muscarinic cholinergic receptors were structurally homologous. The subsequent cloning of the genes for the beta adrenergic receptor and the muscarinic cholinergic receptors clearly established the presence of major sequence and structural homology among these receptors. The review evidence presented by Venter *et al.* (1988) also indicated that adrenergic and cholinergic receptors were ancient proteins whose existence dated back, at least, some 600 million years. Is it possible that the serotonergic system, albeit within different species, might show the same degree of homology? Certainly, classical muscarinic receptors have been found in *Aplysia californica* (Murray *et al.*, 1985). The muscarinic receptors in this gastropod exhibited stereospecificity in the binding of the isomers of quinuclidinyl benzoate. Muscarinic agonists (e.g. carbachol) and antagonists (e.g. atropine) were found also to have IC_{50} values (concentration required to inhibit 50% binding)

similar to those of mammals. Possibly this also could be the case for the 5-HT receptors and their basic structure could have been highly conserved throughout evolution: a speculation that it is a member a G-protein super family would explain the close links with adenylyl cyclase and cAMP. The differential evolution of the fundamental 5-HT receptor within the different phyla could possibly have resulted in duplication and mutation, leading to changes in amino acid sequences. These changes in the amino acid sequence would therefore account for the different affinities shown by the 5-HT receptors, within the different species, to the different 5-HT receptor agonists and antagonists. This hypothesis would explain why none of the 5-HT receptors within the different tissues in *Helix* were susceptible to inhibition by the specific 5-HT receptor antagonists which have been identified because of their inhibitory actions against vertebrate 5-HT receptor types.

Characterization of 5-HT receptors in *Helix*

The lack of any specific 5-HT receptor antagonists to the 5-HT response in the three different tissues of *Helix* made difficult the characterization of the receptors. Criticisms of the use of antagonists to characterize and classify 5-HT receptors have, however, been advanced by Leff and Martin (1988). In their review they examined whether tryptamine analogues could provide a more quantitative, and therefore secure, basis for 5-HT receptor classification. This entailed the extensive use both of agonist and antagonist compounds. They argued that the complex variety of pharmacological actions of 5-HT necessitated reliable receptor classification. However, in bioassays, which are essential to classification, many of the conventional 5-HT receptor probes demonstrated variable pharmacodynamics and failed to fulfil theoretical criteria for valid use. Non-surmountable antagonism and variable affinity in different assays were often evident. They suggested that whether such phenomena represented 5-HT receptor heterogeneity, or problems, such as multiple actions, with the probes were not always clear. Their approach showed that the same receptor type occurring in different tissues could be defined with quantitative consistency using tryptamines when conventional antagonists failed.

From the agonist data presented in this investigation, the three preparations showed some differences in the rank order of agonist potencies.

For the identified neurones in the visceral ganglion, it was difficult to obtain an accurate value of agonist potency unless the potency was assumed to be relative to the amplitude of the 5-HT-evoked response. Other features of the iontophoretic technique also could have affected the 5-HT-evoked response in the same way as the potency. The differences observed in agonist potency in the three preparations from *Helix* could possibly be due to the different techniques utilised in the investigation. An alternative hypothesis could be that the 5-HT receptors within *Helix* show a similar heterogeneity of receptors to that which has been proved to occur in vertebrates.

The previous studies which have been undertaken to attempt to characterize the 5-HT receptors in different molluscs have shown varying affinities for the 5-HT receptor agonists and antagonists that have been tested. This has, in part, been dependent on the species tested and on the methodology utilized. In this way no molluscan 5-HT receptor has been truly characterized.

Methysergide, an LSD derivative and the other ergot derivatives (such as ergotamine) that were tested against the 5-HT response in the three preparations of *Helix*, had diverse actions. Despite the fact that methysergide had been described, in the majority of studies, as a competitive antagonist (for example the study by Boyd *et al.* (1985) on *Helix* heart), in the present study methysergide behaved as a partial agonist in *Helix* heart. This result was also mirrored by the other ergot derivatives that were tested on the *Helix* heart although LSD appeared to act purely as an agonist. From the data, however, it was difficult to ascertain whether these compounds were acting directly on the 5-HT receptor under investigation. This was because they demonstrated a high degree of agonist activity and their block of the 5-HT response in *Helix* heart proved to be only minimal. The potent agonist activity of LSD has been well documented but, more importantly, can this high degree of agonism be attributable to activation of a 5-HT receptor? Could LSD and the other ergot derivatives possibly be exerting their effects through an "ergot" receptor present in *Helix* tissue? A specific receptor blocking drug for either the 5-HT receptor or the putative ergot receptor will be necessary to test this hypothesis.

Conclusion

The 5-HT receptors within *Helix* cannot be characterized according to the classes of 5-HT receptor types which have been described in vertebrates: they are unique. The *Helix* 5-HT receptors show little affinity for any of the specific 5-HT receptor antagonists which have been used to characterize, and subsequently classify, the vertebrate 5-HT receptors. It has become clear in this investigation that to define the 5-HT receptors within *Helix*, solely in terms of their currently available agonist and antagonist binding is not possible. Ultimately they must be defined in terms of their established structure, by means of gene cloning technology. Only in this way will the extent of homology of the 5-HT receptors within different species become apparent.

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