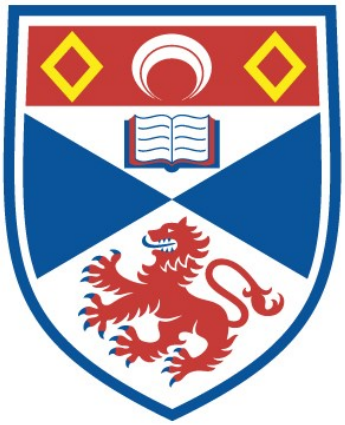
A PHYSIOLOGICAL AND PHARMACOLOGICAL STUDY OF 5-HYDROXYTRYPTAMINE ON HEARTS OF MOLLUSCA BIVALVIA

Caroline Bruce

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



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A PHYSIOLOGICAL AND PHARMACOLOGICAL STUDY OF 5-HYDROXYTRYPTAMINE ON HEARTS OF *MOLLUSCA BIVALVIA*

by Caroline Bruce, B.Sc.

Submitted for the degree of Ph.D. - February 1996

TL C64

Dedication

I would like to dedicate this thesis to:

- Professor Morley Sutter, my mentor and friend, who was the first person to encouraged me to undertake a Ph.D. and whose love of Pharmacology and Medicine has had a major influence on my life,

- Dr. Marion Evans whose friendship and support helped me so much, and whose sense of humour has always made any problem seem smaller,

- my parents and sisters, for their support and encouragement throughout the years.

Declaration:

(i) I **Caroline Bruce**, hereby certify that this thesis, which is approximately 42,000 words in length, has been written by me, that it is the record of work carried out by me and that it has not been submitted in any previous application for a higher degree.

Date 31/1/96 Signature of candidate

(ii) I was admitted as a part-time research student in April 1987 and as a candidate for the degree of Ph.D. in April 1987; the higher degree for which this is a record was carried out in the University of St. Andrews, the University of British Columbia and the University of Florida between 1987 - 1994.

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(iii) I hereby certify that the candidate has fulfilled the conditions of the Resolution and Regulations appropriate for the degree of Ph.D. in the University of St. Andrews and that the candidate is qualified to submit this thesis in application for that degree.

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ABSTRACT

The question under examination is whether there are various 5-HT receptor types present in the hearts of different species of molluscan bivalves.

In order to examine this question, the actions of 5-HT and some analogues on the hearts of several species of bivalve molluscs were investigated in two different preparations. The effects of 5-HT and analogues were examined on isolated whole ventricle preparations and on the unitary currents of patch-clamped myocytes from some of the same species.

The 5-HT receptor activated in *Mya arenaria* and *Mercenaria mercenaria* myocytes produced a decrease in K^+ unitary current activity across the membrane, which correlated with the effect seen by elevation of cAMP intracellularly. This fits well with the excitation of the whole hearts by 5-HT, and the earlier literature.

In *Geukensia demissa* the presence of another 5-HT receptor was observed which when activated also causes an increase in K^+ unitary current activity; which was not mimicked by elevation of intracellular cAMP. This also correlated with some of the mixed effects of 5-HT on the whole hearts. However, 5-CT and 5-MEOT caused a decrease in K^+ unitary current activity; which also correlated with the inhibition of the whole hearts by 5-CT and 5-MEOT. The effects observed with 5-HT, 5-CT, 5-MEOT in *Geukensia demissa* appear to be linked to the activation of an intracellular pathway independent of cAMP.

The results described from the whole heart and the myocytes experiments suggest that there are in fact more than one conformation of 5-HT receptor present in the hearts of bivalves.

TABLE OF CONTENTS

ABBREVIATIONS: UNITS AND MEASUREMENTS

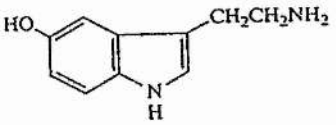
M	molar
mM	millimolar
μm	micrometer
g	gram
mg	milligram
μg	microgram
ng	nanogram
L	litre
ml	millilitre
μl	microlitre
v	volume
w/v	weight per volume
A	amp
pA	picoamp
kHz	kilohertz
V	volts
mV	millivolts
I	current
V_e	electrode holding potential
V_r	resting membrane potential
V_m	patch membrane potential (V_r-V_e)
min	minute
s	second
ms	millisecond
ps	picasecond
r	radius
cm	centimetre
mm	millimetre
w	weight
G	gravity

ABBREVIATIONS : GLOSSARY OF CHEMICAL NAMES

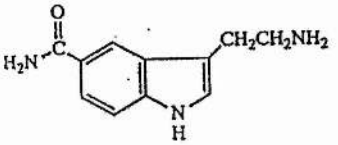
5-HT	5-hyroxytryptamine
5-CT	5-carboxamidotryptamine
5-MEOT	5-methoxytryptamine
8-OH-DPAT	8-hydroxy-2-(di-n-propylamino)tetraline
α-Me-5-HT	α-methyl-5-hydroxytryptamine
2-Me-5-HT	2-methyl-5-hydroxytryptamine
5-MG	5-methoxygramine
7-MT	7-methyltryptamine
UML	1-methyl-d-lysergic acid butanolamide
ICS 205-930	tropisetron(3α-Tropanyl)-1H-3-carboxylic acid
GR 38032F	ondansetron
MDL-72222	1αH,3α,5αH-tropan-3-yl-3,5 dichlorobenzoate
RU-24969	5-methoxy-3(1,2,3,6-tetrahydro-4-pyridinyl)-1H-indole
MK 212	6-chloro-2-(1-piperazinyl)pyrazine
LSD-25	(+)-lysergic acid diethylamide
ACh	acetylcholine
Bz	Benzoquinonium
4AP	4 aminopyridine
TEA	tetraethyl ammonium
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid
cAMP	Adenosine 3': 5' cyclic monophosphate
cGMP	Guanosine 3':5' cyclic monophosphate
8-bromo-cAMP	8-bromo adenosine 3'5'cyclic monophosphate
IP_3	inositol triphosphate
G_s	stimulatory G protein
G_i	inhibitory G protein

Structure of 5-Hydroxytryptamine and some analogues

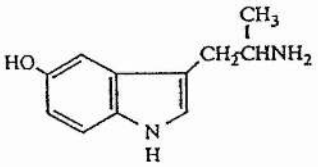
5-HT



5-CT



α-Me-5-HT



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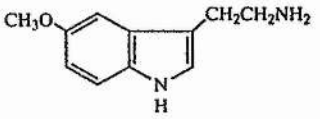

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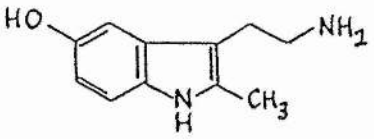

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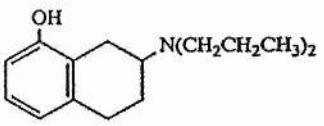

STRUCTURE OF SOME 5-HT ANTAGONISTS

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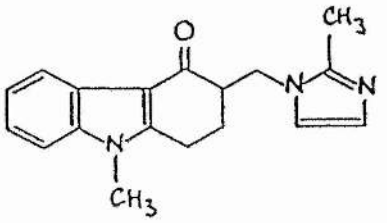

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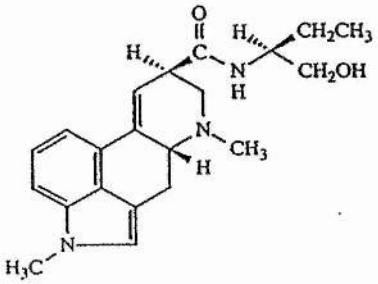

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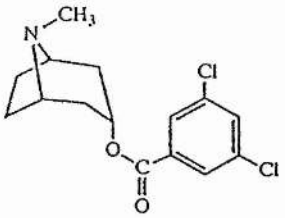

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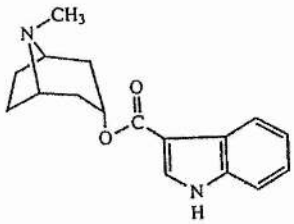
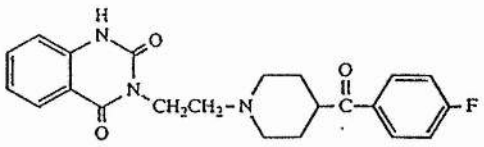
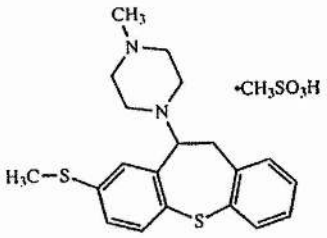


Ketanserin



Methiothepin



A PHYSIOLOGICAL AND PHARMACOLOGICAL STUDY OF 5-HYDROXYTRYPTAMINE ON HEARTS OF *MOLLUSCA BIVALVIA*

INTRODUCTION

5-Hydroxytryptamine(5-HT) is 3-(β-aminoethyl)-5-hydroxyindole and is found throughout the animal and plant kingdoms. It occurs in vertebrates, tunicates, molluscs, arthropods, coelenterates and even in fruits and nuts. It is also present in numerous venoms including those of the common nettle, wasps and scorpions, Walker(1985).

Numerous synthetic and/or naturally occurring analogues of 5-HT have varying degrees of pharmacological activity. Noteworthy are the "tryptamines" of plant origin known for their hallucinogenic properties (e.g. bufotenine and psilocine). 5-HT is referred to as an autocoid (a local hormone), a "neurohormone" or a neurotransmitter; this emphasises the wide distribution and physiological action of this chemical throughout nature. In man 5-HT is found in platelets, enterochromaffin cells throughout the gastrointestinal tract and in several regions of the brain and nervous system. 5-HT is usually synthesised *in situ* from tryptophan; hydroxylated to 5-hydroxytrytophan (5-HTP) and decarboxylated to 5-HT, Garrison (1990).

The structure of 5-HT agonists and antagonists used in this study are given on pages 10 and 11.

5-HYDROXYTRYPTAMINE RECEPTOR CLASSIFICATION

Over a hundred years ago an endogenous vasoconstrictor substance was detected in serum, Stevens & Lee (1884), Brodie (1900). The substance was named thrombocytin and vasotocin by these different workers, Welsh (1953). Vialli and Erspamer

(1933) in a study of the mammalian gut identified a substance derived from enterochromaffin cells of the mucosa, which they called "enteramine". Rapport et al. (1947) first coined the name "serotonin" for a vasoconstrictor amine present in serum; they subsequently identified the ligand as 5-hydroxytryptamine, Rapport et al. (1948). Serotonin and enteramine were found to be the same compound, Erspamer and Asero (1952).

In 1951 5-HT was synthesised commercially by Abbott Laboratories. John Welsh, in the period between 1951-1953, was the first to study the excitatory action of 5-HT on the heart of *Mercenaria (Venus) mercenaria,* Twarog (1988). Twarog and Page(1953) using the heart of *Mercenaria* as a bioassay demonstrated the presence of 5-HT in mammalian brain.

5-HT is involved in numerous physiological functions in vertebrates. In the periphery it has effects on smooth muscle, both in the vascular beds and in the gut, Saxena et al. (1989) and Saxena and Villalon (1991). 5-HT has been claimed to be one of several neurotransmitters active in a variety of CNS pathways involved in sleep, learning, behaviour and feeding, Zifa and Fillion (1992).

Gaddum and Picarelli (1957) were the first to attempt to classify the different 5-HT receptors. They described two receptors for 5-HT involved in the control of guinea pig gut smooth muscle contraction. The "D" receptors, 5-HT receptors blocked by Dibenzyline (phenoxybenzamine) and the "M" receptor, a 5-HT receptor blocked by morphine. This classification remained relatively unchanged for 20 years, due mainly to the lack of selective agonists and antagonists.

Throughout the 1970s binding studies (with labelled [^{3}H]5-HT, [^{3}H]spiroperidol and [^{3}H]LSD) were used to identify the "5-HT_1" and "5-HT_2" families of receptors in mammals, Peroutka and

Snyder (1979). Thus in the early 1980s a receptor classification evolved based around these two main groups, with more "sub-types" of receptors being identified within these groups e.g. 5-HT_{1A}, Humphrey (1984). However, there was a lack of correlation between the known functional roles of 5-HT with many of the findings from the binding studies.

Bradley et al. (1986) tried to reconcile all the different nomenclatures and establish a classification which would clearly define 5-HT receptors. By correlating the binding site results to functional physiological properties they proposed three major receptor classes:

5-HT"1-like"	- with 4 possible "sub-types", $5\text{-HT}_{1A,1B,1C,1D}$
5-HT_2	- included the old "D" receptor group
5-HT_3	- included the old "M" receptor group.

Since 1986, there have been several changes to this classification mainly due to the development of molecular biological techniques which have yielded new protein sequencing data. Cloning, sequencing and biochemical techniques have helped to clarify some of the differences in receptor sub-types both within and between species. Most of the 5-HT receptors appear to belong to the G-protein coupled receptor family; however, the 5-HT_3 receptor has been identified as a member of the ligand-gated channel family, Derkach et al. (1989).

The multiplicity of receptor subtypes is observed not only in mammals but has been described in molluscs and arthropods as outlined in a review by Hen (1992). This area of research is changing rapidly with "new" receptor sequences appearing in the literature as different tissues and species are examined, Hen (1992). The 5-HT_2 receptor, which was thought to be relatively homogeneous in earlier binding studies, has been shown to have

different structural (sequence) variations in some species, as well as sharing more structural similarity to the 5-HT_{1c} receptor, Hen (1993); the latter is now termed the 5-HT_{2c} receptor.

Table 1, is a modified summary of the 5-HT receptor classification as produced by the Serotonin Club Receptor Nomenclature Committee, Humphrey et al. (1993). Hen (1992,1993) reviewed the molecular biology of the structure of the 5-HT receptors, he emphasises the importance of identifying the commonalities or structural conservation between species. As these sequence conservations are linked to G-protein or other transduction pathways in the future, we will gain better understanding of the function and the "critical" structure of the ligand required to activate the 5-HT receptor(s).

Hoyer et al. (1994) produced a major review, along with seven other researchers from the Serotonin Club, entitled:

VII. International Union of Pharmacology Classification of Receptors for 5-Hydroxytryptamine (Serotonin).

They reviewed the 5-HT receptor literature to date against their "Criteria for receptor characterisation". Of particular interest was the review of the 5-HT_4 receptor properties, and those of the newly identified but not completely characterised receptors which they called "5-ht_5, 5-ht_6 and 5-ht_7" (as the receptors had not been fully investigated). The relevance of these newly described receptors will be discussed late in the thesis.

TABLE 1

5-HT RECEPTORS (current literature)

NOMENCLATURE	AGONISTS	ANTAGONISTS	TRANSDUCTION/EFFECTORS
*5-HT_{1A}	8-OH-DPAT/5-CT	?	G protein / Adenylyl cyclase ⇑/⇓
*5-HT_{1B}	5-CT	CP93129	G protein / Adenylyl cyclase ↓
*5-HT_{1D}	5-CT	sumatriptan	G protein / Adenylyl cyclase ↓
*5-HT_{1E}	?	?	G protein / Adenylyl cyclase ↓
*$5\text{-HT}_{1F(1EB,5\text{-HT6})}$	?	?	G protein / Adenylyl cyclase ↓
5-HT_4	5-MEOT	GR113808	G protein / Adenylyl cyclase ↑
5-HT_{2A}	α-methyl 5-HT	ritanserin	IP3 /DG
5-HT_{2B}	α-methyl 5-HT	ritanserin	IP3 /DG
$5\text{-HT2}_C(5\text{-HT}_{1C})$	α-methyl 5-HT	LY53857	IP3 /DG
5-HT_3	2-methyl 5-HT	tropisetron	cation channel

*5-HT_1 receptors (different species) now include 5-HT_{1}nonA, non B, NonC:

Drosophila* - $5\text{-HT}_{dro1,dro2A,dro2B}$ (Hen 1992):Lymnaea* 5-HT receptor cloned named 5-HT_{lym} (Sugamori et al. 1993)

5-HT IN INVERTEBRATE SPECIES

The study of 5-HT in invertebrates began with Erspamer and Ghiretti(1951) who identified enteramine in the salivary glands of *Octopus vulgaris, Eledone moshata* and the hypobrachial body of *Murex trunculus* and *M. brandaris*. Erspamer and Ghiretti's (1951) study of the action of enteramine on "the heart of molluscs" showed it to be a potent agonist in two species, *Helix pomatia* and *Octopus vulgaris*. Enteramine was later identified as 5-HT.

In 1951, Welsh and Twarog began their investigations into 5-HT activity on the heart of the mollusc *Mercenaria(Venus) mercenaria,* as well as other molluscan smooth muscle preparations, Welsh and Twarog (1960). Their experiments, as reviewed by Twarog (1988) and Greenberg (1988), showed 5-HT to have a potent excitatory effect on the heart of the clam *Mercenaria mercenaria,* increasing both the strength and frequency of the heartbeat. Because this effect was selective for small amounts of 5-HT, the isolated heart of this clam became a useful bioassay preparation for 5-HT. Throughout the 1950s the clam heart preparation was used as a bioassay tool in the investigation of 5-HT, Twarog and Page(1953), Gaddum and Paasonen (1955), Welsh (1953,1957).

Welsh and Moorhead (1960) studied the distribution of 5-HT in invertebrates. They identified the presence of 5-HT in approximately 60 different species of invertebrates. It became apparent that this chemical played a significant role throughout the animal kingdom, and in particular the molluscan phylum. More 5-HT was found per unit weight of tissue in ganglia of the bivalve molluscs than in any other neuronal tissue examined.

Cottrell and Laverack (1968) in their review of invertebrate pharmacology discussed the extensive literature published on 5-HT from 1948-1968. They clearly outlined to date the major research that had led to the identification of 5-HT as an important neurotransmitter and neurohormone in invertebrates.

5-HT AS A NEUROTRANSMITTER IN MOLLUSCAN SPECIES

Welsh (1953) proposed that 5-HT mediated the effect of the cardioexcitatory nerves in *Mercenaria mercenaria,* he then demonstrated the presence of 5-HT in ganglia from *Mercenaria mercenaria,* Welsh (1957). Wright et al. (1962) showed that Methysergide(UML) could block the cardioexcitatory effect produced by the stimulation of the excitatory nerves to the heart of *Mercenaria mercenaria.* Twarog (1954) suggested a role for 5-HT as a peripherally acting neurotransmitter in the mussel *Mytilis edulis* because 5-HT had an inhibitory (relaxing) effect on the byssus retractor muscle of this species.

In accord with the proposed neurotransmitter role, 5-HT was detected in neurones of several species of molluscs. Loveland (1963) showed the presence of 5-HT in the cardiac nerves of *Mercenaria mercenaria;* Falck and Owman (1965) demonstrated the presence of 5-HT in several gastropod neurones, by histochemical localisation of 5-HT with fluorescent techniques. Rozsa and Perenyi (1966) demonstrated that 5-HT was released from the cardiac nerve of *Helix pomatia* when electrically stimulated. Cottrell and Osborne (1969) using a fluorescence technique, demonstrated the presence of 5-HT in nerve fibres found throughout the ventricles of *Helix pomatia.*

Electrophysiological studies of the Giant Serotonin Cells (GSC) in *Helix pomatia* showed that each cell made synaptic connections with other neurones in the buccal ganglia. By pharmacological methods, Cottrell and Macon (1974) had identified 5-HT as the transmitter substance at these synapses. Morphine, Methysergide and 4-BromoLSD reduced the response of the buccal neurones to 5-HT.

EFFECTS OF 5-HT ON NEURONES IN MOLLUSCS

Kerkut and Walker (1961,1967), Gershenfeld and Tauc (1961), showed 5-HT to have a potent effect on central neurones of gastropod molluscs, Gerschenfeld (1973). 5-HT was found to excite some neurones but to inhibit others. The study of 5-HT application on neurones improved with the introduction of "iontophoresis", a technique of localising drug application to individual neurones.

Gershenfeld and Stefani (1966) demonstrated that CILDA cells from a gastropod responded to minute amounts of 5-HT, the response being antagonised by UML; when 5-HT was applied iontophoretically to different neurones, some were depolarised (excited), while others were hyperpolarised(inhibited). Walker et al. (1972) showed 5-HT to be excitatory on a single neurone in *Helix aspersa*.

Gershenfeld and Paupardin-Tritsch (1974 a, b) studied the actions of 5-HT by examining its effects on ionic conductance across the cell membrane; they examined the ionic mechanisms and attempted to characterise the receptors involved. These studies used two gastropod species, *Helix aspersa* and *Aplysia californica*. Their results showed three distinct types of excitation (depolarisation) due to 5-HT; two types, one "slow" the other "fast", involved an increase in Na^+ conductance. The

third type was caused by a decrease in K^+ conductance. They also identified three types of inhibition (hyperpolarization) due to increased membrane conductance in response to 5-HT; two types due to either Cl^- or K^+ conductance, the third type to a decrease in both Na^+ and K^+ conductance.

The study also characterised, using antagonists, four of the receptors involved in the actions of 5-HT on the neuronal membranes. Tables(2A,& 2B) of the results from this study are summarised below. However, the antagonists used in this study (see Table 2 B) were far from being selective for the 5-HT receptors:

TABLE 2A.

RECEPTOR	ACTION	IONIC MECHANISM	ANTAGONIST
A	Fast depolarisation	Increased Na^+	non selective
A'	Slow depolarisation	Increased Na^+	Bufotenine
B	Slow hyperpolarization	Increased K^+	non selective
C	Fast hyperpolarization	Increased Cl^-	non selective
α	"?" depolarisation	Decreased K^+	no antagonist
β	"?" hyperpolarization	Deceased Na^+ & K^+	no antagonist

TABLE 2B.

Antagonist	**A**	**A'**	**B**	**C**
(+)-TC(curare)	blocks	none	none	blocks
LSD 25	blocks	none	blocks	blocks
Tryptamine	blocks	none	blocks	blocks
7-MT	blocks	none	none	none
Bufotenine	blocks	none	blocks	none
5-MG	none	none	blocks	none
Neostigmine	none	none	none	blocks

Note: No specific antagonists were found for the responses linked to a conductance decrease.
These tables were taken from Gerschenfeld and Paupardin-Tritsch (1974a).

PHYSIOLOGY OF 5-HT IN NEURONES OF MOLLUSCS

A link between 5-HT effects on cAMP levels within the cell and the modulation of certain ionic conductances across the cell membrane has been reported in many studies on gastropod neurones, Klein and Kandel (1978), Levitan (1974), Drummond et al. (1980) and Deterre et al. (1981).

The study of the effect of 5-HT on second messenger pathways was aided by the addition of the "patch-clamp" electrophysiological technique, Hamill et al. (1981). Investigators were now able to examine small patches of membrane in isolation while applying drugs, second messengers, or kinases to the inner or outer surface of the membrane patch.

Siegelbaum et al. (1982), using cell-attached patches of *Aplysia californica* sensory neurones, identified a potassium current affected by 5-HT via a second messenger pathway. They demonstrated the similarity between the effect produced by 5-HT to that of elevating intracellular cAMP directly. This serotonin sensitive potassium channel (named the "S-K^+" channel) had several novel properties:

a) the channel was active at the cell's resting potential; gating was only moderately affected by membrane potential,
b) the activity did not appear to be dependent on intracellular Ca^{++},
c) intracellular injections of cAMP cause prolonged complete closures of the channel, similar to the effect produced by extracellular application of 5-HT.

Closure of this channel could account for electrophysiological changes seen with both 5-HT or cAMP elevation: namely a decrease in membrane conductance, a long-lasting depolarising synaptic potential and a delay in repolarization of the action potential.

Shuster and Siegelbaum (1987) later showed this channel was not blocked by either TEA or 4-AP applied to the outer membrane surface. They reviewed the characterisation of the S-K^+ channel found in *Aplysia californica* sensory neurones and described the similarity between the patch-clamp single S-channel and the macroscopic S-current earlier characterised by their study on whole cell voltage.

Benson and Levitan (1983) reported the effect of 5-HT on an anomalously rectifying K^+ current in the *Aplysia californica* R15 neurone; 5-HT increased the K^+ conductance and the response was also linked to the cAMP pathway. This study showed that 5-HT could produce an opposite effect on conductance, on a different neurone in the same species, by modulation of a K^+ current (via cAMP pathways).

Lotshaw et al. (1986) also studied the *Aplysia californica* R15 neurone and showed that 5-HT can produce both excitation and inhibition; cGMP inhibited one ion current that cAMP activated. They concluded "these findings emphasise the intricacy of regulatory pathways which contribute to fine tuning of neuronal electrical activity".

Brezina et al. (1987) reported that FMRFamide modulated the same S-channel but in the opposite way to 5-HT. This opening of the S-K^+ channel by FMRFamide appeared to be linked to a cAMP independent pathway. Piomelli et al. (1987) found that 12-hydroperoxyeicosatetetraenoic acid (12-HPETE) proved to be very effective in mimicking FMRFamide. Volterra (1990) reviewed these findings and proposed a molecular model for the antagonist modulation of the S channel by 5-HT and FMRFamide.

It should be noted here that Cottrell et al. (1984) had shown that FMRFamide could also cause suppression of a K^+ current in *Helix aspersa* neurones.

Furukawa and Kobayashi (1988a) identified two K^+ channels in the excitatory neurones to the heart of the snail *Achatina fulica;* both channels were 5-HT sensitive. They called the channels SL and SS (Serotonin Large and Small). The SL K^+ channel had many characteristics similar to the *Aplysia* S-K^+ channel described earlier. The SS K^+ channel was thought to be voltage and Ca^{++} dependent. 5-HT decreased the K^+ current activity of both channels causing depolarisation of the cells and one of the responses was blocked by UML.

Shuster, Camardo and Siegelbaum (1991) later compared the characteristics of both the S-K^+ channel and a voltage and Ca^{++} dependent K^+ channel blocked by TEA in *Aplysia californica* neurones. They concluded that *Aplysia* sensory neurones contain two prominent distinguishable classes of K^+ channels. The gating properties of the S-K^+ channels contribute to an outward repolarising current over a wide range of membrane potentials. They proposed that the modulation of the S-K^+ channel by neurotransmitters contributes to changes in both resting potential and action potential duration.

EFFECT OF 5-HT ANALOGUES ON MOLLUSCS

With the increasing number of new 5-HT agonist analogues described in the mammalian literature, some researchers decided to apply these analogues to invertebrate preparations. Murakami et al. (1986,1988) studied the byssus retractor muscle of *Mytilus edulis* using agonists and antagonists of 5-HT. They suggested that 5-HT receptors in this tissue had more

similarity to the 5-HT_{1A} mammalian receptor than to the 5-HT_2 receptor.

Ram et al. (1987) reported that the 5-HT receptor in the buccal muscle of *Aplysia californica* had characteristics similar to 5-HT_{1A} (as defined by the mammalian classification).

Walker and Vehovszky (1990) studied the action of the newer 5-HT agonists and antagonists in the following preparations:
Helix neurones, excited or inhibited by 5-HT; *Helix* heart and pharyngeal retractor muscle. They concluded that invertebrate 5-HT receptors cannot be easily classified in terms of the vertebrate 5-$HT_{1,2}$ and 3 nomenclature. Tryptamine analogues were active in all the *Helix* preparations, while non-tryptamine compounds demonstrated low potencies. They identified several of the most active agonists which were:
5-carboxamidotryptamine(5-CT),
α-Methyl-5-HT(α-Me-5-HT),
5-methoxytryptamine(5-MEOT).
Several antagonists showed some degree of selectivity. They were MDL-72222, cinanserin and ketanserin; UML demonstrated agonist properties. Tryptamine itself selectively reduced 5-HT excitatory responses on *Helix* central neurones.

Walcourt-Ambakederemo and Winlow (1993,1994) studied pharmacologically (using antagonists) the cerebral giant cells (CGC) and buccal ganglia cells from *Lymnaea stagnalis*. They reported the possible existence of more than one 5-HT receptor type; identifying one receptor with 5-HT_{1A} properties and another with 5-HT_2 characteristics.

In the literature there are a few pharmacological studies using a variety of specific 5-HT analogues and antagonists, applied to isolated organ or neuronal preparations of

invertebrates. However many of the more recently described 5-HT analogues have not yet been studied in detail in any membrane patch-clamp experiments on invertebrate neurones.

5-HT IN OTHER PHYLA OF INVERTEBRATES

5-HT appears to play a different role in the invertebrate peripheral tissue than in that of mammals; 5-HT is the bioamine in the invertebrates that often serves a similar physiological role to that of noradrenaline in the vertebrates.

There is evidence for the presence, release, and physiological role of 5-HT in peripheral and central synapses in the invertebrates. The complex and diverse role of 5-HT and other neurotransmitters in invertebrates was thoroughly reviewed by Leake and Walker (1980), Walker (1984 a, b,), Walker and Holden-Dye (1989). In a review by Walker (1985), "The Pharmacology of Serotonin Receptors in Invertebrates" there is an extensive discussion of the diversity and structure-activity studies carried out on many different tissues from invertebrate species.

Kerkut et al. (1967) identified 5-HT in giant neurones (Retzius cells) in the ganglia of a leech, *Hirudo medicinalis*. This was one of several different invertebrate neuronal preparations used to examine the action of 5-HT in the late 1960s and 1970s. Kerkut and Walker (1967) showed that 5-HT has a direct inhibitory action on the Retzius cells in the leech. This effect was associated with an increase in conductance to chloride ions, Walker and Smith (1973).

Smith and Walker (1974) went on to examine the structure-activity relationships of 5-HT and some analogues on these cells. 5-HT was found to be the most potent ligand, however α-methyl 5-hydroxytryptamine (α-Me-5-HT) was a potent agonist in

this tissue. The antagonists used were dibenamine and UML; however these compounds were shown to have direct actions themselves. Morphine and atropine were also found to antagonise the effect of 5-HT; morphine also had direct inhibitory effect.

5-HT has been shown to act in many neuronal pathways such as those described in insects by Evans (1980) and in Fiddler Crabs by Fingerman et al. (1974,). 5-HT was shown to have an effect on the control of secretions in insects; Berridge, Berridge and Prince (1970, 1972) showed that the salivary gland in the blowfly *Calliphora* was under the control of 5-HT. Furthermore in the same species, one 5-HT receptor appears to activate cAMP, while another one is linked to the IP3-calcium pathway, Berridge and Heslop (1981).

Recently molecular structures for several 5-HT receptors identified from *Drosophila melanogaster* have been reported in the literature. 5-HT_{dro2A} and 5-HT_{dro2B} reportedly have more similarities to the 5-HT_1 type receptors, and are negatively linked to cAMP pathways, Saudou et al.(1992). The 5-HT_{dro1} has been positively linked to cAMP pathways and is suggested as having a possible role in modulation of circadian rhythms, Saudou and Hen (1994).

ACTION OF 5-HT ON THE HEARTS OF MOLLUSCS

Greenberg (1960 a, b) was the first to study how altering the structure of the 5-HT molecule affected the mechanical activity of hearts of different bivalve species. He showed that the excitation seen with 5-HT on *Mercenaria mercenaria* hearts could be reduced or blocked by UML and 2-bromo-lysergic acid diethyl amide (LSD). Leake, Evans and Walker (1971) showed the same antagonism of the excitatory action caused by 5-HT on the heart of the gastropod *Patella vulgata*; however UML

had a direct inhibitory action of its own on the heart of this species.

Greenberg (1960a) and Painter and Greenberg (1982) had observed different responses and sensitivity to 5-HT and analogues among several species of bivalve molluscs. One anomaly was seen with LSD, which was exquisitely potent on the heart of *Mercenaria mercenaria* (in the *f*M range), causing prolonged excitation followed by an extensive period of insensitivity to 5-HT. Greenberg (1960 b) confirmed earlier reports of an inhibitory effect of LSD applied to the heart of some species of bivalve, e.g. *Cardium edula* (Gaddum and Paasonen, 1955). Greenberg (1965,1969) also found that 5-HT produced a depression of rate and/or tone in the hearts of several other bivalve species, *Clinocardium nuttalli, Cyrtopleura costata, Anodonta grandis* and *Modiolus (Geukensia) demissus*.

However it is important to mention that Benzoquinonium(Bz) was frequently used in many of these studies, to block any possible background effect of Acetylcholine (ACh). This fact will be referred to in the discussion of the results in Part I.

The inhibitory effect of 5-HT on the heart of *Modiolus (Geukensia) demissus* was examined in detail by Irisawa et al. (1973). They concluded that the inhibition, membrane conductance change and hyperpolarisation caused by 5-HT were virtually independent of extracellular Cl^- or Na^+; the membrane conductance changes induced by 5-HT were possibly due to changes in K^+ permeability.

Koch and Greenberg (1981) examined calcium fluxes in the heart of *Geukensia demissus;* they correlated "excitation" caused by 5-HT with a net decrease in calcium efflux, thereby increasing intracellular calcium. However, they reported that

the "cardioinhibition" effect seen with 5-HT in this species, was accompanied by no significant change in calcium flux.

Painter and Greenberg (1982) undertook an extensive study of the action of 5-HT and FMRFamide on the hearts of 50 different bivalve species. They found the predominant effect of 5-HT to be excitation. However, there were several species, *Geukensia demissus, Lampsiis clabornensis, Villosa villosa, Trachycardium egmontianum* and *Rangia cuneata* which showed either a predominant inhibitory or a mixed response to 5-HT.

STUDIES OF 5-HT RECEPTOR(S) IN THE HEARTS OF MOLLUSCS

Kiss and Rozsa (1978) tried pharmacologically, by using antagonists, to differentiate 5-HT receptors in the hearts of *Helix pomatia*. They examined differences between the depolarisation (excitation) seen with low doses of 5-HT and hyperpolarization (inhibition) seen at high concentrations. They showed that morphine and nicotine blocked the "depolarising" effect of 5-HT. UML, curare and atropine blocked the "hyperpolarising" effects allowing the "depolarising" effects to prevail. The lack of specific selective antagonists to differentiate between these responses is apparent. Kiss and Rosza (1978) concluded, "the complex and sometimes opposing action of 5-HT on the same cell membrane could be explained by the presence of different 5-HT receptors probably not in the physical but rather in the functional sense".

Kawakami and Kobayashi (1984) showed that UML antagonised the effect of 5-HT in a gastropod heart, *Rapana thomasiana*. Akagawa et al. (1987) went on to study the response of the heart of *Achantina fulica* to 5-HT. They used the antagonists UML and cinanserin to try and dissect out different

physiological effects. They reported that 5-HT demonstrated dual effects on the heart of this species: potentiation of beat (both in frequency and amplitude) and post-potentiative periodic arrest of the beat. The ability to block these effects separately with different antagonists led them to suggest that `the two actions of serotonin are mediated by separate receptors'.

EFFECT OF 5-HT ANALOGUES ON THE HEARTS OF MOLLUSCS

Reports, such as that of Peroutka (1988) and Saxena et al. (1989), of new 5-HT agonist and antagonist ligands and the identification of distinct 5-HT receptors in vertebrates, led some workers to re-examine 5-HT receptors in invertebrates.

Boyd et al. (1985) were the first to systematically examine some of these ligands to try to classify 5-HT receptors in a molluscan heart. The species studied was *Helix aspersa*. Among the analogues used were 5-HT, RU24969, MDL-72222, MK212, and 8-OH-DPAT (see page 9 for full chemical names). Several analogues produced excitation but were in the order of 100 times less potent than 5-HT. While 8-OH-DPAT behaved as an agonist (less potent than 5-HT), it was noted that this agent and RU24969, antagonised the effect of 5-HT subsequently tested. UML had an agonist excitatory effect but also acted as an antagonist to 5-HT, thus exhibiting partial agonist characteristics. Ketanserin, a 5-HT_2 antagonist, demonstrated very weak antagonist action to 5-HT in this tissue. They concluded there was more similarity between the 5-HT receptor in the gastropod heart to the "5-HT_1" mammalian receptor group, than the 5-HT_2 receptor.

Walker and Vehovszky (1990) studied the response of *Helix* hearts to several 5-HT analogues, they found that 5-

methoxytryptamine (5-MEOT) was slightly more potent than 5-HT. 5-Carboxyamidotryptamine (5-CT), and α-Me-5-HT were marginally less potent than 5-HT. However these analogues were more selective than some used in earlier studies by this group.

Buckett et al. (1990) studied the pharmacology of 5-HT in *Lymnaea stagnalis* hearts. The action of several antagonists on the heart from this species re-emphasises the differences which exist between species. The antagonists UML, ketanserin and cinanserin all showed agonist properties; however, some antagonism of 5-HT by cinanserin was observed.

The most recent extensive screening of "new" 5-HT analogues on gastropod hearts was by Cadogen and Humphrey (1992). They tried to classify the 5-HT receptor mediating cardioexcitation of *Helix aspersa* hearts using a wide variety of the agonists and antagonist developed for mammalian 5-HT receptor research. They reported that both 5-CT and α-Me-5HT were active agonists, though not as potent as 5-HT. Several agonists and antagonists behaved like partial agonists. Cadogen & Humphrey (1992) stated that they had not found any distinctly selective antagonist; they concluded that the cardioexcitation produced by 5-HT on *Helix* hearts, did not fall into any distinct classification category similar to those of the vertebrate 5-HT receptors.

PHYSIOLOGICAL ROLE OF 5-HT IN THE HEARTS OF MOLLUSCS

Cottrell & Osborne (1969) were the first to show that 5-HT stimulated adenylyl cyclase in *Spisula solida* hearts; they concluded that cAMP might be involved in the response of the heart to 5-HT.

Higgins (1974) studied the actions of 5-HT on the intracellular levels of adenylate and guanylate cyclases; he

showed increased cAMP levels in the heart of two molluscan species exposed to 5-HT, *Mytilis edulis* and *Modiolus (Geukensia) demissa*. Higgins noted that in *Modiolus* hearts, often inhibited by 5-HT, the levels of cAMP did not increase significantly. He pointed out that cAMP, or phosphodiesterase inhibitors, had no apparent direct effect on the mechanical activity of these isolated bivalve ventricles. Higgins (1977) went on to link the tachyphylaxis induced by prolonged exposure to 5-HT with a desensitisation of the myocardial adenylate cyclase and a decrease in the intracellular cAMP in *Mercenaria mercenaria*.

Paciotti and Higgins (1985) showed potentiation of the 5-HT effect on myocardial contractility in *Mercenaria mercenaria* by forskolin, an agent which elevates intracellular cAMP.

Wollemann and Rozsa (1975) reported 5-HT activation of adenyl cyclase results in elevated cAMP in the hearts of *Anodonta cygnea*. Sawada et al.(1984) examined the effect of 5-HT on the ventricles of *Aplysia californica* and demonstrated a link between the increased contractility, elevated cAMP and intracellular Ca^{++} movement. The effect was blocked by UML.

ELECTROPHYSIOLOGICAL STUDIES OF HEART CELLS FROM MOLLUSCS

The first report of patch-clamp studies of myocytes from molluscan hearts came from the group of Brezden et al. (1986) and Sigurdson et al. (1987). They identified a K^+ channel in *Lymnaea stagnalis* heart cells, which appeared to be stretch sensitive. This channel is now known as the stretch activated potassium (SAK^+) channel. Vandorpe and Morris (1992) went on to identify similarities between the SAK^+ channel found in *Lymnaea stagilis* heart and neurones to those of the *Aplysia californica* neuronal $S\text{-}K^+$ channel, discussed earlier in the

Introduction, Siegelbaum et al. (1982) and Shuster et al. (1985, 1991).

This study is important because it was the first which linked a K^+ channel characterised in a mollusc heart to a molluscan neuronal K^+ channel, which is known to be affected by 5-HT and is well characterised in the literature.

Sugamori et al. (1993) have identified the molecular structure of a 5-HT receptor found in the heart and neurones of *Lymnaea stagnalis* which they have named 5-HT_{lym} . This receptor has a structure with many similarities to the 5-HT_{dro2B} receptor; however its functional intracellular coupling has not been analysed. Saudou and Hen (1994) link this receptor to the general 5-HT_1 family.

5-HT RECEPTOR TYPES IN THE HEARTS OF *BIVALVIA*

Ever since the findings of Painter and Greenberg (1982) which illustrated the wide differences between species of bivalves in the response of their hearts to 5-HT; the question as to the uniformity of the 5-HT receptors in the hearts of the bivalve species has waited to be answered.

The antiquity and evolutionary isolation of molluscs, might explain how differences in receptor conformation might evolve within many of the sub-classes in this phylum. Greenberg (1965) and Wilkens and Greenberg (1973) have described pharmacologically distinct types of ACh receptors in the hearts of different bivalve species. Perhaps similar evolutionary events have led to differences in 5-HT receptors.

This introduction has reviewed those studies which the author feels are relevant to the question under study: are there various 5-HT receptor types present in the hearts of different species of molluscan bivalves? Could the study of

selective 5-HT agonists in species with known differences in their response to 5-HT, help to identify or characterise the 5-HT receptor(s) further?

I should emphasise that the new selective ligands have been designed for mammalian receptors. However the comparison between the pharmacological profile of the receptors in different species and the intracellular mechanism of action may identify similarities and differences among the 5-HT receptors.

Eventually the sequence of the corresponding receptor proteins might provide further insights into the determinants of 5-HT-binding specificity and the receptor(s) classification throughout the invertebrate and vertebrate populations.

A fitting summary to my introduction on the 5-HT receptor, as the numbers of receptors identified increase annually, is taken from the introduction by Hoyer et al. (1994):
"Protein receptors that mediate the actions of 5-HT have existed in the membranes of a variety of animal cell types for millions of years..... It would seem likely that during such a long period of time, there has been ample opportunity for mutation and consequent evolutionary acceptance of multiple variants of receptors for all of these older neurotransmitters and hormones".

TABLE 3

CLASSIFICATION OF MOLLUSCAN BIVALVES USED IN PART I AND PART II

Mollusca		SOURCE & STUDY LOCATION
CLASS	*Bivalvia*	
SUB CLASS	*Pteriomorphia*	
Family	*Mytilidae*	
Mytilis californianus	(a)	U.B.C.
Geukensia demissa	(b)	Whitney Laboratory
SUB CLASS	*Heterodonta*	
Family	*Venaridae*	
Saxidomus giganteus		U.B.C.
Mercenaria mercenaria		Whitney Laboratory, & U.B.C.
Family	*Myidae*	
Mya arenaria	(c)	St.Andrews, Whitney Laboratory, & U.B.C.
Family	*Carditidae*	
Clinocardium nuttalli		U.B.C.
Trachycardium egmontianum		Whitney Laboratory

(a) Collected by Seacology Ltd. from coastal British Columbia, for the University of British Columbia, Canada

(b) Whitney Marine Laboratory, University of Florida, USA

(c) St. Andrews University, Scotland

PART I

ACTION OF 5-HT AND ANALOGUES ON THE ISOLATED HEART

INTRODUCTION:

Molluscan hearts are very sensitive to 5-HT, usually showing marked cardioexcitation; however some hearts are inhibited and some hearts show responses which are a mixture of excitation and inhibition. In reviewing earlier literature it became apparent there were different effects seen with 5-HT on the hearts from a variety of molluscan species.

In Part I, I described studies of the action of 5-HT and some of the more recently introduced selective "mammalian" agonists on the hearts of the following bivalve species: *Mercenaria (Venus) mercenaria*, Mya arenaria*, Geukensia (Modiolus) demissa*, Saxidomus giganteus, Clinocardium nuttalli and Mytilus californianus.*

Experiments were carried out on isolated heart preparations using cumulative doses of the agonists. The species were chosen after screening species reported extensively in the literature (as identified by the asterisks) as well as other local species. I identified species whose hearts showed only excitatory responses to 5-HT, as well as those which showed inhibition or more complex mixed responses.

METHOD

The hearts from bivalves, collected from various locations (see Table 3), were removed and placed in artificial sea water (ASW, see Table 4). The digestive tract, which runs through the ventricles of all species used in this study, was carefully removed. As shown in Diagram 1, 5-0 braided silk suture thread was tied at the proximal and distal ends of the ventricles

which included small amounts of the atria (auricles). One end of the heart was attached to a mounting hook in a 25 ml organ bath, the other end was attached to a Grass FT03 force transducer. The resting tension exerted on the ventricles was 0.2 - 0.5g (depending on the size of the heart). The organ bath was cooled by a water jacket and maintained at 16-18°C for species collected from cold water environments. Those hearts from species found in warmer waters were studied at room temperature 20-22°C, (Diagram 1). The heart tissue was constantly aerated by a small stream of air bubbled through the bathing solution.

The force transducer was attached to a Grass Polygraph Model 79 to record changes in force and rate of the heartbeats. The effects of the drugs on the activity of the ventricles was monitored. 5-HT or its analogues were administered in a cumulative dose regime. Increasing concentrations were added to the bath at intervals (60 - 120s) when each response had stabilised. This method was employed after verifying that the cumulative dose response curve (DR) was the same as the (DR) using multiple single doses, see Figure 1 (b). The cumulative method provided a complete dose response curve for each compound in a short period of time.

Often hearts were found to beat irregularly or very slowly. Since I could not pretreat with ergotamine or add Benzoquinonium to the bath, I decide to use the technique suggested by Gaddum and Paasonen (1955). They found that after the Mg^{+} concentration was lowered in their ASW, hearts beat more rapidly and regularly.

Diagram of Organ Bath set-up

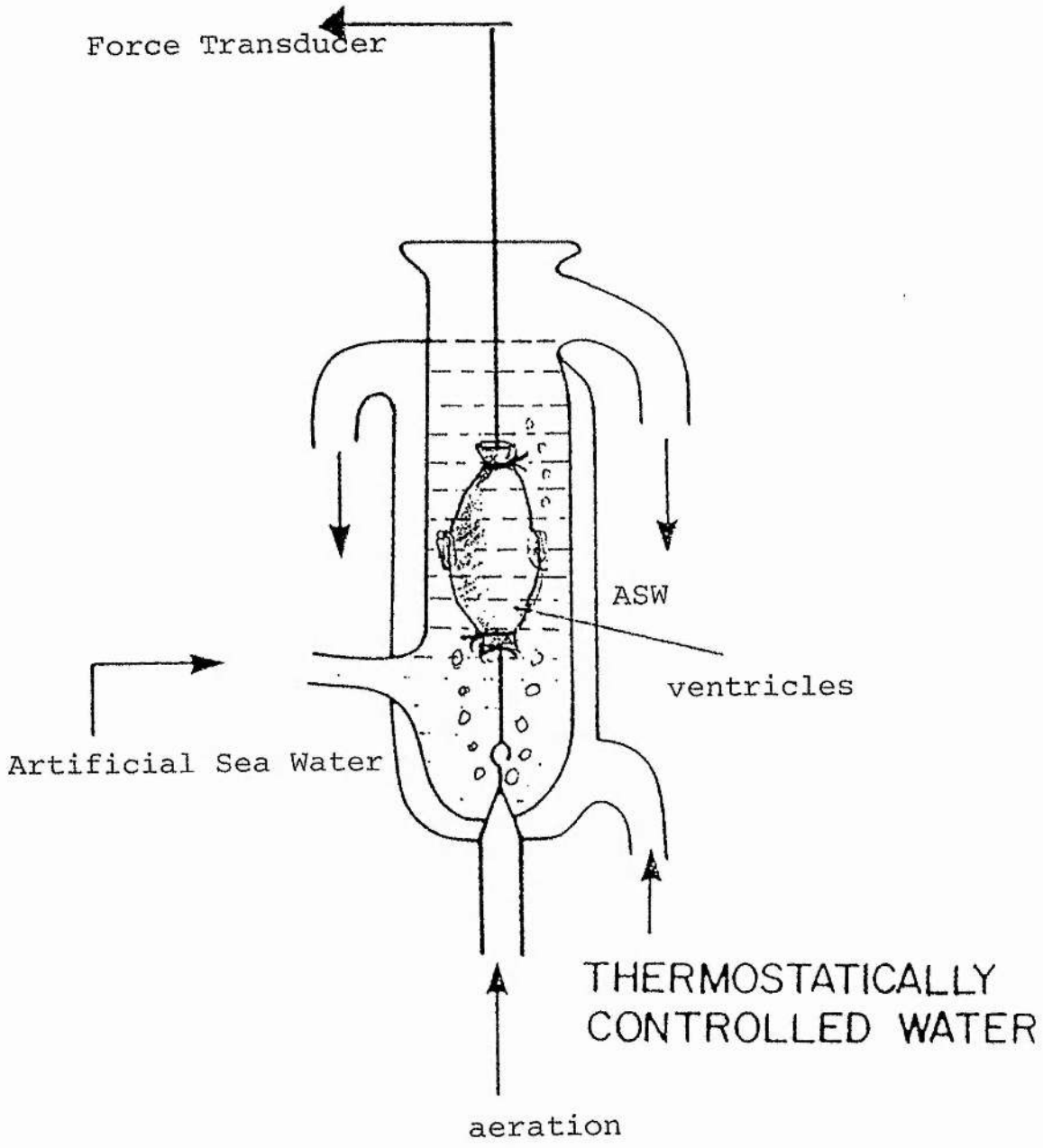


Diagram 1

The diagram illustrates the isolated ventricles suspended in aerated artificial sea water (ASW) in an organ bath.
The thermostatically controlled water passes through the bath jacket to control the temperature of the ASW in the bath.

Table 4

Artificial Sea Water(ASW) used in Part 1

	Regular ASW (Welsh & Taub, 1948)	**Low Mg^+ ASW** (Gaddum & Paasonen, 1955)
	ASW g/L	**(Low Mg^+)ASW g/L**
NaCl	30.0	23.0
KCl	0.9	0.65
$CaCl_2$	1.1	1.1
$MgCl_2.6H_2O$		1.0
$MgSO_4.3H_2O$	3.5	
Na_2SO_4		4.0
$NaHCO_3$	as required to (buffer to pH = 7.4)	

ASW(low Mg^+) was used in most of the experiments, in this ASW the hearts would usually beat faster and more regularly, the rate was often increased by 10-20%, see figure 1(c). No difference was observed in the response of the hearts (in the low Mg^+ ASW) to 5-HT.

Antagonists were added to the bath in single doses at least 2-4 min before addition of the agonist dose. The antagonists were used at concentrations in the range of 0.1-10 μM. Thus several agonists and an antagonist could be examined on one heart within a reasonable time.

AGONISTS	**SOURCE**
5-hydroxytryptamine creatine sulphate(5-HT)	Sigma
5-carboxamidotryptamine(5-CT)	Glaxo
2-methyl-5-hydroxytryptamine(2-Me-5-HT)	Glaxo
5-methoxytryptamine(5-MEOT)	RBI
α-methyl-5-hydroxytryptamine(α-Me-5-HT)	RBI
8-OH-DPAT	RBI

ANTAGONISTS	
Methysergide-hydrogenmaleinate(UML)	RBI
Methiothepin(MT)	Glaxo
Benzoquinonium(Bz)	Stirling-Winthrop
Ketanserin	Janssen
Retanserin	Janssen
GR38032F	Glaxo
ICS 205-930	RBI
MDL-72222	RBI

The main species examined were:

Mya arenaria *Saxidomus giganteus*

Mercenaria mercenaria *Geukensia demissus*

Some data are also presented for *Clinocardium nuttalli*, and *Mytilus californianus*.

Initially hearts from the different species were screened with 5-HT, several DR regimes were tested with washing and a

rest period (>20 min) between each regime. This identified any desensitisation which might affect subsequent drug application. 5-HT or its analogues were applied to the bath in a different order between hearts. Individual DR curves were plotted from the results of each cumulative drug application.

From the individual DR curves, the results for each group of hearts from the same species were analysed. The percentage increase (or decrease) in response to a ten fold change in concentration of a drug was tabulated. The mean (and standard error) of the percentage (%) change of tension was plotted against each 10 fold concentration increase of drug.

The EC_{50} for a drug was tabulated from each individual DR curve. The mean EC_{50} (K_{EC50}) for the drug in each species was then calculated, see Table 5. The EC_{50} is the molar concentration of an agonist that produces 50% of the maximal possible (excitatory) effect of that agonist. The term IC_{50} was used to differentiate when the effect of an agonist caused a decrease (inhibition) in the force of contraction.

The EC_{50} (or IC_{50}) value was used to compare the potency of each analogue and expressed as a ratio of the potency of 5-HT in that species. 5-HT thus has a potency of 1 in each species and the activity, or potency, of an analogue is expressed as a ratio relative to 5-HT, see Table 6.

RESULTS - PART I

Tables 5 and 6, show the K_{EC50} and Potency ratios of the responses to 5-HT and several analogues on the hearts of three different bivalve species (the major focus of the studies that I have reported in this section).

TABLE 5

K_{EC50} for 5-HT and Analogues

Species	5-HT (n=20)	α-Me-5HT (n=5)	5-MEOT (n=5)	5-CT (n=5)
Mya	3.90×10^{-7} (M)	4.30×10^{-8} (M)	3.50×10^{-7} (M)	6.98×10^{-7} (M)
Mercenaria	2.33×10^{-7} (M)	3.38×10^{-8} (M)	1.65×10^{-7} (M)	7.33×10^{-7} (M)
Saxidomus	5.34×10^{-7} (M)	3.30×10^{-8} (M)	8.25×10^{-7} (M)	$3.3 \times 10^{-9*}$ (M)

*K_{IC50}

TABLE 6

Potency Ratio of 5-HT to Analogues

Drug	*Mya*	*Mercenaria*	*Saxidomus*
5-HT	1.0	1.0	1.0
α-Me-5HT	0.11	0.17	0.06
5-MEOT	0.9	0.71	1.54
5-CT	1.9	3.2	-0.006(Inhib)*

RESULTS - PART 1

In the following section I have shown the responses of the isolated hearts to 5-HT, the analogues and antagonists, for each species.

Mya arenaria

Please see **Figures 1(a),(b),(c),(d),and (e)** for the Graphs and Traces.

In every *Mya arenaria* heart tested, 5-HT was found to be excitatory, with an $EC_{50} = 3.9 \times 10^{-7}$ M. An increase in both rate and force was seen every time. In figure (1a) the responses of 10 hearts to 5-HT, are plotted along with other analogues tested on some of the same hearts. Not all of the analogues could be tested on each heart; usually one or two analogues and 5-HT were tested in different order on the hearts.

I had found that some hearts tested with more than 3 drugs, with full cumulative DR curves, showed changes in the control baseline which often led to irregular or biphasic beats. It was then difficult to assess the changes due to a drug; the number of agonists tested on each heart were generally limited to three.

On the hearts of *Mya arenaria* α-Me-5-HT was excitatory (with an $EC_{50} = 4.3 \times 10^{-8}$ M) and more potent than 5-HT. 5-MEOT was excitatory and similar in potency to 5-HT. 5-CT was excitatory with an $EC_{50} = 7 \times 10^{-7}$ M, but less potent than 5-HT. 8-OH-DPAT was excitatory but less potent than 5-HT. LSD-25 was usually more potent than 5-HT; however the excitation persisted for long periods. It was noted that subsequent doses of 5-HT appeared to be less potent. Whether this was due to an

antagonist action of the LSD or a desensitisation of the receptor to 5-HT was not clear.

None of the agonists tested were found to be inhibitory; however several of the antagonists did show some degree of inhibition themselves. UML caused some negative inotropic and chronotropic effects, see Figure 1(d). In the traces shown, several other agents blocked some of the excitatory effects of 5-HT. MDL-72222 was inhibitory on the hearts. Both Methiothepin and UML when added to the bath at $1x10^{-6}$ M concentration, demonstrated some antagonism to 5-HT.

Benzoquinonium (Bz) a compound known to antagonise acetylcholine(ACh) effects, was found to be excitatory when added to the bath. The drug did not appear to alter the potency of any subsequent 5-HT doses. However the percentage increase in force of contraction was usually less, see Figure 1(c). This may be due to the increase in amplitude caused by Bz itself (at concentrations of $1x10^{-6}$ M).

GR38032F, at a dose of $1x10^{-5}$ M had excitatory effects and appeared to exert a weak potentiation of the 5-HT effect. Ketanserin had no effect as an antagonist. Thus the only direct inhibitory effects seen on the hearts of *Mya arenaria* were those seen with some of the higher doses of antagonists, in particular MDL 72222. Such effects may have had little to do with the drug's activity at the 5-HT receptors.

None of the 'selective' mammalian 5-HT receptor antagonists examined at bath concentrations of 10^{-8} M or 10^{-7} M, demonstrated any degree of selective antagonism of the 5-HT action on these hearts.

Mya arenaria

Figure 1(a)

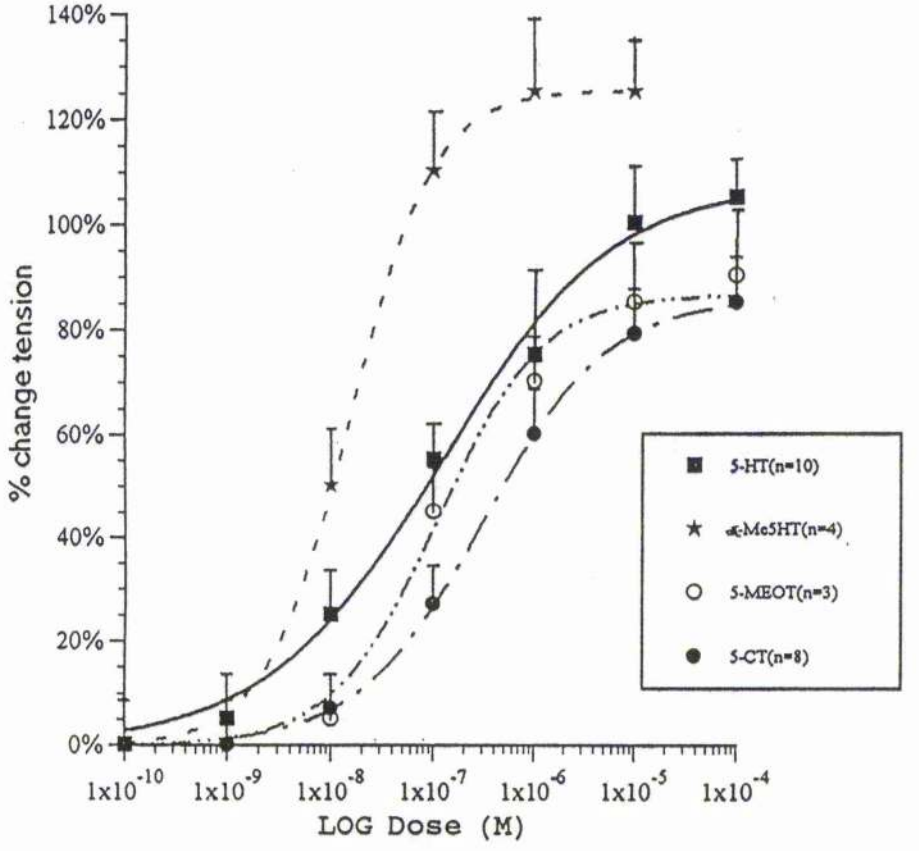
A plot of the mean percentage (%) change in tension(g) against the bath concentration of 5-HT, or the analogue, on several hearts (error bars = S.E. of the mean). The data for each drug were obtained from the individual DR curves as described in the Method section. Not all analogues could be tested on the same heart. The individual traces show typical responses of the hearts to each drug:

(a) shows the response of a single heart to 5-HT and 5-CT.

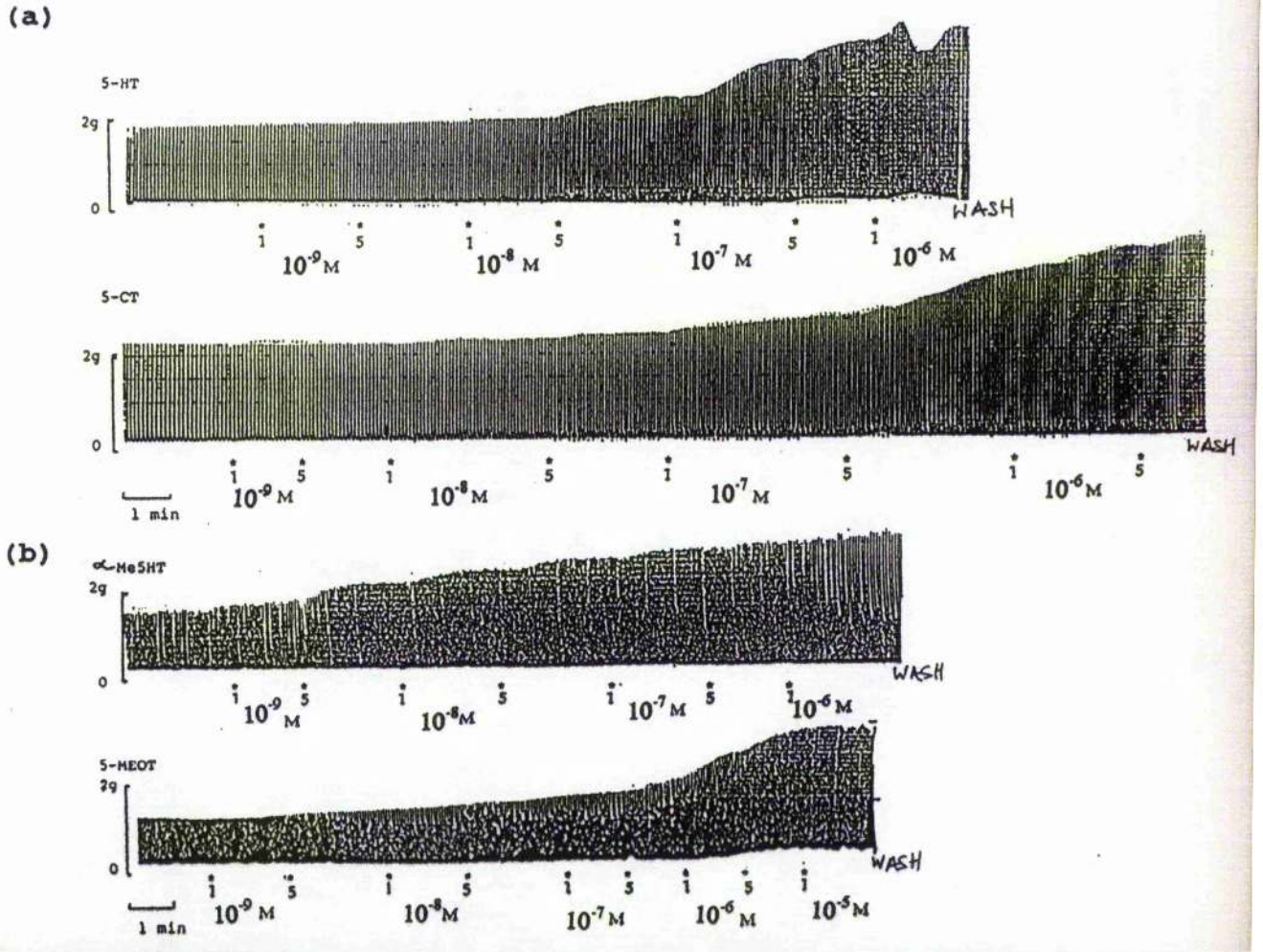
(b) shows the response of a single heart to two other analogues, α-Me-5-HT and 5-MEOT.

Asterisks show where each dose was added to the bath.

Effects of 5-HT and analogues on the heart of *Mya arenaria*



Individual Traces



Mya arenaria

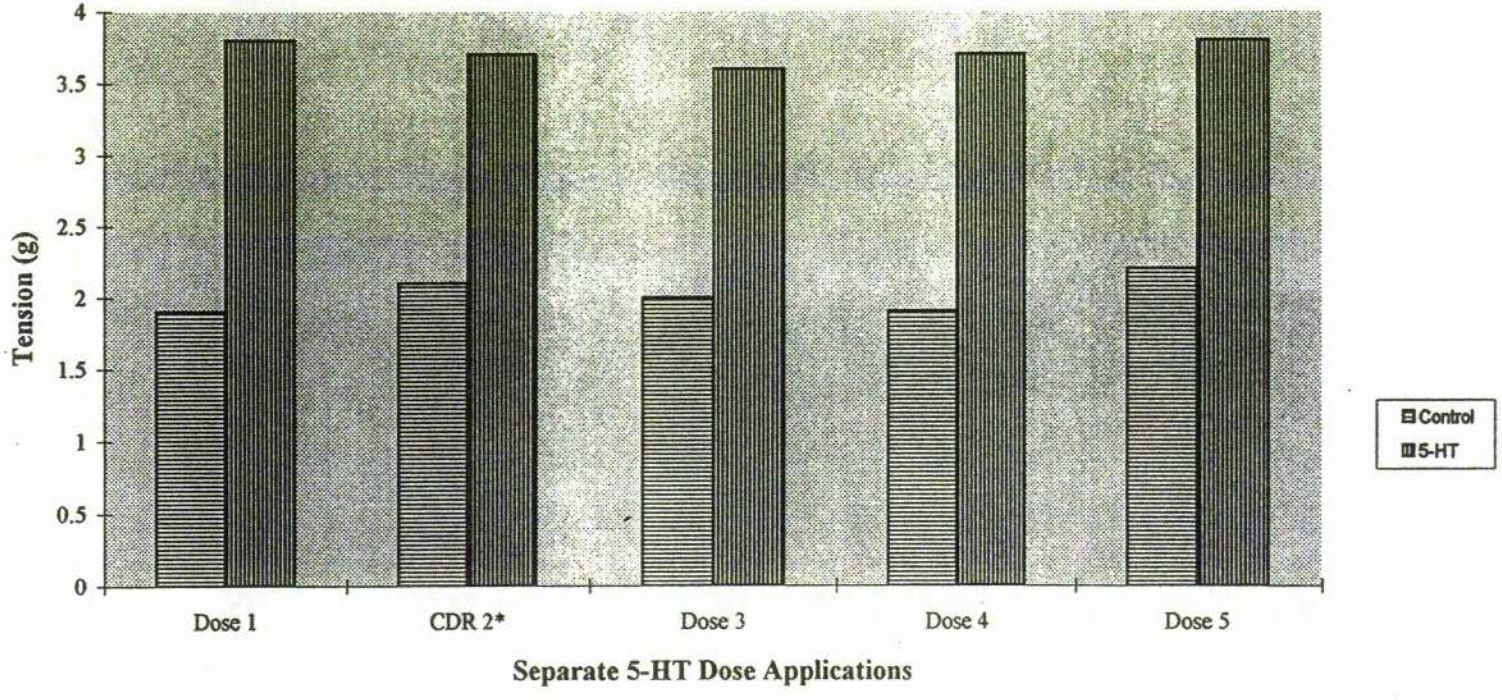
Figure 1(b) The bar graph shows the effect of $1x10^{-6}$ M 5-HT on one heart. The single dose applications were administered at 20 min intervals with multiple washes in between, see Doses 1, 3, 4, and 5.

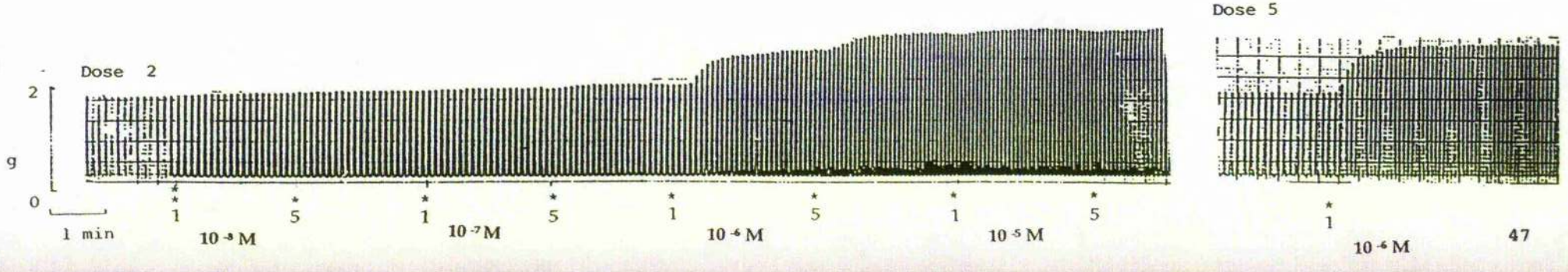
Dose 5 - the final application of a single dose of 10^{-6} M 5-HT.

CDR 2* - the trace shows the response of the same heart to cumulative doses of 5-HT, the asterisks identify the cumulative application of 5-HT as well as the bath concentration.

When compared, the single dose applications gave similar results to the cumulative DR, there was no desensitisation due to the repetitive application of the drug, or time.

Comparison of the Effect of Single Doses to Cumulative Doses of 5-HT on the heart of *Mya arenaria*





Mya arenaria

Figure 1(c)

Traces a) and b) are from a *Mya arenaria* heart originally bathed in regular ASW which was then changed to low Mg^{+} ASW. A dose of $1X10^{-6}$ M 5-HT was applied as shown by the asterisk in both types of ASW. The only difference in response seen between the two traces was heart rate, which was elevated throughout by the low Mg^{+} ASW.

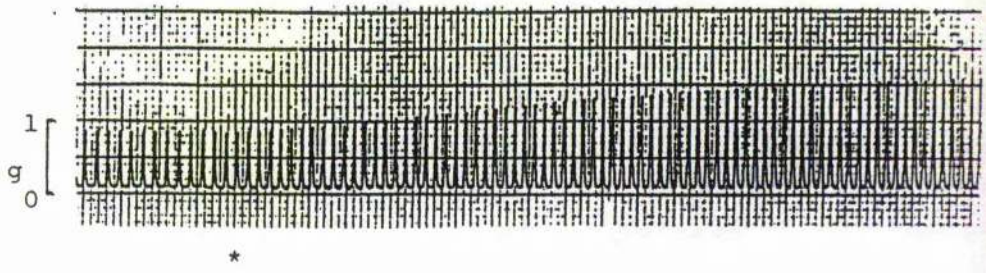
Traces c) and d) are from another heart and show the effect of single doses of 5-HT and Benzoquinonium (Bz).

Trace e) shows another heart in the presence of Bz ($1x10^{-6}$ M). The subsequent percent increase in force in response to 5-HT was smaller, but the maximum force was increased in the presence of the Bz and the potency was unchanged.

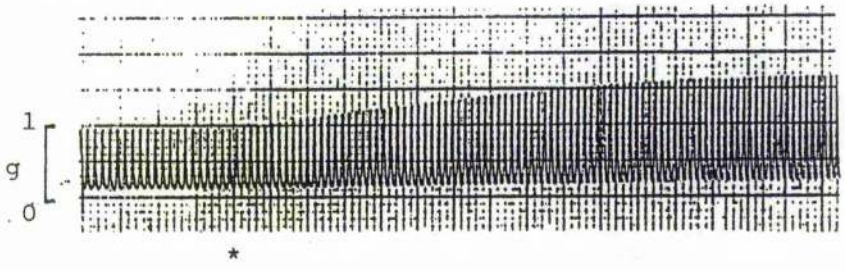
Figure 1(c)

Individual Traces - *Mya arenaria*

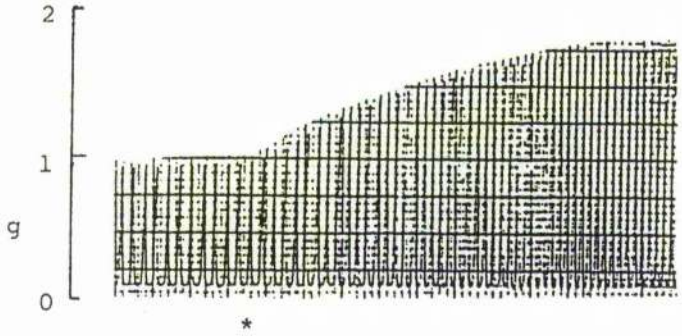
a)Single Dose of $1x10^{-6}$ M 5-HT in Regular ASW



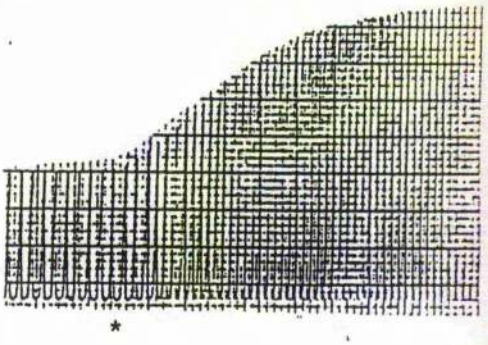
b) Single Dose of $1x\ 10^{-6}$ M 5-HT in ASW (Low Mg^{+})



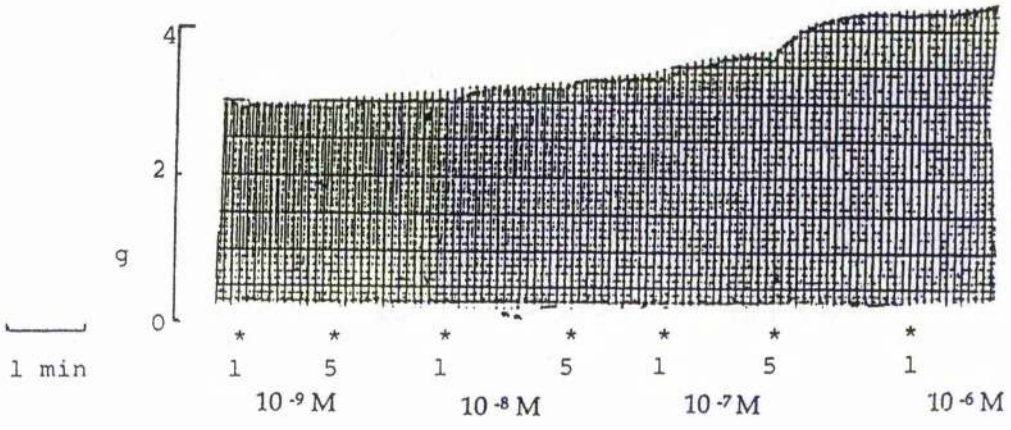
c) Single Dose of $5x10^{-6}$ M 5-HT



d) Single Dose of $1x10^{-5}$ M BZ



e) Trace of responses to cumulative doses of 5-HT in the presence of $1x10^{-6}$ M Bz



Mya arenaria

Figure 1(d)

Cumulative DR curves of the percent changes in tension in two hearts of *Mya arenaria* in response to 5-HT, alone and in the presence of either UML or methiothepin. The bath concentrations of the antagonists were $1x10^{-6}$ M.

The traces show the response of hearts to single doses of 5-HT or 5-CT, before and after the same antagonists.

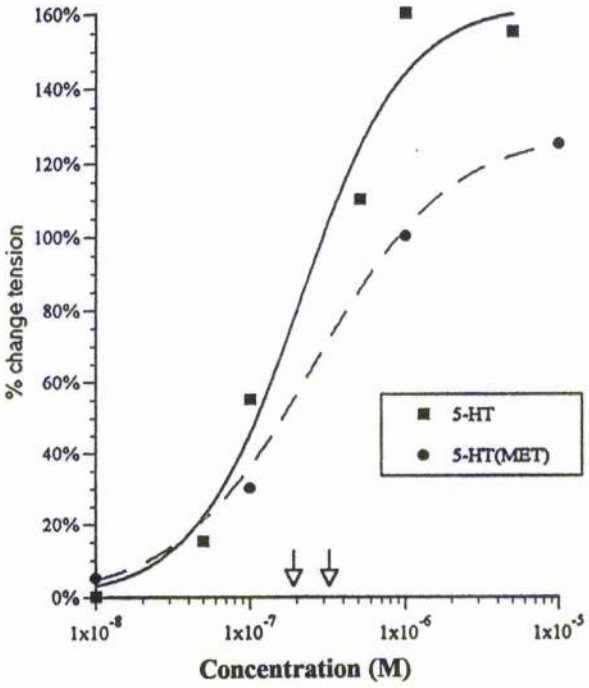
Trace (a) Heart 1 - the effect of $1x10^{-6}$ M UML on a single dose of $5x10^{-6}$ M 5-HT.

Trace (b) Heart 2 - the effect of the same concentration of UML on a single dose of $1x10^{-6}$ M 5-CT.

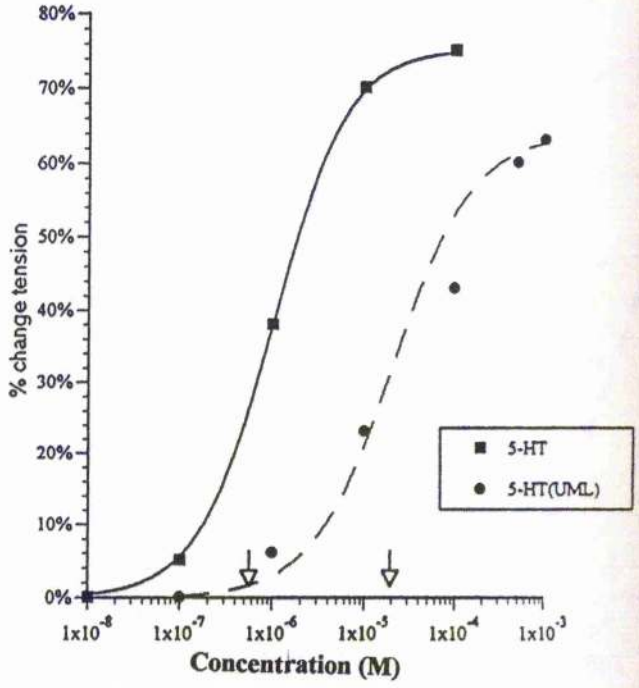
Trace (c) Heart 3 - the effect of $1x10^{-6}$ M Methiothepin on a single dose of $1x10^{-6}$ M 5-HT.

Antagonism of 5-HT effect on the heart of *Mya arenaria*

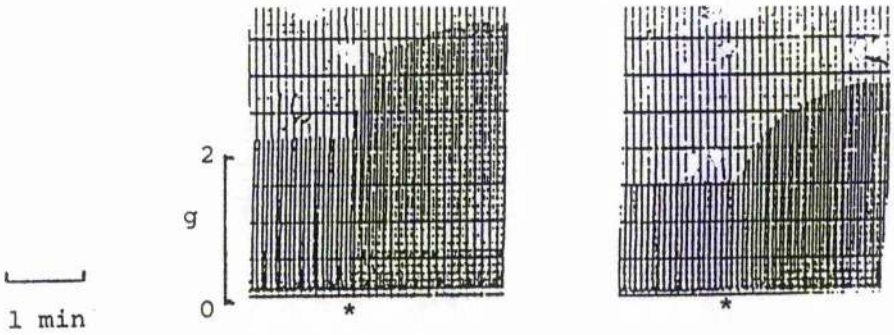
Methiothepin antagonism of 5-HT



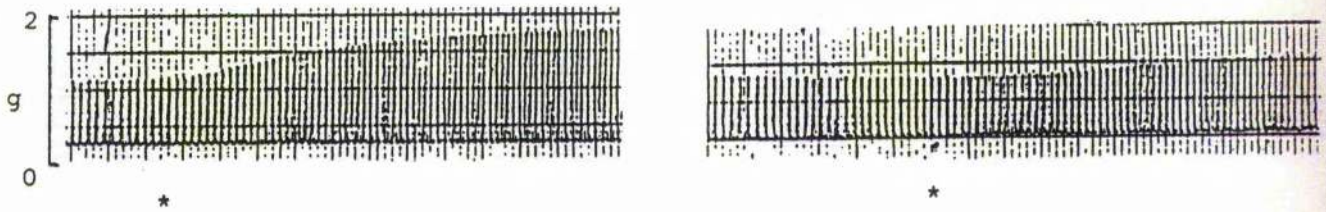
UML antagonism of 5-HT



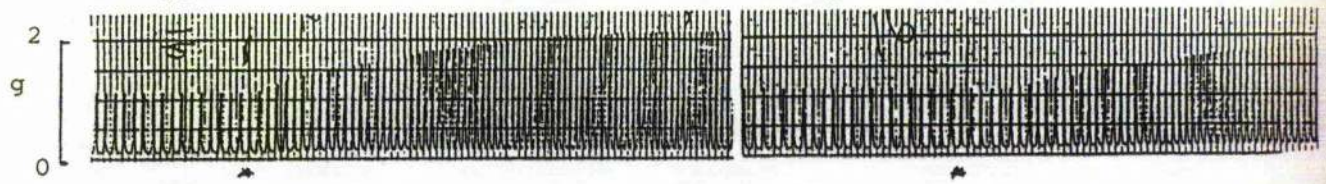
Trace (a) - A Dose of 5X10⁻⁶ M 5-HT, alone and in the presence of 1x10⁻⁶ M UML



Trace (b) - A Dose of 1X10⁻⁶ M 5-CT, alone and in the presence of 1x10⁻⁶ M UML



Trace (c) - A Dose of 1x10⁻⁶ M 5-HT, alone and in the presence of 1x10⁻⁶ M Methio.



Mya arenaria

Figure 1(e)

(a) The excitatory effect of a dose of 5-HT. After washing, GR38032F was added to the bath (note the excitatory effect). The same dose of 5-HT was the added in the presence of the GR38032F.

(b) Some small degree of direct inhibition by ketanserin, but little antagonism of the response to 5-HT.

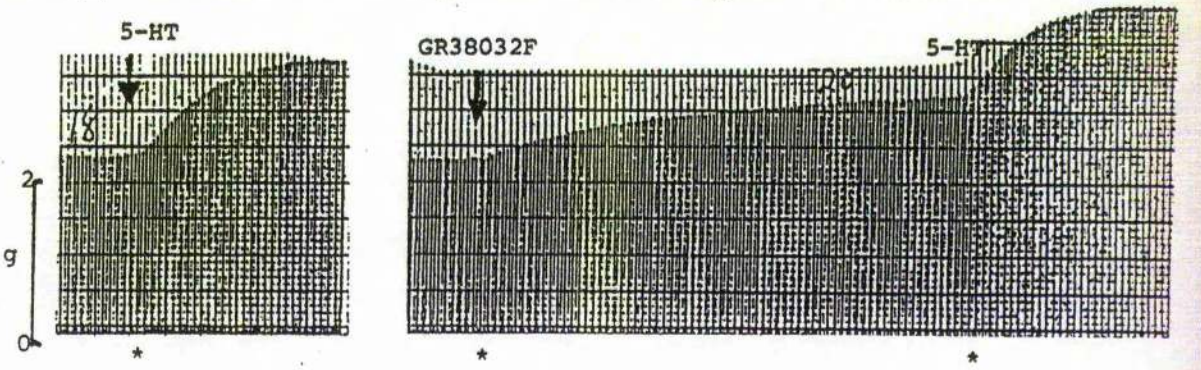
(c) The long-lasting effect of LSD-25 on inotropy. There is a reduction of the size of response to a subsequent dose of 5-HT. This could be due to desensitisation, or a lasting partial agonist effect of the LSD-25, or the force of contraction might still be close to the maximum force obtainable.

(d) The inhibitory effect of $1x10^{-5}$ M MDL-72222.

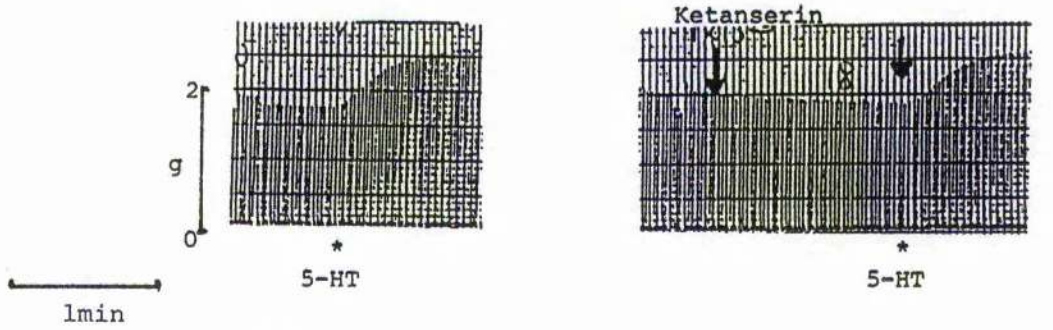
(e) The response of the heart to 5-HT in the presence of 10^{-6} M MDL 72222, which antagonised a high dose of 5-HT ($1x10^{-5}$ M).

Effect of Partial Agonists and Antagonists on the Heart of Mya arenaria

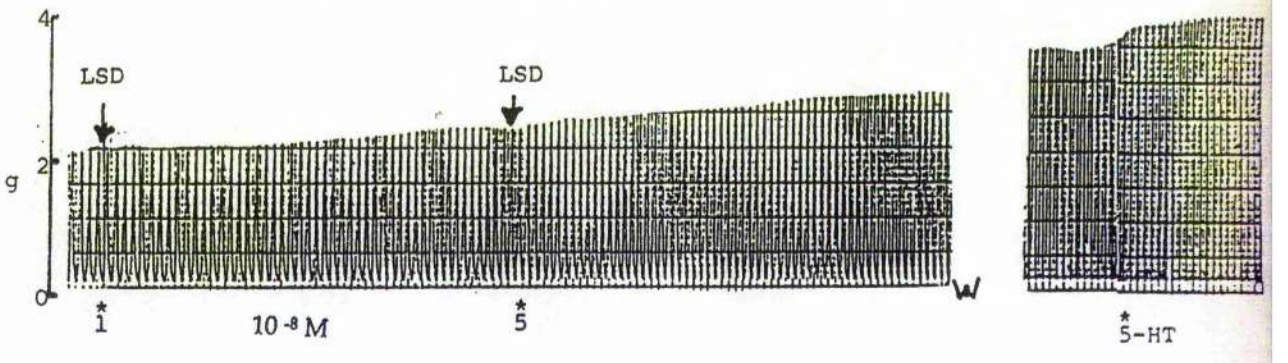
Trace (a) Dose of $5x10^{-6}$ M 5-HT, before and in the presence of $1x10^{-5}$ M GR38032F



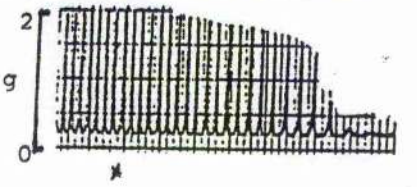
Trace (b) Dose of $1x10^{-6}$ M 5-HT, before and in the presence of $1x10^{-5}$ M ketanserin.



Trace (c) Effect of LSD-25 on a subsequent dose of $1x10^{-6}$ M 5-HT



Trace (d) Effect of a single dose of $1x10^{-5}$ M MDL 72222



Trace (e) Effect 1x10-5 M 5-HT in the presence of 1x10-6 M MDL 72222

Mercenaria mercenaria

Please see **Figures 2 (a),(b),(c),(d),and (e)** for the Graphs and Traces. While 5-HT was predominantly excitatory on the hearts of this species, lower doses occasionally showed weak inhibition. However as the dose was increased both rate and force of contraction was increased, EC_{50} = $2.33x10^{-7}$ M. α-Me-5-HT was found to be purely excitatory (EC_{50} = $3.4x10^{-8}$ M) and more potent than 5-HT. 5-MEOT was excitatory and approximately equipotent to 5-HT. 2-Me-5HT was less potent (EC_{50} = $6x10^{-7}$ M) than 5-HT on the hearts of this species. 5-CT was less potent (EC_{50} = $7.4x10^{-7}$ M) than 5-HT at producing excitation of contraction. However, occasionally higher doses caused increased rate and the heart would stop beating in contracture.

8-OH-DPAT was excitatory (EC_{50} = $5x10^{-6}$ M) but less potent than 5-HT. However, this drug appeared to act as a partial agonist, see figure 2(d). This drug always caused a prolonged and sustained increase in force which persisted even after multiple washes, as well as a prolonged rest period of 30-45 minutes. When subsequent doses of 5-HT or 5-CT were tested they appeared to be effectively blocked (or the heart was desensitised to them), see figures 2(b) and 2(d).

UML (as reported in the Introduction) was an antagonist in *Mercenaria mercenaria* as was seen with *Mya arenaria*. Methiothepin was a weak antagonist to 5-HT but more effective at antagonism of the 5-CT response. Both of these two antagonists were only weakly effective in reducing the excitation caused by α-Me-5-HT. Bz did not antagonise any of the 5-HT responses but did show an excitatory effect on force as seen in *Mya arenaria*. The potency of 5-HT and analogues remained the same or was greater in the presence of Bz.

Effect of 5-HT and analogues on *Mercenaria mercenaria* hearts

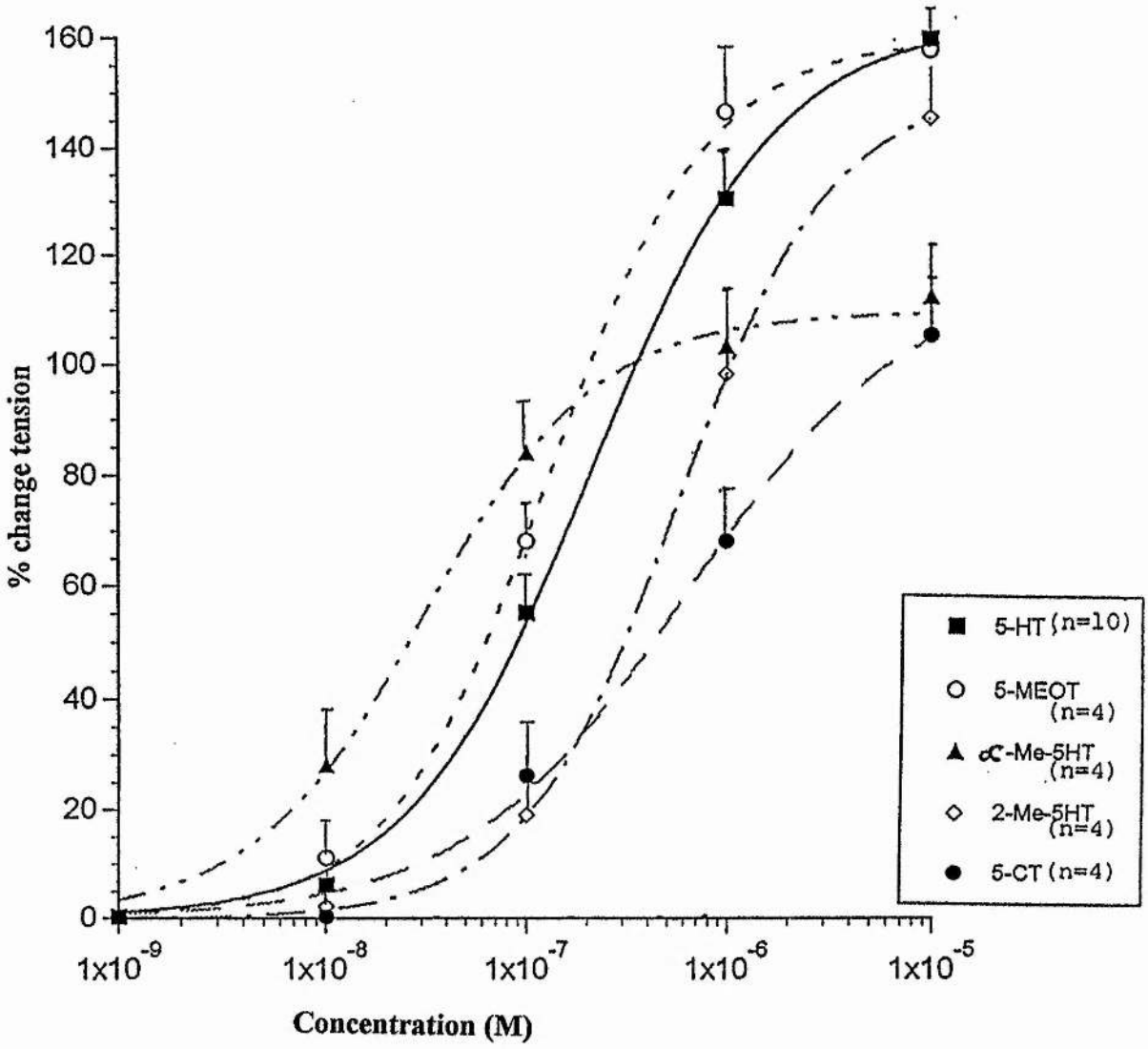


Figure 2(a) This Graph illustrates the effect of 5-HT and some analogues on the hearts of *Mercenaria mercenaria*. The graph was plotted as outlined in the Method section, error bars = S.E. of mean.

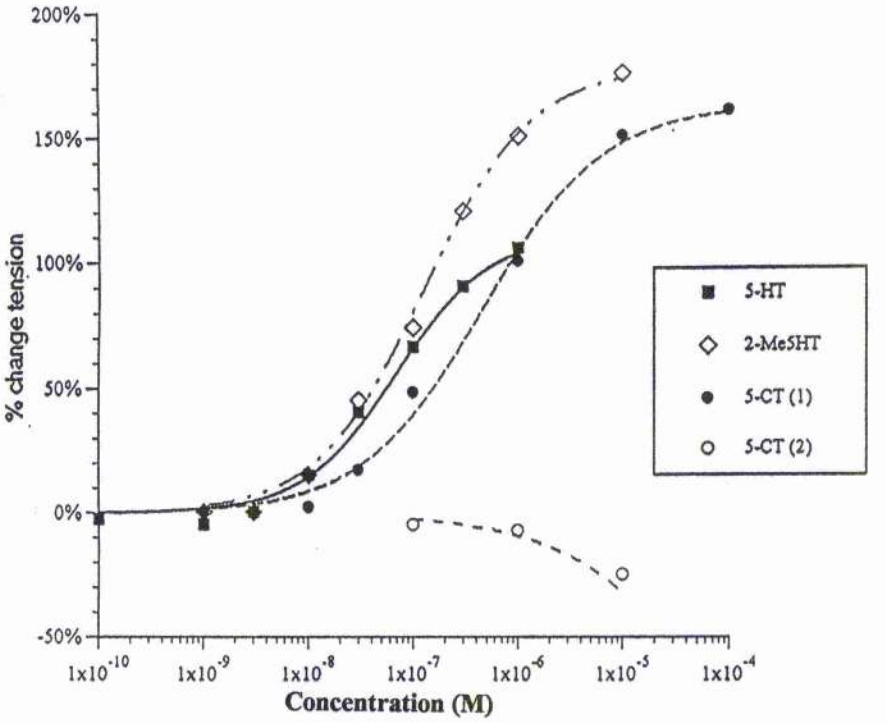
Mercenaria mercenaria

Figure 2(b)

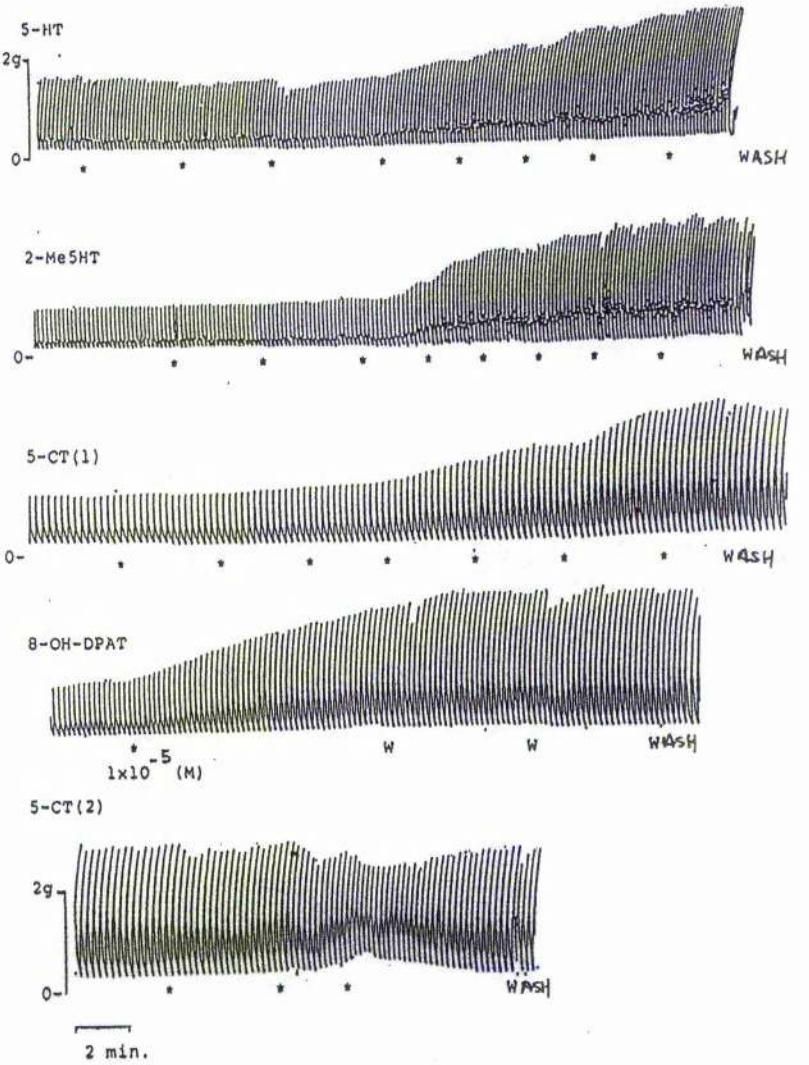
The graph shows the DR Curves from one heart tested with 5-HT and several analogues. The asterisks on the individual traces indicate where each dose was added to the bath (see the graph for the bath concentration of each drug).

The Log bath concentration of the drug is plotted against percent change in tension. Note the prolonged excitation and apparent blocking effect of a single dose ($1x10^{-5}$ M) of 8-OH-DPAT when a second DR response curve was repeated with 5-CT.

Effect of 5-HT and analogues on a *Mercenaria mercenaria* heart



Individual Traces showing a heart's responses to 5-HT and analogues



Effect of two 5-HT analogues on a *Mercenaria mercenaria* heart

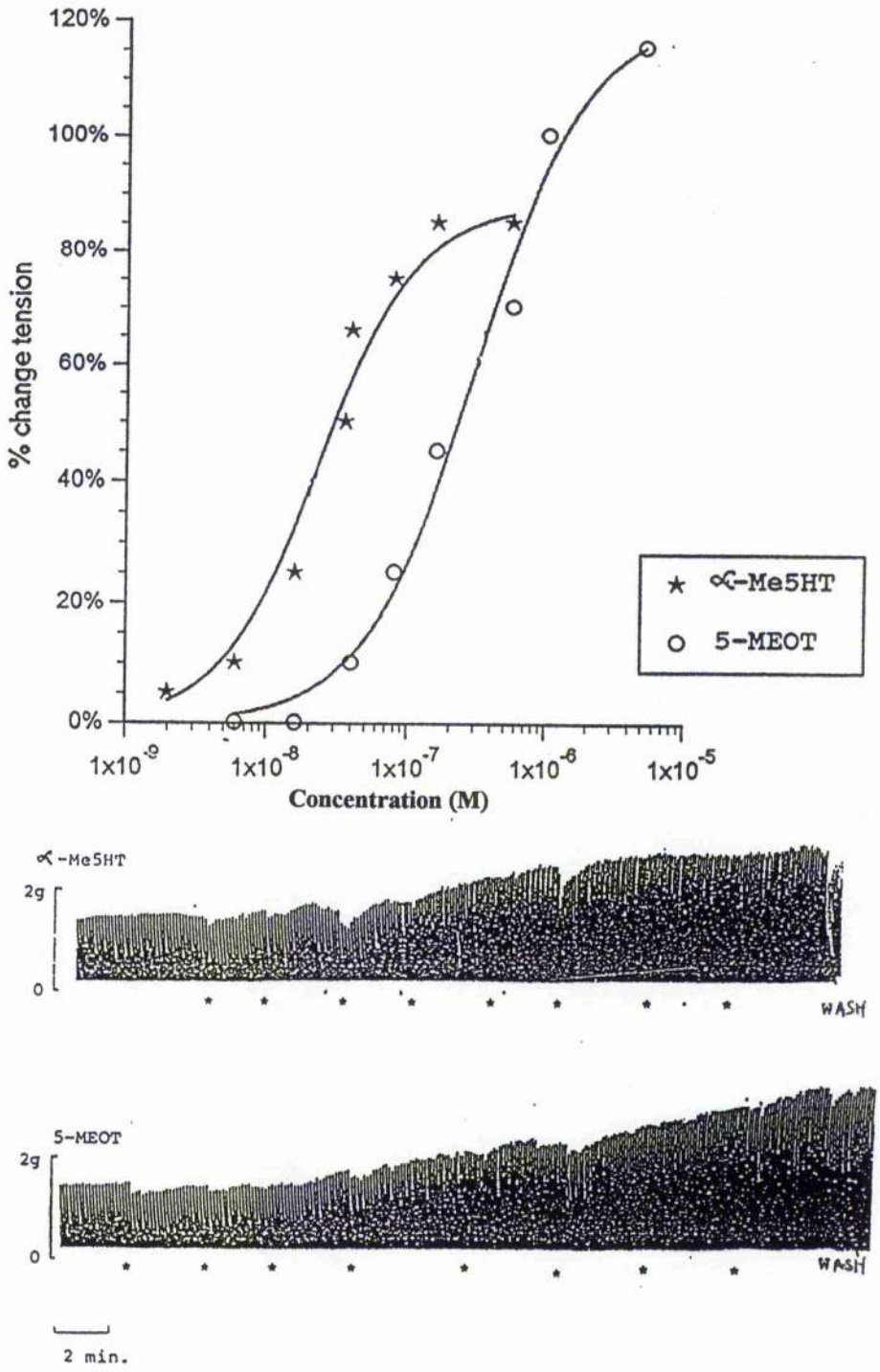


Figure 2(c) Shows a DR plot and the individual traces from one heart in response to two 5-HT analogues. The asterisk indicates the addition of the dose to the bath, the concentrations are shown in the graph.

α-Me-5-HT was more potent than 5-HT or 5-MEOT. Note: In this heart 5-MEOT was equipotent to 5-HT(curve not shown).

Mercenaria mercenaria

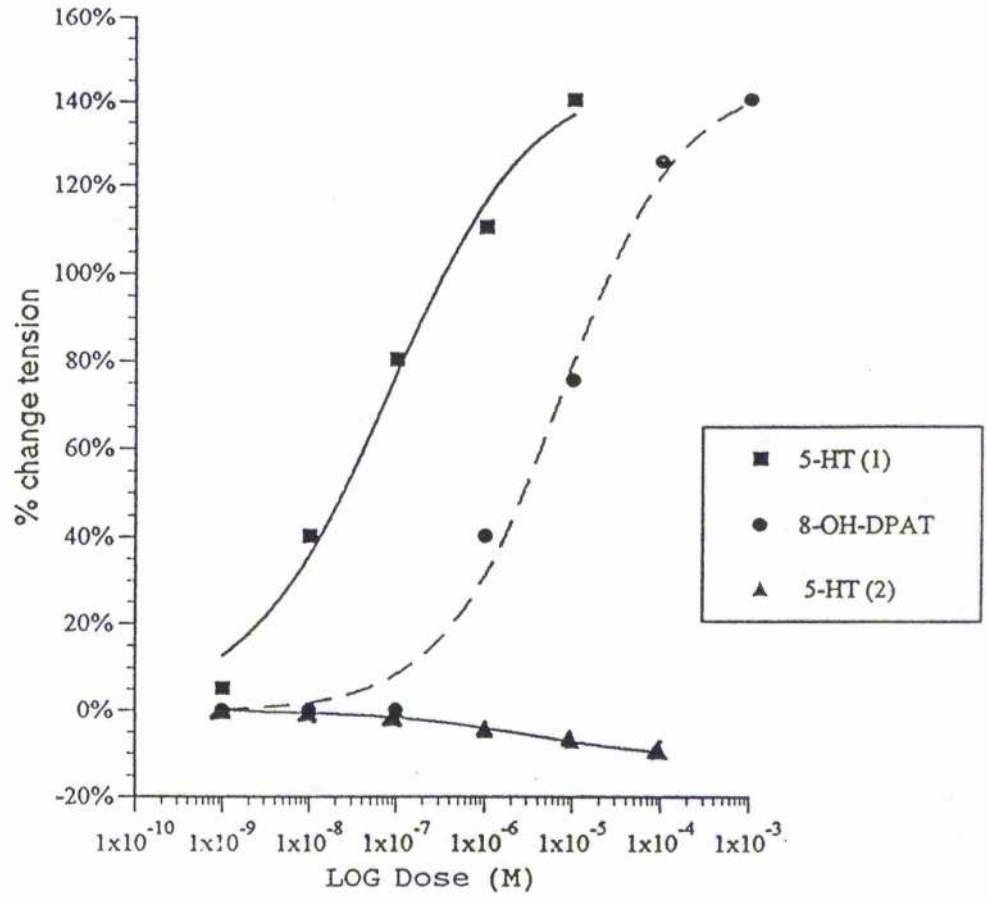
Figure 2(d)

The effect of 5-HT on a heart before and after a cumulative dosing regime with 8-OH-DPAT. The asterisks below each trace show where the dose was added to the bath; the bath concentrations can be seen in the graph.

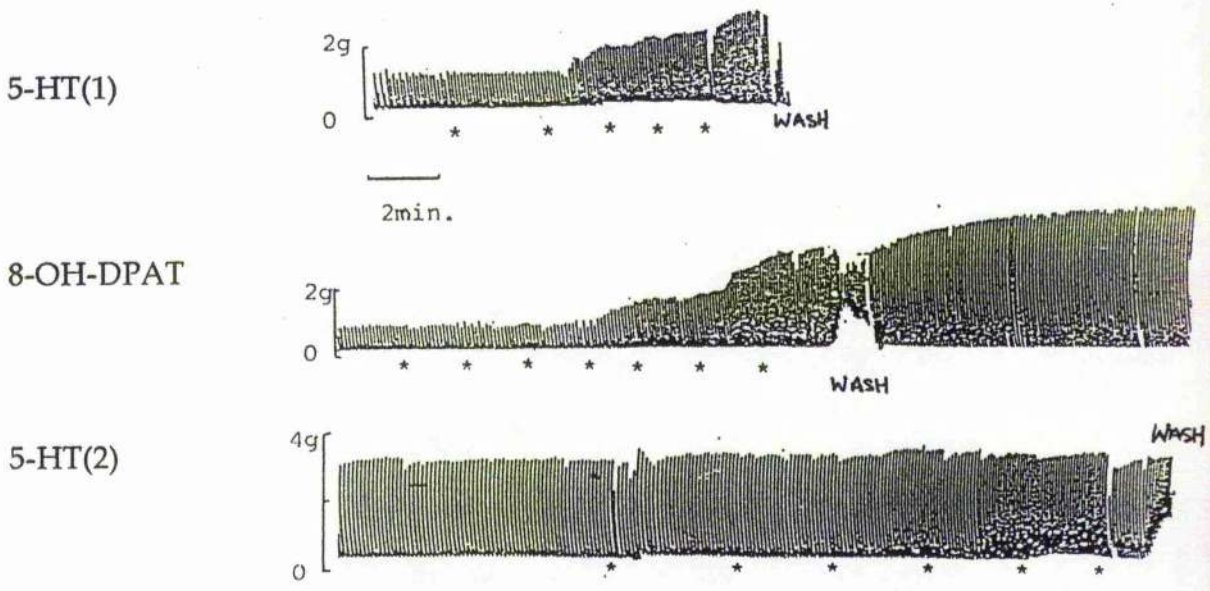
The prolonged excitation effectively inhibited any subsequent response of the heart to 5-HT. Although 5-HT was far more potent than 8-OH-DPAT, it should be noted that 8-OH-DPAT showed the same or greater efficacy as 5-HT. The effect was difficult to wash off.

In the third trace, which was made more than 45 min after the cumulative DR to 8-OH-DPAT, the excitatory effect of 5-HT was not observed. The heart was still beating with a much greater force, see the scale, after more than 45 minutes and multiple washes.

Effect of 8-OH-DPAT on the heart of *Mercenaria mercenaria*



Effect of cumulative doses of 5-HT on the same heart, before and after 8-OH-DPAT



Mercenaria mercenaria

Figure 2(e)

The graph shows the response of one heart to 5-HT, before and after a prolonged exposure to a high dose ($1x10^{-6}$ M) of 5-HT. There was a two fold change in the response (EC_{50}) to a second cumulative regime of 5-HT doses given only 20 minutes later. This may be due to some degree of desensitisation after the extended exposure to 5-HT, but this was markedly less than the desensitisation shown in Figure 2(d).

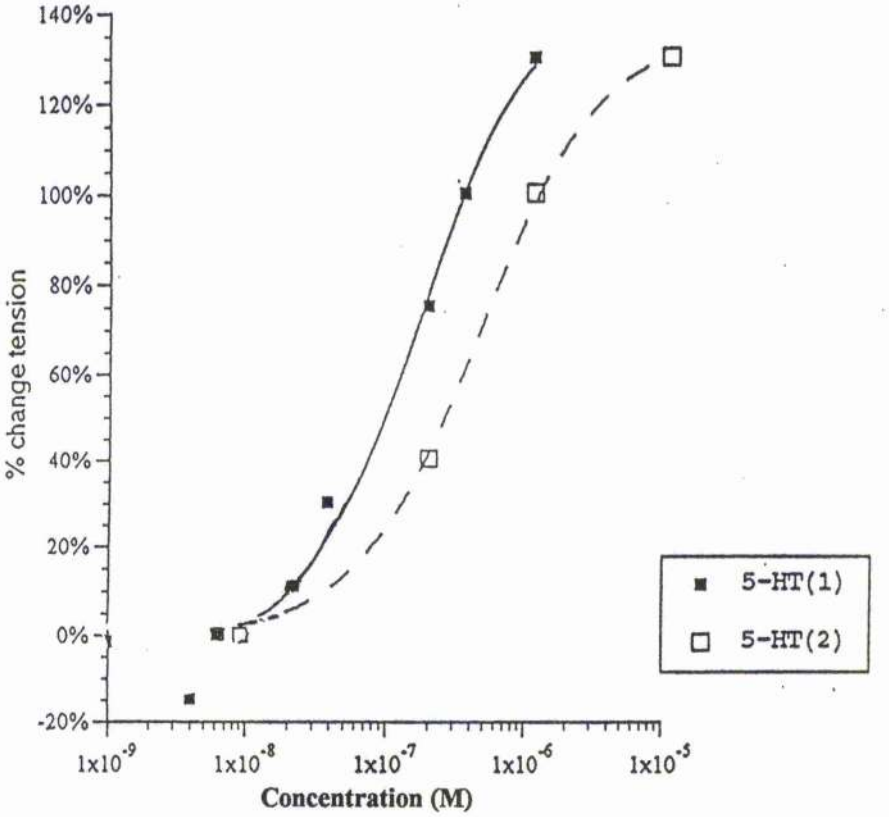
Asterisks show where the doses were added to the bath. The concentrations in the bath are shown in the graph.

Trace (a) cumulative DR to 5-HT, which is plotted in the graph as 5-HT(1). Note that the initial dose caused some inhibition.

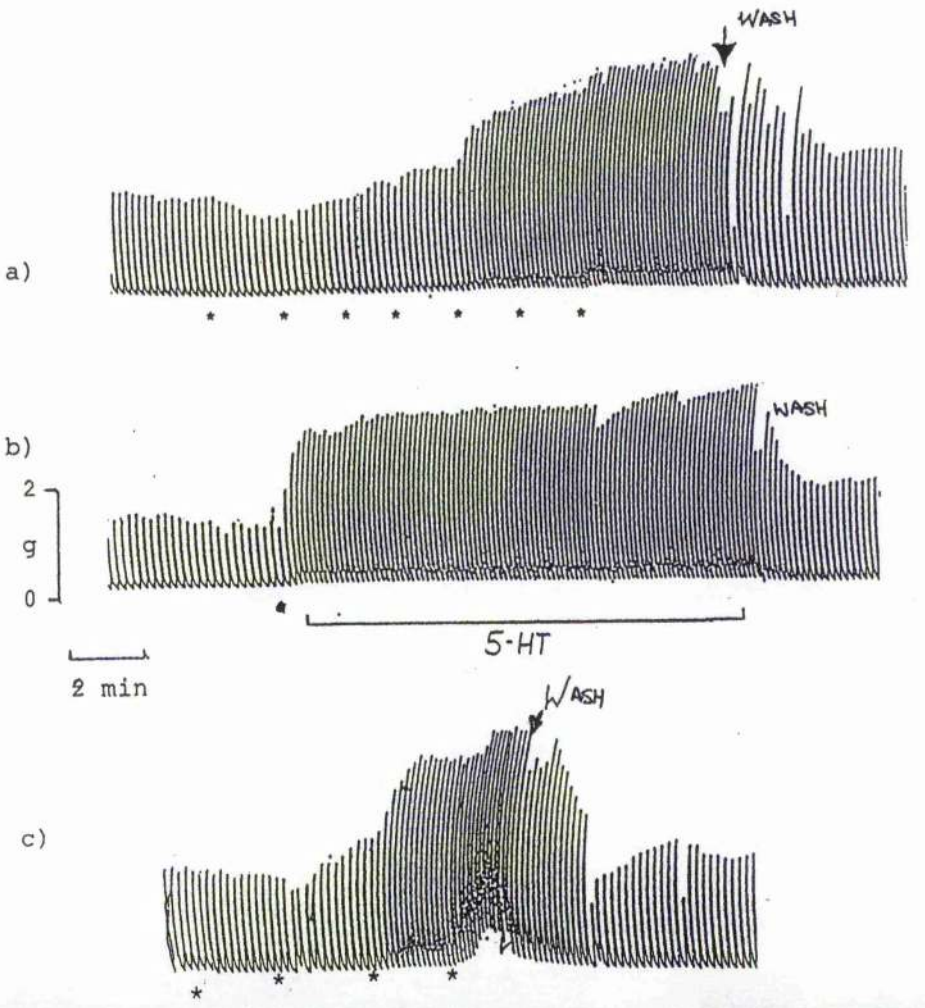
Trace (b) the effect of a single dose of 5-HT ($1x10^{-6}$ M) left in contact with the heart for 20 minutes.

Trace c) cumulative DR to 5-HT plotted as 5-HT(2). Heart rate did appear to increase more with this application of 5-HT.

Prolonged exposure to 5-HT on a *Mercenaria mercenaria* heart



Individual Traces on a heart with repeat dosing of 5-HT



Saxidomus giganteus

Please see **Figures 3(a),(b),(c),(d),(e) and (f)** for the Graphs and Traces.

The effect of 5-HT and some of the analogues on *Saxidomus giganteus* were very interesting. More than 150 different hearts were examined with different agonists and antagonists for both 5-HT and ACh; some of the key results are presented here.

5-HT was found to be predominantly excitatory (EC_{50} = $5.4x10^{-7}$ M) in this species; however weak inhibition was usually observed with the initial low doses see Figure 3(a). α-Me-5-HT was purely excitatory on both force and rate (EC_{50} = $3.3x10^{-8}$ M) and was more potent than 5-HT.

5-MEOT was excitatory (EC_{50} = $8.3x10^{-7}$ M) but less potent than 5-HT. Occasionally high concentrations led to either inhibition of contractions or erratic slower beating.

5-CT was found to be a very potent inhibitor (IC_{50} = $3.3x10^{-9}$ M); occasionally large doses might cause excitation (only if the heart had not stopped completely with the lower doses).

8-OH-DPAT was less potent (EC_{50} = $1x10^{-5}$ M) than 5-HT but produced a prolonged excitatory effect and a subsequent 'blocking' effect as described in *Mercenaria mercenaria*.

UML usually an antagonist, had a strong inhibitory effect itself but was less potent than 5-CT, see Figure 3(a). This could be due to a partial agonist effect on the same receptors which produced inhibition with 5-CT. The responses to 5-CT were slightly diminished (antagonised) after UML had been tested on the heart.

2-Me-5-HT was inhibitory with low doses and sometimes excitatory at the higher concentrations. ICS 205-930 was the only drug to show weak antagonism of this response.

Both methiothepin and Bz were effective antagonists of the inhibitory responses seen with 5-CT, see Figure 3(d). Neither ketanserin or ritanserin antagonised any of the analogues. The effect of BZ was to enhance the excitatory potency of 5-HT on the heart. Figure 3(e) shows that the presence of Bz in the bath did not change the efficacy of 5-HT, but it did change the potency, the EC_{50} for 5-HT went from $1x10^{-7}$ M to $1x10^{-9}$ M.

I tried to examine this effect seen with Bz further to see whether Bz was directly affecting the 5-HT or 5-CT receptor(s), or the ACh receptor and intracellular pathways. Or was the activation of this 5-HT receptor causing release of ACh, the effect of which was now blocked by the Bz. I was unable to find another ligand other than methiothepin which was as effective as Bz at blocking the 5-CT inhibitory effect.

I looked at other ACh antagonists to try to identify an agent which could effectively block the inhibitory effect of ACh so that I could compare its effect when 5-HT or 5-CT was added to the bath. I found that the inhibitory response to ACh was at least 10 fold less potent than that of 5-CT. As ACh was considerably less potent as an inhibitor than 5-CT, it was unlikely that the inhibition caused by 5-CT was due to release of ACh.

Saxidomus giganteus

Figure 3(a)

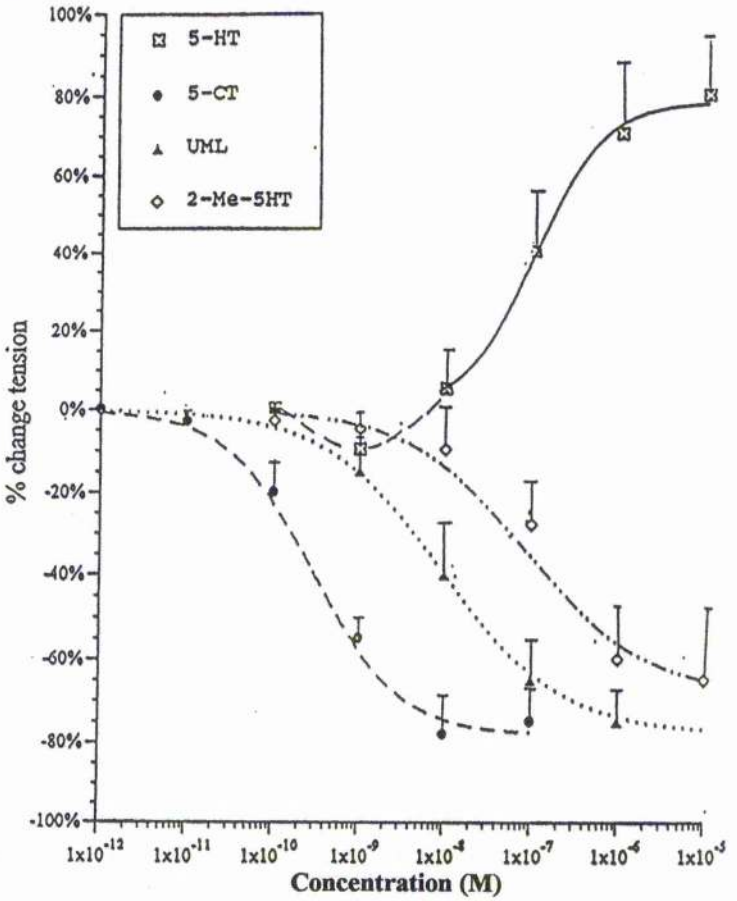
This graph shows the results from 5 hearts all tested with 5-HT, 5-CT and UML. The mean (and S.E.) percent change in tension was plotted against 10 fold changes in drug concentration. The curve which shows the effect of 2-Me-5HT is from 5 different hearts.

Traces (a) (b) and (c) show the cumulative DR recordings from one heart in response to 5-HT, 5-CT and UML.

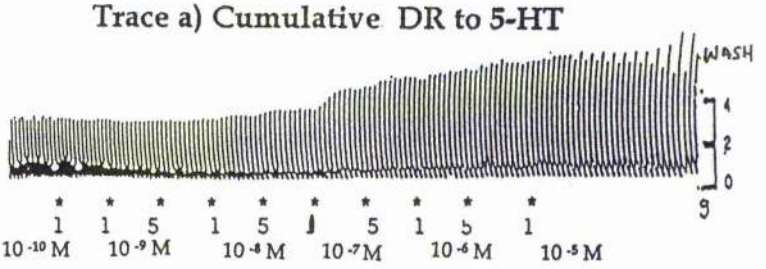
Trace (d) shows another heart's response to 2-Me-5-HT.

Asterisks identify where the dose was added to the bath, the drug concentration is identified beneath the individual Traces.

Effect of 5-HT and analogues on the hearts of *Saxidomus giganteus*

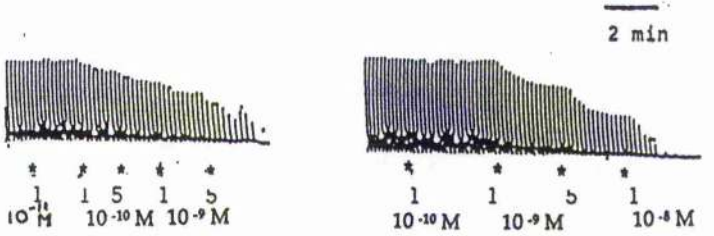


Trace a) Cumulative DR to 5-HT

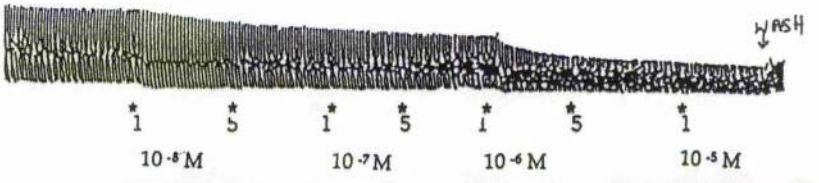


Trace b) Cumulative DR to 5-CT

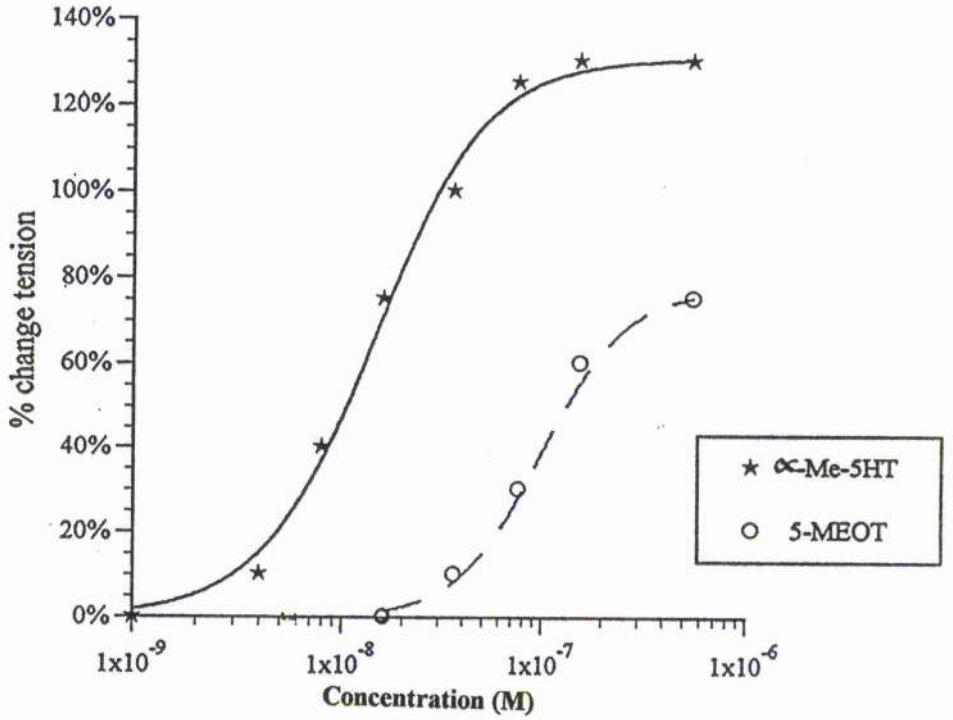
c) Cumulative DR to UML



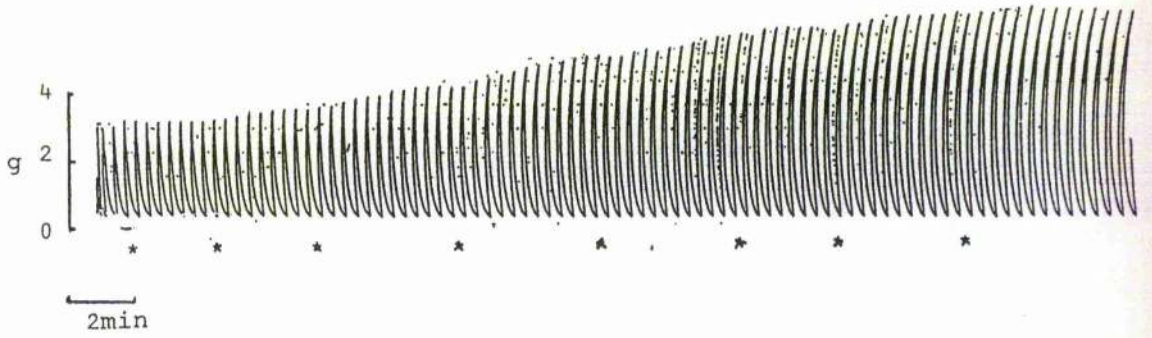
Trace d)Cumulative DR to 2-Me-5-HT (on a different heart)



Effect of two 5-HT analogues on the hearts of *Saxidomus giganteus*



Trace a)



Trace b)

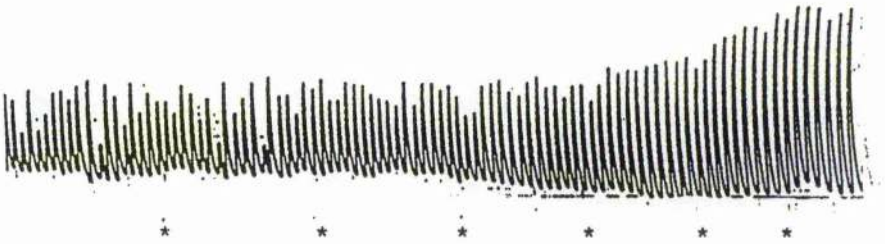


Figure 3(b) A DR plot of α-Me-5-HT and 5-MEOT on two hearts, and the recorded traces.

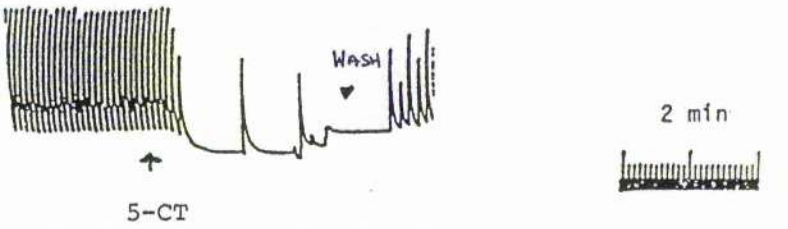
Trace a) cumulative DR showing the effect of α-Me-5HT.

Trace b) cumulative DR showing the effect of 5-MEOT.

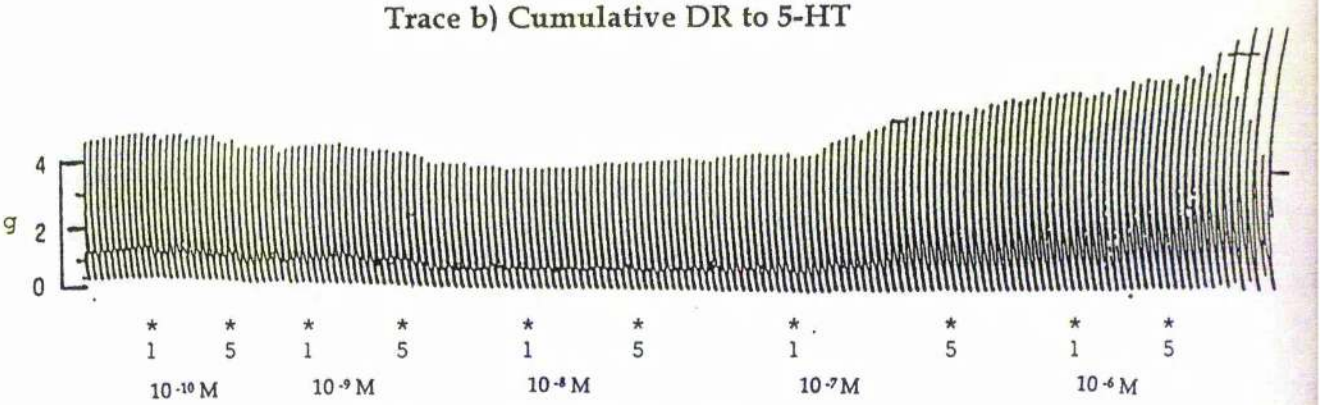
The *asterisks* identify where each dose was added to the bath, see the graph for the bath concentration of each application.

5-HT and analogues on a single *Saxidomus giganteus* heart

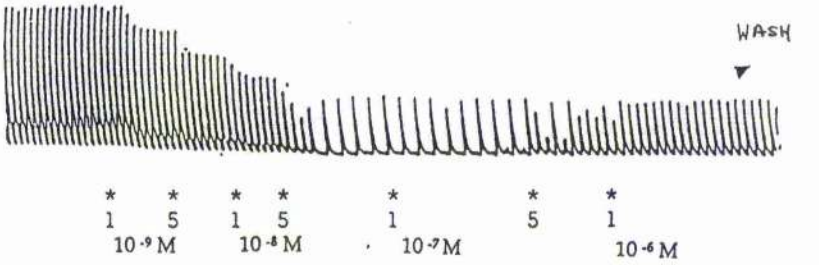
Trace a) Single dose $5x10^{-9}$ M 5-CT



Trace b) Cumulative DR to 5-HT



Trace c) Cumulative DR to UML



Trace (d) Cumulative DR to 5-CT before and in the presence of UML

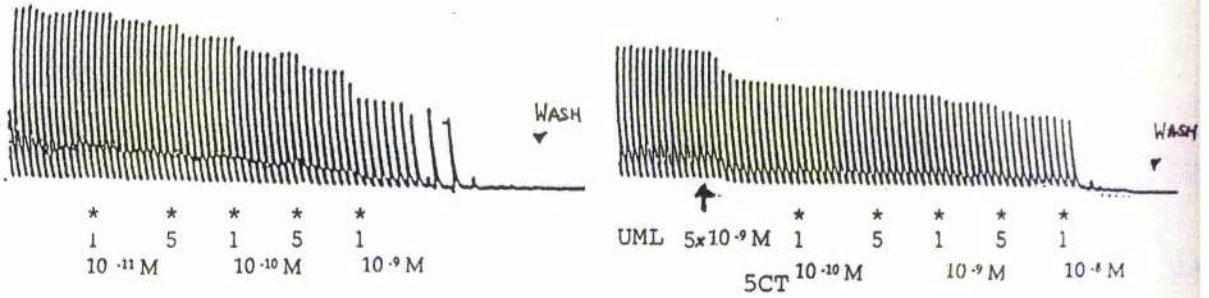


Figure 3(c) Traces (a)-(d) shows the effect of a single dose of 5-CT as well as cumulative DR of 5-HT, UML and 5-CT, before and in the presence of a low dose of UML, on one heart.

Saxidomus giganteus

Figure 3(d)

This figure shows DR experiments from two different hearts:

Heart 1

(a) The response of the heart to 5-HT.

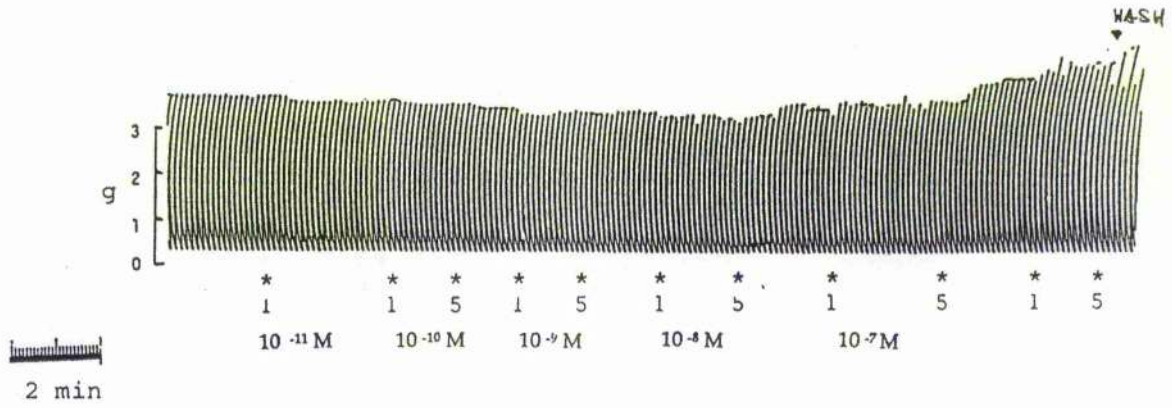
(b) The response of the same heart to 5-CT, alone and in the presence of methiothepin in the bath.

Heart 2

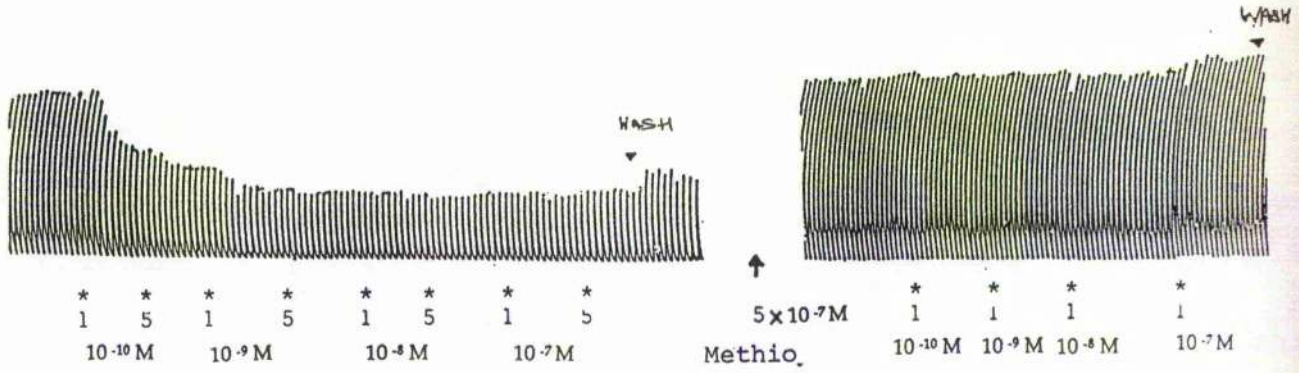
(c) Two cumulative DR traces with 5-CT, alone and in the presence of Benzoquinonium ($1x10^{-6}$ M) in the bath.

Effects of 5-HT, analogues and antagonists on *Saxidomus giganteus* hearts

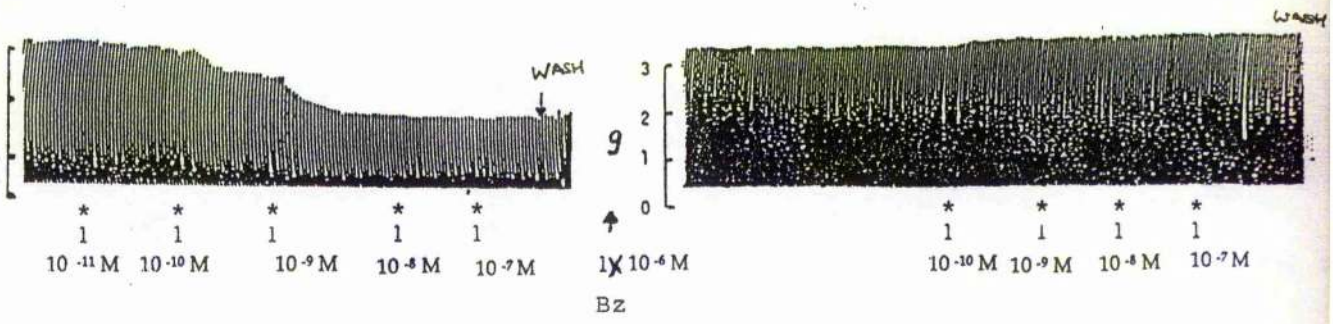
Trace a) Cumulative DR to 5-HT



Trace (b) Cumulative DR to 5-CT alone and in the presence of Methiothepin



Trace (c) Cumulative DR to 5-CT alone and in the presence of 1x10^{-6} M BZ



Effect of Benzoquinonium on *Saxidomus giganteus* heart's response to 5-HT

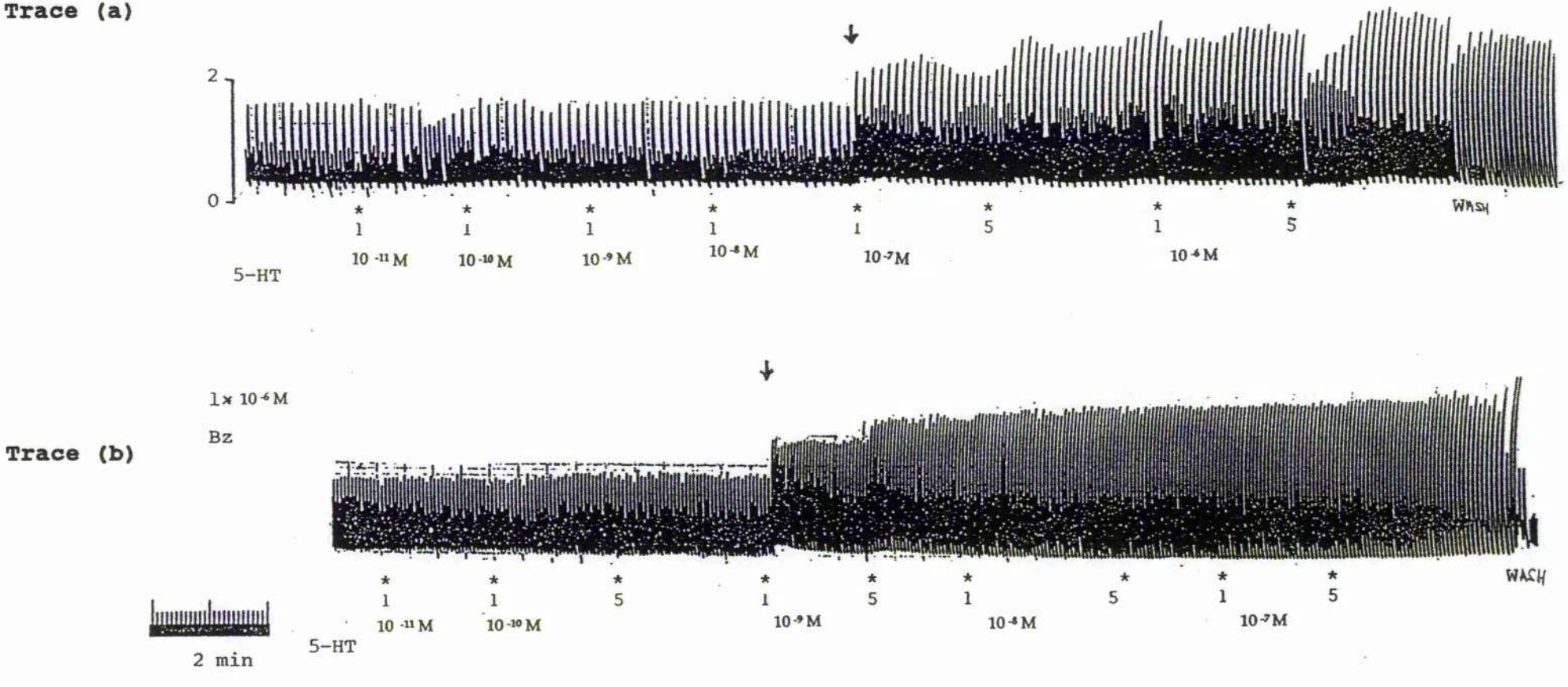


Figure 3(e) (a) Response of a heart to 5-HT; (b) The response of the same heart to 5-HT after Bz was added to the bath. Note: the response to 5-HT is more potent with Bz in the bath and the heart rate was increased and was more stable. The arrows identify the EC_{50} of the response to 5-HT in both traces.

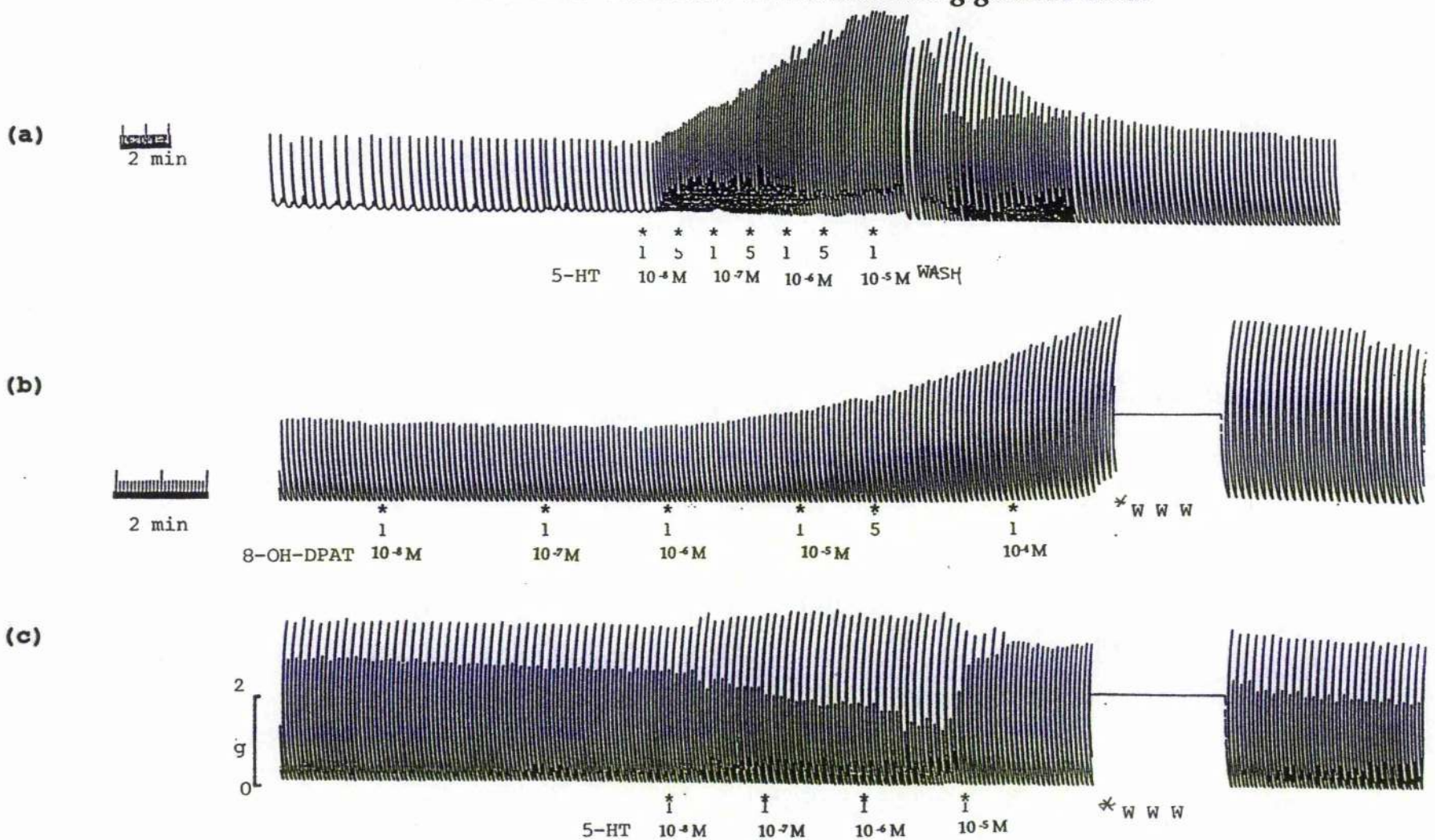


Figure 3(f) Cumulative DR to 5-HT in (a) and (c), before and after 8-OH-DPAT (b). Note: even after many washes, prolonged excitation remains and the subsequent doses of 5-HT are effectively blocked.

Geukensia demissa

Please see **Figures 4 (a),(b), and (c)** for the Traces.

5-HT caused a mixed response on the hearts of this species, low doses sometimes produced a small increase in force of contraction initially; higher doses lead to inhibition of force but this was often accompanied by an increase in frequency of contraction. With the variation in responses and the shifting baseline (diastolic tone) it was not possible to produce meaningful DR curves for 5-HT and the analogues tested.

There appeared to be some seasonal variation between the degree of inhibition or excitation observed, this was corroborated by Dr. Michael Greenberg who had noticed this in some of his earlier studies on this species. 5-HT would cause total inhibition in about 10% of the hearts tested, less than 10% of the hearts would demonstrate only increased force and frequency. However α-Me-HT, a potent excitatory agent on all hearts from the other species tested, also acted similarly on the hearts of *Geukensia demissa,* see Figure 4(a).

5-CT and 5-MEOT were predominantly inhibitory on force of contraction; however 5-MEOT did sometimes cause an increase in rate, whereas 5-CT generally decreased both force and frequency. 5-CT was consistently more potent as an inhibitory agent than 5-MEOT (see figures 4(a)and 4(b)).

8-OH-DPAT was similar to α-Me-HT, regarding increased chronotropy but much less potent; but it did not cause the prolonged excitation after washout as seen in several of the other species described.

Because of the mixed responses it was difficult to accurately assess antagonist activity. Bz did not appear to have any marked effects.

The effect of three 5-HT analogues on a *Geukensia demissa* heart

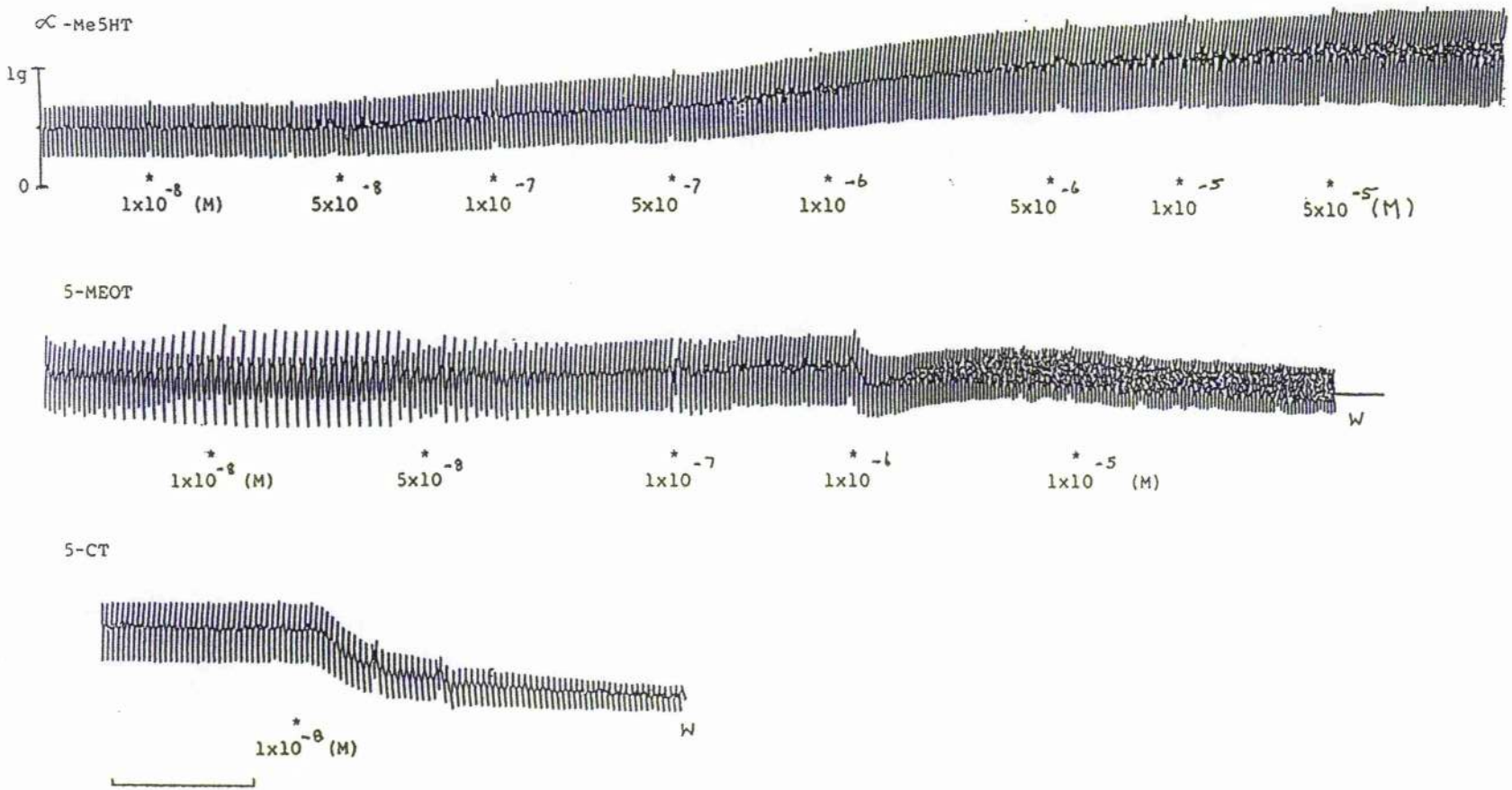


Figure 4(a)

Trace a) α-Me-5HT acted as a potent excitatory agonist causing increased inotropy.
Trace b) 5-MEOT was inhibitory on force of contraction, but rate did increase.
Trace c) 5-CT was inhibitory on both the force and the rate of contraction.

The effects of 5-HT and 3 analogues on a *Geukensia demissa* heart

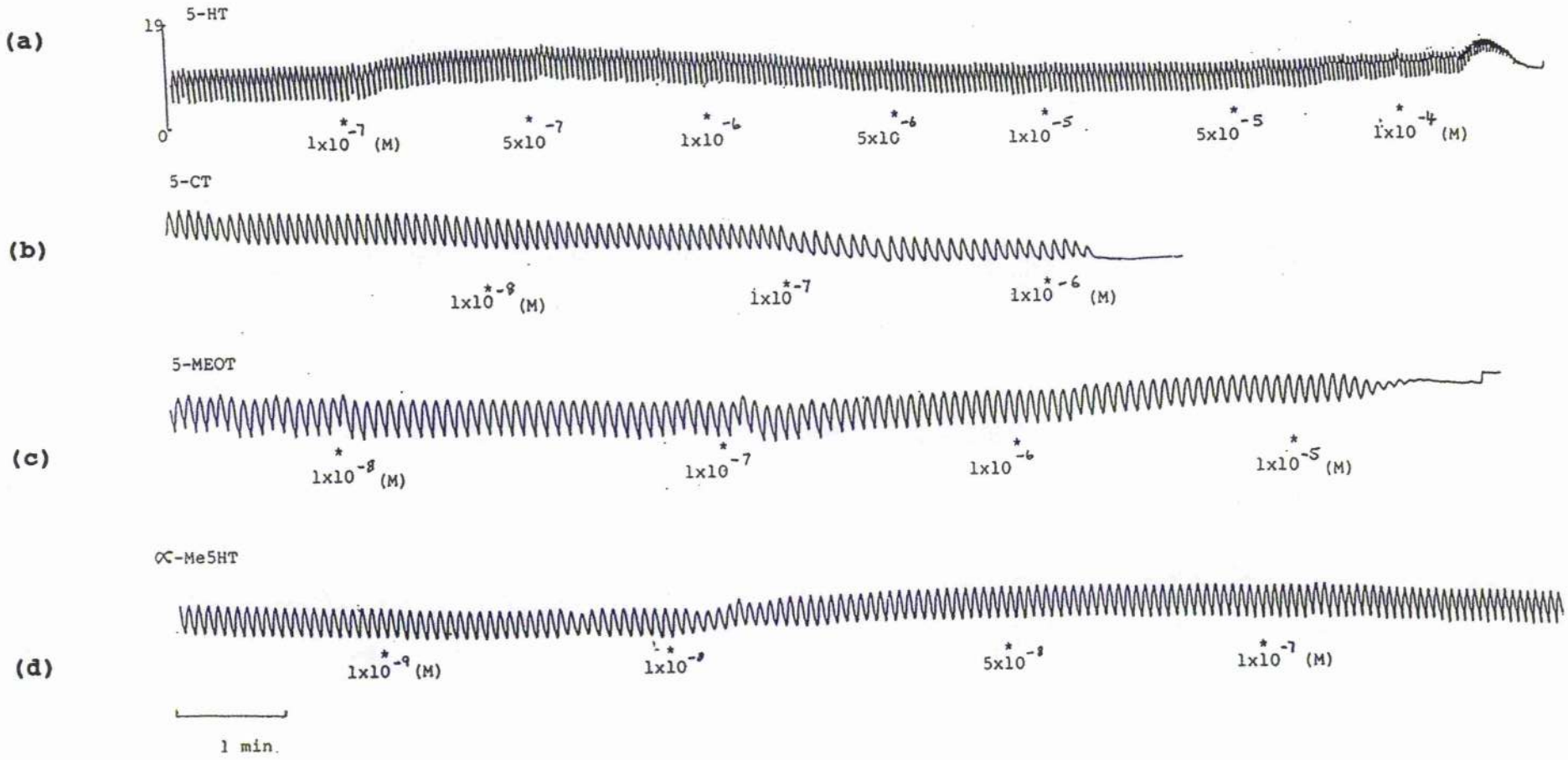


Figure 4(b) This heart showed a mixed response to 5-HT. 5-CT was clearly inhibitory and 5-MEOT was inhibitory but less potent than 5-CT. α-Me-5-HT caused increase of both rate, force and tone.

The response of *Geukensia demissa* hearts to 5-HT and 8-OH-DPAT

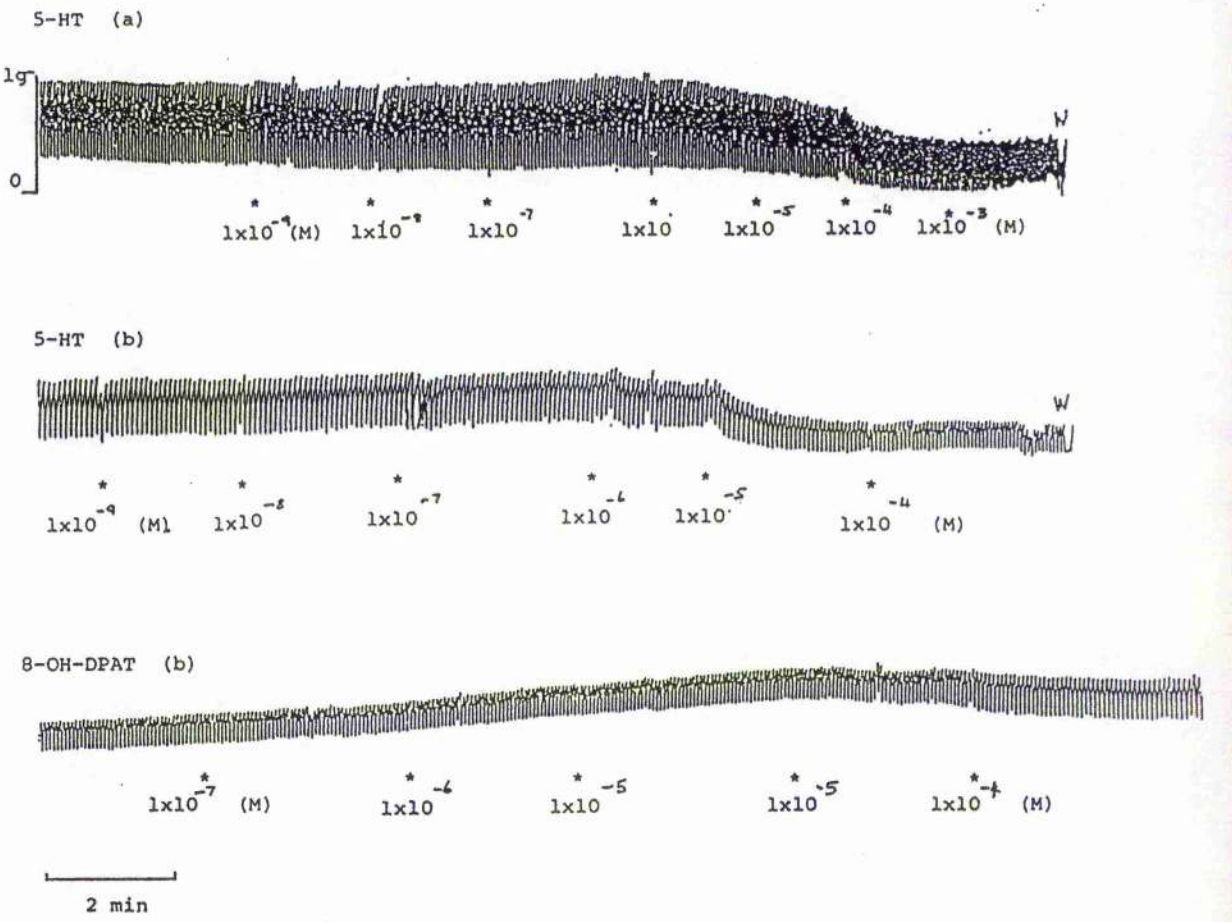


Figure 4(c)

(a) and (b) The mixed response of two hearts to 5-HT. Note in (a) the heart showed some positive inotropy initially, but this was inhibited by higher doses, while the rate still increased. However, in (b) we see a predominantly inhibitory response.

(b) and (c) The response of the same heart to both 5-HT and 8-OH-DPAT. 8-OH-DPAT weakly increased rate and force, but the effects diminished after washout.

Results - Other Species

Summarised below are some of the results observed on two of the other species tested:

Mytilis californianus

Please see **Figure 5 (a)** for the Graph. 5-HT, 5-CT, and Methysergide (UML) were all found to cause an increase in rate and force. The rank potency was 5-HT > 5-CT > UML > 8-OH-DPAT. Methiothepin was found to be a weak antagonist at a concentration of $1x10^{-6}$ M. The hearts from this species were more easily desensitised after long exposure to either 5-HT, or some of the analogues, compared to other species I tested. There also appeared to be a desensitisation of the hearts to dopamine which occurred after several cumulative DR curves to 5-HT. Although this was not investigated further it has been reported in other tissues in the literature previously, Peters (1989).

Clinocardium nuttalli

Please see **Figure 6 (a)** for a Graph and Traces.

Clinocardium nuttalli was very similar to the bivalve *Saxidomus giganteus* in its response to 5-HT and analogues. However, the response to 5-HT was more unpredictable in this species than in *Saxidomus giganteus*, a greater degree of inhibition was seen with the initial doses, but higher doses would usually produce some excitation; in some hearts no excitation was seen at all.

In *Clinocardium nuttalli* 5-CT always caused an inhibitory response. 2-Me-5-HT showed a weak inhibitory response,

although it was not a potent agent. Similar to the results seen on *Saxidomus giganteus*, Bz and methiothepin appeared to antagonise the inhibitory responses produced by 5-CT and 5-HT.

Clinocardium nuttalli was not easily obtained in Vancouver, so I was not able to test these hearts with all analogues and antagonists. However, α-Me-5-HT was tested on one heart and observed to cause only excitation.

The Effect of 5-HT and analogues on a *Mytilis californianus* Heart

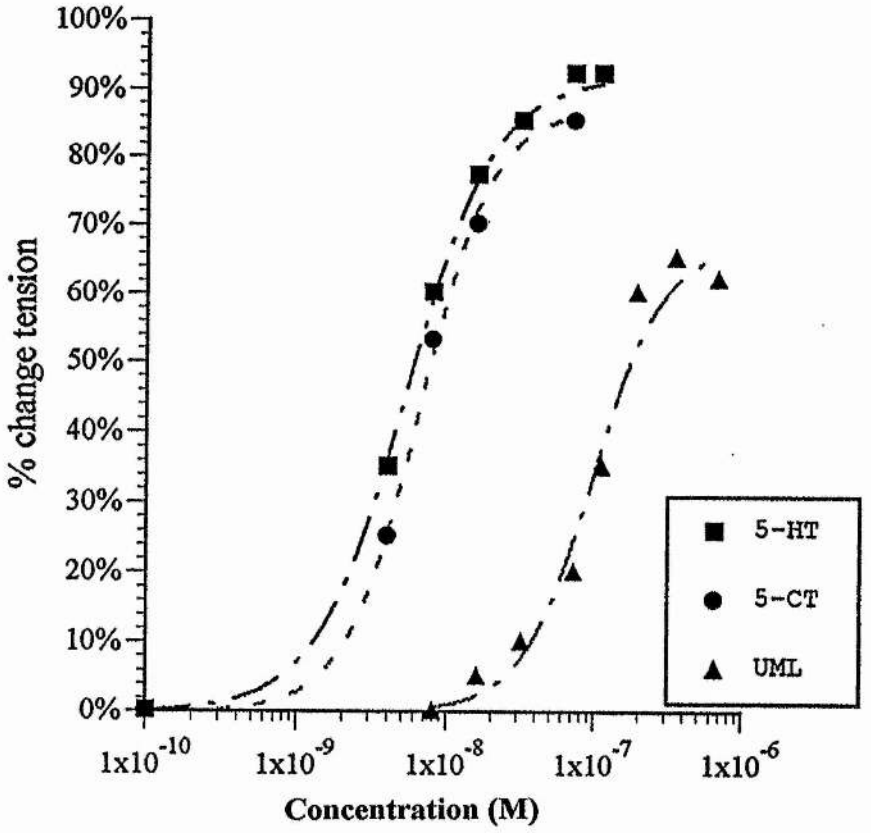


Figure 5(a) A plot of cumulative DR to 5-HT, 5-CT and UML shows that 5-CT was approximately equipotent to 5-HT. UML acted as a partial agonist, showing less potency and efficacy.

Clinocardium nuttalli

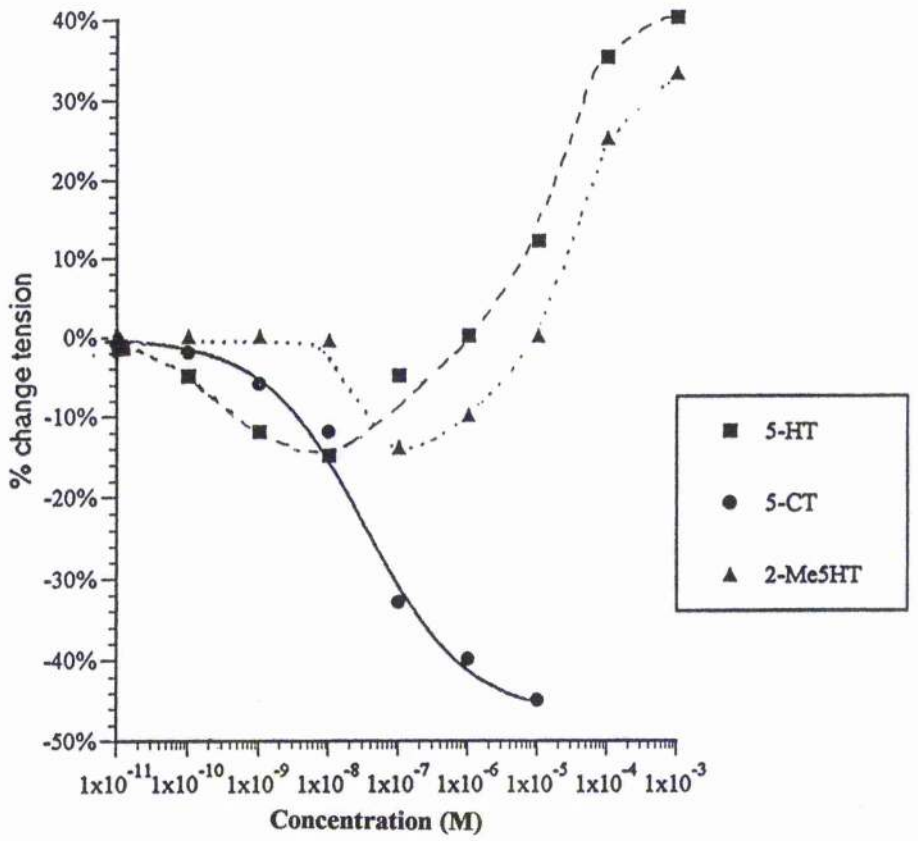
Figure 6(a)

The plot shows the cumulative DR curves in response to three drugs : 5-HT, 5-CT and 2-Me-5-HT.

(a),(b),(c) Cumulative responses to the application of each of the three drugs. The asterisks identified where the doses were added to the bath; the graph identifies the bath concentration after each drug application.

(d) The response of the same heart to 5-CT in the absence and presence of $1x10^{-6}$ M Benzoquinonium.

The Effect of 5-HT and analogues on a *Clinocardium nuttalli* heart



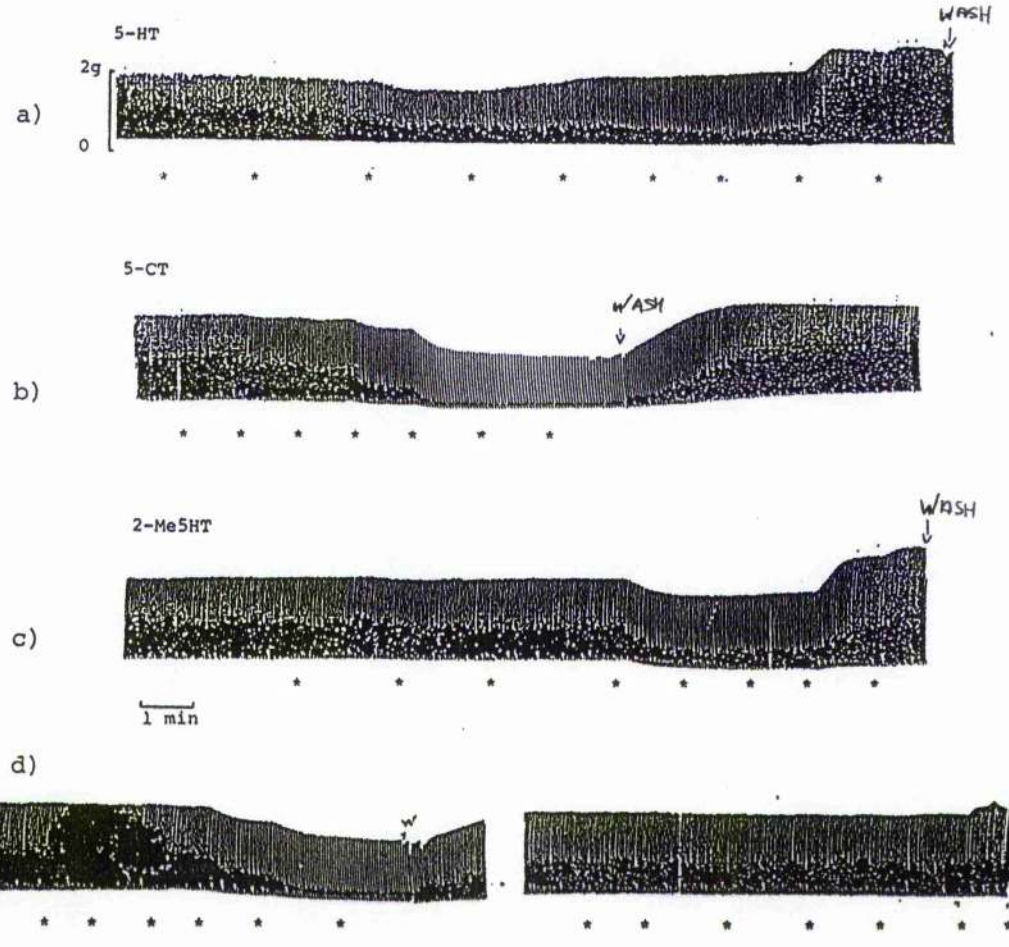


Table 7

Part I - Summary Table of Effects of 5-HT and Analogues

DRUG	*Mya arenaria*	*Mercenaria mercenaria*	*Saxidomus giganteus*	*Geukensia demissa*	*Mytilis californianus*	*Clinocardium nuttalli*
5-HT	excite	excite	inhibit +/- excite	inhibit/mixed	excite	inhibit +/- excite
α-Me-5-HT	excite	excite	excite	excite	excite	not tested
5-CT	excite	excite	inhibit	inhibit	excite	inhibit
5-MEOT	excite	excite	weak excite	inhibit/mixed	not tested	not tested
2-Me-5-HT	not tested	excite	inhibit +/- excite	not tested	weak excite	inhib/excite
8-OH-DPAT	excite	excite/persist agonist	excite/persist agonist	weak excite	weak excite	not tested

DISCUSSION - PART I

Table 7 summarises the results with 5-HT and analogues on the hearts of six different species. The action of each analogue will be discussed separately.

THE ACTION OF 5-HT ON THE HEARTS OF BIVALVES

In the extensive FMRFamide and 5-HT screening study by Painter and Greenberg (1982) they reported that the hearts of a few species including *Geukensia demissa* and several of the *Carditidae* species were either inhibited or showed a mixed response to 5-HT. It should be noted that Bz was not used in this study.

As with their findings, I found that the responses to 5-HT were predominantly excitatory on both the rate and force of contraction of the hearts. However, in several species *Saxidomus giganteus* and *Clinocardium nuttalli* inhibition was seen with the initial lower doses, often followed by excitation with higher doses (causing the biphasic DR curve to 5-HT). The response seen in *Geukensia demissa* was a more complex mix; the force of contraction of the heart was almost always reduced by higher doses of 5-HT, but this was often accompanied by an increase in rate.

THE ACTION OF 5-CT

5-CT was shown to be excitatory in *Mya arenaria, Mercenaria mercenaria,* and *Mytilis californianus;* in those hearts which were excited by the drug it was usually found to be less potent than 5-HT. However 5-CT was shown to reduce the force of contraction of the hearts of *Saxidomus giganteus,*

Geukensia demissa, Clinocardium nuttalli (and *Tresus capex* in experiments not reported here). In those hearts which were inhibited by 5-CT, methiothepin and Bz were often shown to antagonise this effect. 5-CT is an agonist which has been linked, in the literature on mammalians, to $5\text{-HT}_{1(A,B,D)}$ receptors, Hoyer et al. (1994).

The potent inhibition seen with 5-CT on the hearts of *Saxidomus giganteus* and *Clinocardium nuttalli* was completely abolished by Bz in these two species. In the presence of Bz, 5-CT became a weak excitatory agonist but was far less potent than 5-HT. This suggests there may be more than one type of 5-HT receptor in these molluscan hearts since another analogue α-Me-5-HT was found to be a selective and potent excitatory agonist in the all hearts studied.

THE ACTION OF α-Me-5-HT

α-Me-5-HT was an excitatory and potent agonist on <u>all</u> the hearts tested from *Mya arenaria, Mercenaria mercenaria, Geukensia demissa and Saxidomus giganteus*. This compound is known to be an agonist on the 5-HT_2 and 5-HT_{1C} (or 5-HT_{2C}) receptors in the mammalian classification and is also suggested as having some 5-HT_4 agonist activity, Hoyer et al. (1994). However, although α-Me-5-HT was often more potent as an excitatory agent than 5-HT, neither ketanserin nor retanserin (selective 5-HT_2 antagonists) appeared to be selective antagonists to α-Me-5-HT in any of the hearts tested (at concentrations of $1\text{x}10^{-6}$ M).

A recent study by Goldberg et al. (1994) which examined the 5-HT receptors involved in early embryonic behaviour of a pulmonate gastropod *Helisoma trivolvis* showed α-Me-5-HT to act as a partial agonist. The antagonist mianserin but not

ketanserin showed the most selective antagonist activity to 5-HT on this receptor (however 5-CT was also a potent agonist). They suggested that possibly it was the size of the ketanserin molecule which somehow prevented its activity on their preparation since the egg capsule membrane was left intact in their experiments. They concluded from their findings that the characteristics of the 5-HT receptor were distinct from those previously characterised in vertebrate or invertebrate systems. They suggested that complementary molecular, bioactivity and binding studies are needed to elucidate the evolution of serotonin receptors.

THE ACTION OF 5-MEOT

5-MEOT has been classified as an agonist at 5-HT_4 receptors (mammalian) in the literature, Hoyer et al. (1994). However, it should be remembered that in earlier studies, before the identification of a 5-HT_4 receptor, this analogue was shown to have some activity at the 5-$HT_{1\text{-like}}$ receptors, Bradley et al. (1986).

In the hearts of *Mya arenaria, Saxidomus giganteus* and *Mercenaria mercenaria* this agent was found to be excitatory but usually less potent than 5-HT. In *Geukensia demissa* hearts, where 5-CT was inhibitory, 5-MEOT appeared to act in a similar though less potent manner. However, it is interesting to note that in *Saxidomus giganteus* the effect of 5-MEOT was definitely excitatory, while 5-CT demonstrated a potent inhibitory action.

In their review Hoyer et al. (1994) point out that in the literature from vertebrate studies on 5-HT_4 receptors, both 5-MEOT as well as α-Me-5-HT have been shown to act as potent agonists at this receptor. This introduces further confusion as α-Me-5-HT has previously been suggested as a potent agonist

at the 5-HT_2 receptors. However, Hoyer et al. note that absolute potency appears markedly tissue dependent in vertebrates and 5-HT_4 receptor responsiveness may vary even along the course of a piece of intestine.

THE ACTION OF 2-Me-5-HT

2-Me-5-HT, a 5-HT_3 agonist according to the mammalian classification, showed initial inhibition followed by excitation at higher concentrations in *Saxidomus giganteus,* and *Clinocardium nuttalli*. In other species, it showed only weak excitation and was much less potent than 5-HT. The 5-HT_3 receptor, which is described as a ligand-gated receptor in the mammalian literature was not identified in my study. The suggested selective agonist 2-Me-5-HT did not appear to have a potent (or rapid onset) effect in any of the *bivalvia* species examined. The 5-HT_3 antagonist identified through mammalian research, GR38032F now known as ondansertron, acted as a weak agonist in one species, *Mya arenaria*. No selective antagonism of 2-Me-5-HT was observed with this compound.

THE ACTION OF 8-OH-DPAT

8-OH-DPAT is an agonist which is known to effect 5-HT_{1A} receptors as reported in the mammalian literature, Hoyer et al. (1994). This agent was found to be an agonist in most species studied but usually less potent than 5-HT. However this agonist had a prolonged effect on several of the species. After exposure to this agent the hearts of *Mercenaria mercenaria* and *Saxidomus giganteus* continued to beat at close to maximum force. The effect lasted for more than an hour even with multiple washes. When the hearts were beating at this increased force the effect of subsequent doses of 5-HT were

ineffective. This desensitisation may have been due to the fact that the heart was beating at a force which was close to maximum. The mechanism of action which produced this effect is not known.

THE ACTION OF BENZOQUINONIUM

In many of the earlier studies, discussed in the Introduction, the agent Benzoquinonium was often added to the sea water bathing the hearts, to block any response to ACh which might be released. My observations of the action of 5-HT on the hearts of some bivalves identified a difference in the effect in the presence of Bz. In the species I have studied, where an initial inhibitory effect was seen with 5-HT, some other agonists were shown to excite or inhibit selectively. However the selective inhibitory effect was not observed when Bz was added to the sea water. Therefore, I suggest that in some of the earlier studies any inhibitory effect of 5-HT, or its analogues, may have been masked by the presence of Bz.

The mechanism by which Bz antagonised the inhibitory effect is not known, but there are several possible explanations:

a) Bz might act as an effective antagonist at the 5-HT receptor responsible for the inhibitory response to 5-CT.

b) Bz might directly affect a second messenger pathway which is activated in response to 5-CT.

c) Could Bz be blocking the ACh receptors in the heart which would be affected by release of ACh from the heart tissue. Would this explain the increase in tone and rate generally seen when this drug was added to the bath? However, explanation (c) would imply that 5-CT causes the release of ACh which then produces the inhibitory response.

This is an unlikely explanation as the inhibitory DR curves in response to ACh on the hearts of *Saxidomus giganteus* showed ACh to be more than tenfold less potent than 5-CT itself.

Hoyer et al. (1994) reviewed the effects of benzimidazolones and substituted benzamides (''benzamide-binding sites''), which at high concentrations exhibit muscarinic receptor-blocking activity. These compounds in several vertebrate preparations are effective 5-HT_4 receptor agonists and/or partial agonists. Perhaps Bz is acting in a similar manner on some of the hearts from different *bivalvia* species?

THE ACTION OF 5-HT ANTAGONISTS

UML has been reported as an antagonist at both 5-HT_1 and 5-HT_2 receptor sites, although it often shows partial agonist properties on 5-HT_1 receptors, Hoyer et al. (1994).

UML usually demonstrated antagonist action to 5-HT in many of the species tested, however it was shown to have direct effects (acting as a partial agonist) on several species. This made it much harder to quantify as an antagonist. UML was noted to cause a small inhibitory effect at higher concentrations (10^{-5}M) in *Mya arenaria*. MDL 72222 had a weak antagonist action to 5-HT in this species, but caused a potent inhibitory effect itself at a 10^{-5} M concentration.

Methiothepin has been reported to be an antagonist at several of the 5-HT receptor types, Hoyer et al.(1994). Although, weak antagonism to 5-HT was usually seen with methiothepin at pharmacologically high concentrations, $1x10^{-6}$ M or $1x10^{-5}$ M, this agent was the only antagonist which did not appear to show any agonist action on the hearts tested.

Ketanserin and retanserin are described as selective 5-HT_2 antagonists in the mammalian literature, Hoyer et al.(1994). However neither of these agents were found to have selective antagonist action to 5-HT in any of the species tested (at the 10^{-6} M concentration). The compound mianserin was not investigated in these preparations. It should be noted that Goldberg et al. (1994) recently showed selective antagonist action of mianserin to 5-HT in a gastropod preparation, but no antagonist effect was shown with ketanserin.

SUMMARY

From these studies on different species it was apparent that there was more than one type of response to 5-HT and its analogues. In those species which showed a mixed or an inhibitory component in the response to 5-HT, I was able to show a potent inhibitory component with one analogue and a potent excitatory response to another. As shown by the summary of *Saxidomus giganteus* results outlined in Table 7.

This does not necessarily prove that specific mammalian "sub-types" of 5-HT receptors are present in the hearts of bivalves. However it does support my hypothesis that there may be more that one type of 5-HT receptor present in molluscan hearts. Due to the lack of selective antagonists available to study the 5-HT receptor in molluscs, it is not possible at present to further classify the receptor(s) sub-types.

This was the same conclusion reached by Cadogen and Humphrey (1992) after their extensive screening of 5-HT and analogues on the *Helix* heart, as discussed in the Introduction.

This is not too surprising when one considers the evolutionary time separating the *bivalvia* from mammals; it might seem unlikely that the receptor structure would be the

same. However, some or many of the mechanisms of action or second messenger pathways may well have been conserved. For example, in current research examining ion channel structure, scientists have used molecular biology to sequence the channel protein structure. Many have reported similarities in the channel protein sequences between species separated by evolution for more that 100 million years.

Anderson et al. (1993), identified a Na^+ channel in a Jellyfish, *Cyanea capillata* which showed its protein sequence structure had been conserved by up to 55% when compared to specific Na^+ channels found in mammals. If conservation of certain common structure survived through the evolutionary process (over one hundred million years) it would seem probable that certain structural similarities may exist between types of 5-HT receptors found in the hearts of humans, guinea pigs and even molluscs.

The experiments in Part II were designed to try to correlate the actions of 5-HT and analogues on the whole heart to specific cellular changes by examining the effect of 5-HT on the unitary current activity of membrane channels recorded from single myocytes.

Part II

5-HT ACTION ON INDIVIDUAL MYOCYTES

INTRODUCTION

I demonstrated in Part I that molluscan hearts are particularly sensitive to 5-HT, responses are generally excitatory and often showed clear inotropic and chronotropic components. However, I observed that the response of some species, *Geukensia demissa, Clinocardium nuttalli* and *Saxidomus giganteus* to 5-HT and some analogues was inhibitory or was comprised of a mixture of excitation and inhibition.

Studies (referred to in the Introduction) have shown that 5-HT can cause both excitation (depolarisation) or inhibition (hyperpolarisation) of molluscan neurones. Studies also have linked 5-HT to intracellular cyclic AMP pathways and the closing of a K^+ channel (the S-K^+ channel) as well as modulation of other Na^+, K^+ and Cl^- currents. I decided to examine the effect of 5-HT on single myocytes from some of the bivalve species which had shown differing responses, excitation, inhibition or a mixed response.

Using the patch clamp technique (Diagram 2) I examined the action of 5-HT and analogues on the ionic currents in single heart cells (myocytes). The species used in the majority of the patch-clamp studies were *Mya arenaria, Mercenaria mercenaria,* and *Geukensia demissa*. As discussed in the Introduction, these species had been used extensively in whole heart preparations, in sucrose gap electrophysiological and second messenger pathway studies. There is considerable evidence in the literature that *Geukensia demissa is* generally

different in its response to 5-HT in comparison to the other two species, Wilkens (1972), Wilkens and Greenberg (1973).

Myocytes were examined using the cell attached mode (Diagram 2). Because of the "tight" gigaOhm seal, any drug applied to the cell would not affect channels in the patch directly. This allows a small piece of the cell membrane to be studied in isolation. Any effect seen within the patch, in response to the drug applied to the whole cell, must be due to intracellular events affecting changes in the channel activity across the membrane of the patch. This would imply that second messenger pathways are involved.

I studied the action of 5-HT and some key selective analogues (used in Part I) on myocytes from hearts of the following bivalve molluscan species: *Mercenaria mercenaria, Mya arenaria, Geukensia demissa,* and a few hearts from the local (Florida) cockle family, *Carditidae*. The experiments were designed to study the action of 5-HT and its analogues on single dissociated heart cells using patch clamp methodology according to Hamill et al. (1981).

Diagram of the Patch-Clamp Technique

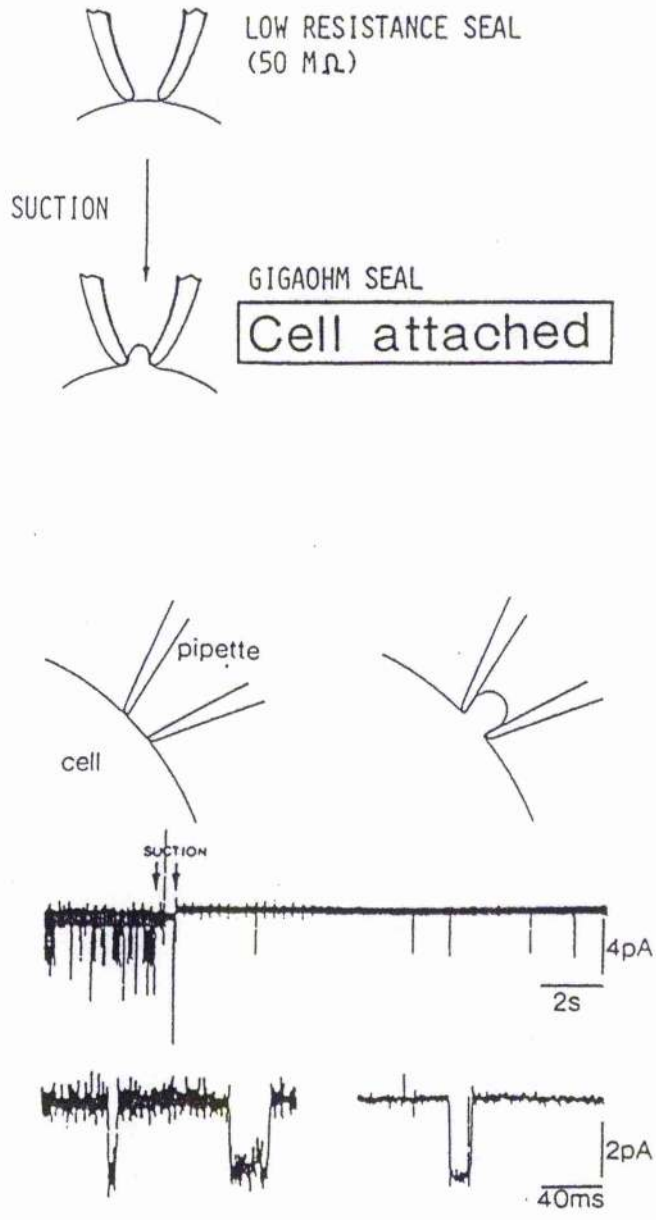


Diagram 2.
This diagram has been modified from Hamill et al. (1981) and shows the technique used to isolate a patch membrane in the cell attached mode. The recording trace shows how with the application of gentle suction to the pipette, a clear ``noise-free'' recording is obtained from the isolated patch.

Mya arenaria myocytes seven days after dissociation

100μ

Fig. II(A) This photograph was taken seven days after the myocytes from *Mya arenaria* ventricles were dissociated. With time the cells become flat and connected up with one another. The myocytes which were connected together would be seen to occasionally beat in unison.

Table 8

Artificial Sea Water (ASW) Composition used in Part II

***Aplysia* Patch-Clamp ASW (Siegelbaum et al. 1982)**

	Normal (10mM K^+) concentration (mM)	High K^+ (100 mM K^+) concentration (mM)
NaCl	460	370
KCl	10	100
$MgCl_2$	55	55
$CaCl_2$	10	10
HEPES	10	10

pH = 7.4 (buffered with NaOH)

Table 9

Preliminary Culture Medium

Heart Cell Growth Medium	**Total Volume = 100 ml**
ASW (Gaddum / Siegelbaum) sterilised	75 ml
Glucose	0.1g
Culture Medium (Wolf & Quimby - Gibco # 350-1835)	25 ml
Gentamycin antibiotic	10 mg/100 ml

Preliminary Experiments: Dissociations were done using both sterile ASW (Gaddum 1955, or Siegelbaum et al. 1982) and growth medium (see Tables 4, 8, and 9). Subsequently, I decided to use the ASW used by Siegelbaum et al. (1982); these researchers had used this ASW composition in cell attached patch-clamp studies of dissociated *Aplysia californica* neurone preparations. I had also compared dissociated myocytes prepared in both Gaddum's or Siegelbaum's ASW, with those kept in sterile filtered sea water collected locally. There was no visible difference seen between the myocytes kept in any of the different sea water preparations. There was no difference in the unitary current data recorded from patch-clamp experiments on myocytes kept in any of the three sea water preparations.

Dissociated molluscan bivalve myocytes can be maintained in culture for several days. With the use of antibiotics and other special media, cultures may be maintained further for weeks. However after 3 days the myocytes became flat and formed thin interconnecting fibres with fibroblasts. It was difficult to patch such cells. Figure II(A), shows *Mya arenaria* myocytes seven days after dissociation. The myocytes have joined and formed a sheet-like configuration which would occasionally beat spontaneously.

After these preliminary experiments I decided to use the ASW(Siegelbaum et al. 1982) without any growth medium or antibiotics. The Siegelbaum ASW has been used in the majority of the studies of dissociated neurones of marine molluscs, Shuster and Siegelbaum (1987). In principle, I had concerns that agents such as antibiotics might affect the cell membrane. I found the best patch-clamp seals were obtained on cells which had been dissociated for 12-48 hours; so there was little need for antibiotic coverage of the cultures over this time.

Table 10

Enzymes used to prepare the dissociated myocytes

Bivalve	Enzyme (a)	Time	Enzyme (b)	Time
Mya arenaria	Trypsin (0.25%)	1 hr	Collagenase	3-4 hr
Mercenaria mercenaria	Trypsin (0.25%)	1 hr	Collagenase	3-4 hr
Mercenaria mercenaria	Protease VIII	3-4 hr		
Geukensia demissa	Protease VIII	4-5 hr		
Trachycardiae	Trypsin (0.25%)	1 hr	Collagenase	3-4 hr

METHODS

DISSOCIATION OF VENTRICLES

The species used in the single cell patch-clamp studies in Part II were: *Mya arenaria, Mercenaria mercenaria, Geukensia demissa* and a small number of hearts of *Trachycardiae* species.

The ventricles and atria were isolated. This minimised contamination by protozoa or bacteria from the gut. The atria were carefully separated from the ventricular tissue. Ventricles were placed in sterile ASW and cut into pieces approximately 2-4 mm square. These pieces were rinsed several times to remove any haemocytes or debris from the fibrous ventricular tissue. The pieces were placed in 35 mm petri dishes containing the dissociation enzyme (see Table 10) dissolved in 2 ml of sterile, filtered ASW (0.2µ filter). This ASW was used for all preparation of the myocytes and during the patch-clamp experiments. The tissue was further cut into approximately 1 mm^3 size pieces under a binocular microscope. The Petri dish was placed on a shaker platform and gently agitated.

I discovered that ventricles from *Geukensia demissa* required different time periods of exposure as well as enzymes for successful dissociation. The enzymes used and periods of exposure used for the different hearts are shown in Table 10. **Note:** Duplicate dissociations of *Mercenaria mercenaria* were done in <u>each</u> of the different enzyme preparations to verify that there was no difference in the current activity observed due to the different enzyme technique required by *Geukensia demissa*.

When the dissociation was completed the cell suspension was triturated gently using a round tipped glass pipette. The 2 ml suspension of cells was added to 18 ml of the ASW and

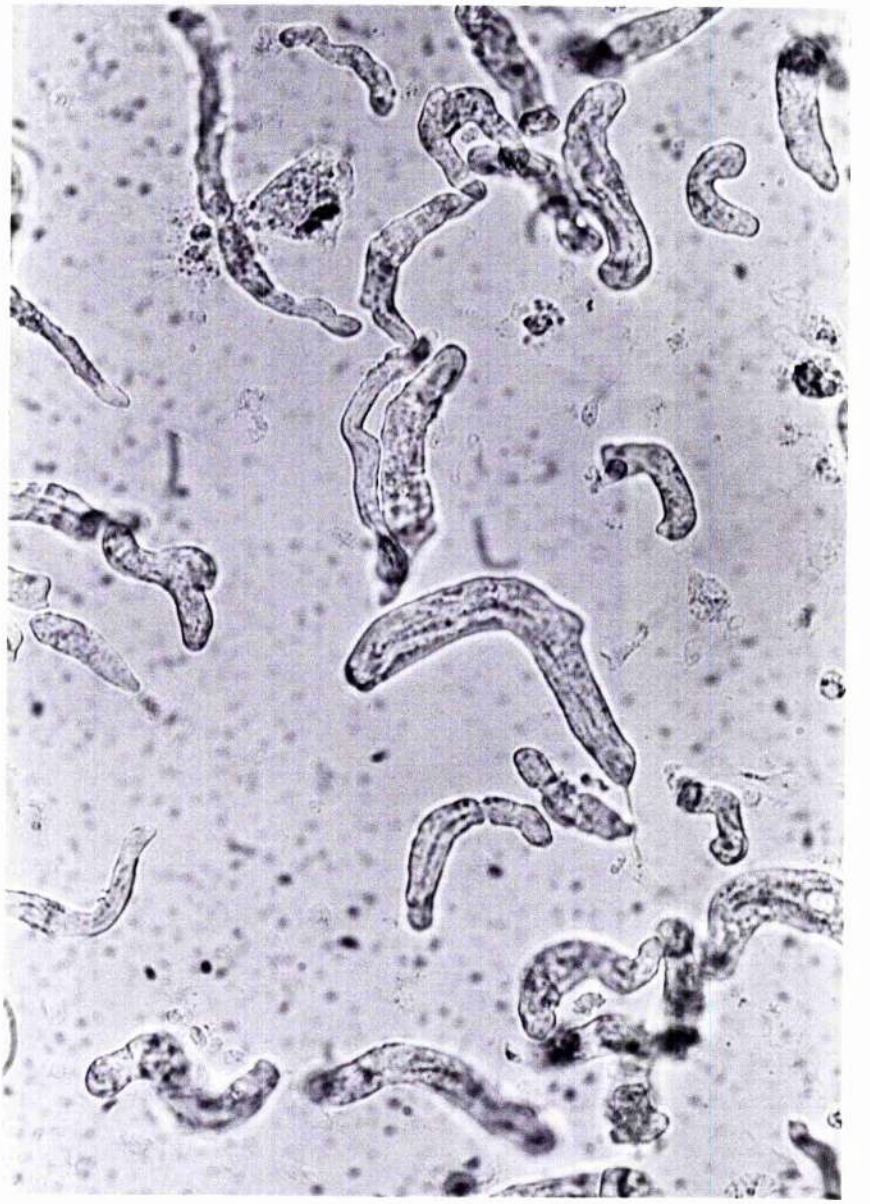
centrifuged at 115 x G for 5 min, Brezden and Gardner(1986). The top 19 ml of the supernatant was discarded and the remaining solution and cells were re-suspended in 15 ml of sterile filtered ASW and centrifuged for a further 10 min. All of the supernatant was discarded. The plug of cells was gently triturated with 1-2 ml of new sterile filtered ASW and plated as large droplets on the prepared coverslips placed in 35 mm Petri dishes. After 30 minutes, approximately 2 ml of sterile filtered ASW was added to the Petri dish. The cells were left to adhere further to the coverslips for at least 6-8 hours before recording.

The photograph shown in Figure II(B), shows *Mya arenaria* cells 12 hours after plating. When viewed under a microscope shortly after plating, many cells could be seen slowly contracting. As the myocytes settled and adhered to the coverslips these spontaneous contractions usually ceased within 12 hours. To get adequate adhesion of the myocytes to the glass coverslip surface, the following procedure was found to be the most successful.

COVERSLIP PREPARATION

Coverslips were placed in 1N HCl for 1 hour then rinsed and soaked in 0.1% Na_2CO_3 for 1 hour. The coverslips were rinsed several times with distilled water, sterilised by autoclaving and then coated with Poly-lysine D (Sigma) 25 µg/ml. After a thorough final rinse with distilled water the coverslips were allowed to dry in the air in a laminar flow hood.

Mya arenaria myocytes



100µ

Figure II(B) Photograph taken approximately 12 hours after the heart was dissociated. The myocytes had become attached to the cover slips, one or two cells could be seen to contract spontaneously.

PATCH-CLAMP RECORDING

Coverslips with the attached cells were placed in a shallow plastic dish and bathed in sterile filtered ASW. The dish was mounted on the stage of a Zeiss Jena (ERGAVAL) inverted microscope fitted with Hoffman optics. The microelectrodes and pipettes were made from Boralex 5061 glass tubing. The reference electrode was a 'silver chlorided' silver wire placed in the bathing ASW. Electrodes were pulled to a diameter of approximately 1 μm by a 2 stage Narashigi PP83 puller then fire polished to a final smooth tip, which gave a bubble test number between 2 and 4.

The electrode was attached to a Dagan 8980(10G) head stage mounted on a Narashigi M103 micro manipulator which was attached to the Dagan 8900 Patch Clamp equipment. Single-channel recordings were performed by conventional patch-clamp methods, Hamill et al. (1981). Recordings from the "voltage-clamped patches" were displayed on an oscilloscope (PM 3215 Philips). The signal was processed by an Analogue/Digital converter (Sony Digital Pulse Code Modulator PCM501E5). Continuous data recordings were stored on Beta tape (Sony SL-2700 Beta Hi-Fi), for analysis at a later date. All data were recorded using a 1 kHz Filter. Rapid voltage stepping from one holding potential to another was applied by a Grass S48(S1U5) Stimulator.

PATCH-CLAMP DATA ANALYSIS

The recorded data were later converted from analogue to digital via the Pulse Code Modulator (PCM501), and the data analysis was done using a Labmaster board (AXON) attached to an ANO 386SX computer loaded with Axoclamp 5 and 5.5 Patch Clamp software. Analysis of the unitary current activity (i) observed

at different electrode holding potentials (V_e), allowed me to plot i/V curves for the unitary currents seen in the patches.

The number, size and open time of these currents was examined, and analysed. The probability of open time was calculated using the method of Vandorpe and Morris (1992). Since I did not know the total number of channels present in a patch, the data were normalised as follows. To express the probability of open time for channels in the patch the Index $NP_{(open)}$ was used; where N is the number of channels open at one Level and $P_{(open)}$ is the fraction of time that the channel is "open". So the formula for open time of channels in a patch would be:

$$\text{Index } NP_{(open)} = N1P_{(open)1} + N2P_{(open)2} + N3P_{(open)3}$$

If the total number of channels in the patch were known this Index could be divided by the total number of channels.

As these experiments used the cell attached configuration I had no way of determining the resting membrane potential of each individual myocyte. Therefore most of the data are expressed in terms of the electrode potential (V_e), which is the holding potential applied to the patch.

IDENTIFYING THE CHARACTERISTICS OF THE UNITARY CURRENTS

After the seal had formed and stabilised (approximately 5 min) the voltage applied to the patch electrode was raised or lowered in 20 mV increments from -100 to +40 mV; the voltage was sustained at these different steps for more than 1 minute, then 10 s intervals were captured on tape for analysis. Every cell that was patched was taken through the full range of electrode potentials at least twice to verify that the seal was holding well and the cell had stabilised.

The K^+ concentrations in the electrode ASW solution were varied from 0, 10, 100 mM to examine changes in the unitary current characteristic.

Several experiments were carried out on myocytes from *Mercenaria mercenaria* and *Geukensia demissa* examining the effect of sudden rapid voltage changes on the patch. While holding the patch at a selected voltage, a 10 s jump in voltage was applied to the patch (either hyperpolarizing or depolarising). This would unmask any differences in the unitary current activity due to sudden voltage changes, allowing comparison with the current activity seen with sustained voltage clamping.

A few experiments were done with either 4AP or TEA in the patch solution, as well as in the bath, to block any unitary current activity via calcium dependent K^+ channels.

APPLICATION OF DRUGS

Drugs were initially applied to the whole bath by perfusion; subsequent experiments used a more selective localised application. This was achieved by the micro manipulation of a pipette, filled with the drug at a concentration of 100 μM, close to the patched myocyte (approximately 100 μ). A small amount of the drug was 'puffed' into the vicinity of the cell by use of a Picospritzer II (made by General Valve).

Bubble tests in mineral oil were performed to verify that the size of the injected pulse (puff) of drug was between 25-50 μ radius; thus the drug had to diffuse into the area around the cell and limited the mechanical stimulation of the cell membrane by the pulse. The drug concentration seen by the whole cell usually 100-200 μ in size itself, with the dilution

caused with diffusion, was estimated to be in the range of 1-10 μM. The photograph in Figure II(C) shows an electrode 'patched' on to a large (~200 μ) *Mercenaria mercenaria* cell. The darker circle around the tip of the second electrode shows the drug (with dye added) which had been released by pulse application in the area beside the cell approximately 3 minutes earlier.

THE DRUG EFFECT ON UNITARY CURRENT ACTIVITY

The drugs which were tested on the dissociated myocytes reported in the Results section were:
5-HT, 5-CT, 5-MEOT, 2-Me-5HT, 8-OH-DPAT,
Forskolin, 8-bromo-cAMP.

Each compound was tested on these three species, *Mya arenaria, Mercenaria mercenaria* and *Geukensia demissa*. Only those experiments where the patch was successfully maintained, from the control period to the drug application and on to the recovery time (>10 min after drug application), were used in the Result section.

It should be noted that 5-HT was tested on only a few *Trachycardiae* myocytes; 4 patches were successfully held to completion of the experiment. No experiments were undertaken to examine the unitary current properties of these myocytes. *Trachycardiae* species proved to be difficult to obtain consistently in Florida during my visits.

A patched *Mercenaria mercenaria* myocyte

Figure II(C)

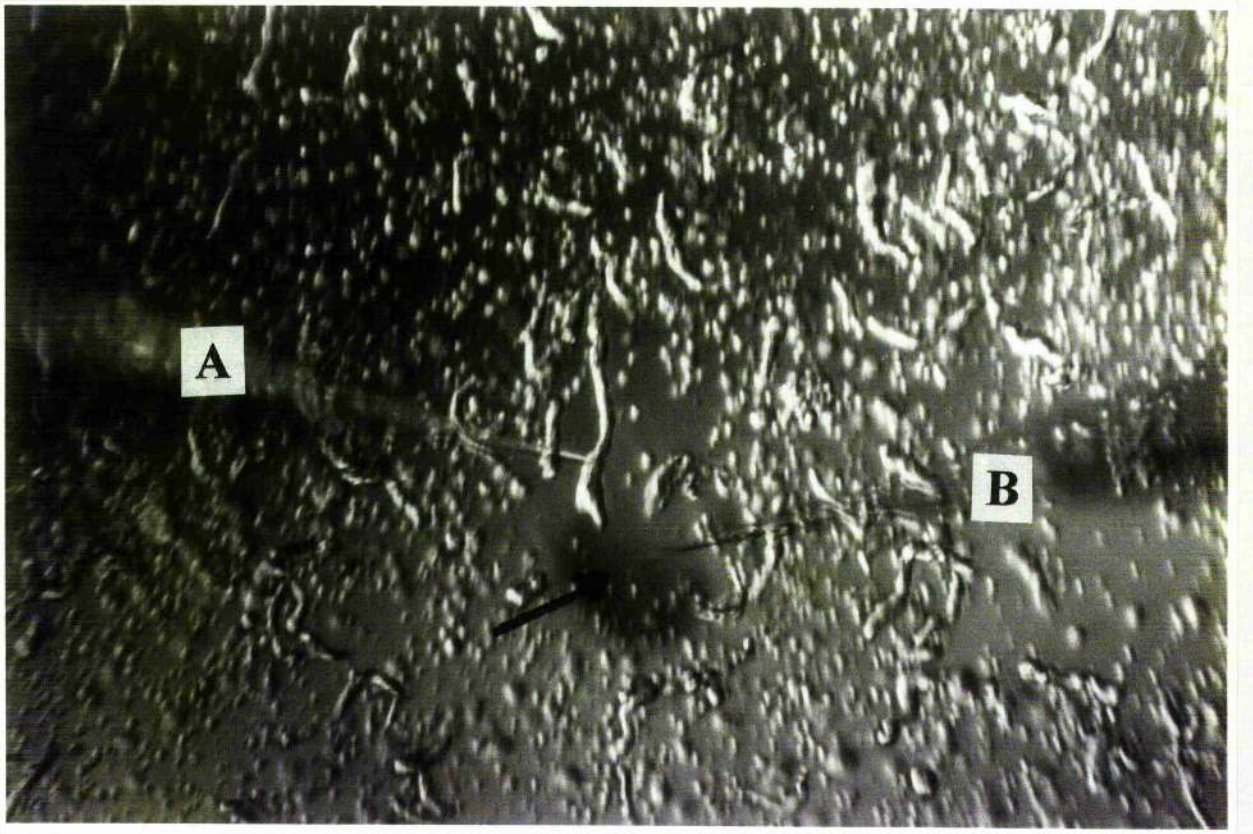
This photograph shows *Mercenaria mercenaria* myocytes which are long and thin. The patch pipette **(A)** is shown attached to a large myocyte, the other pipette **(B)** contained the drug, the dye (dark area) is shown beside the cell. The dark area identified by the arrow shows the area of diffusion of the drug solution (containing the dye) 3 minutes after application by the Picospritzer.

Geukensia demissa myocytes

Figure II(D)

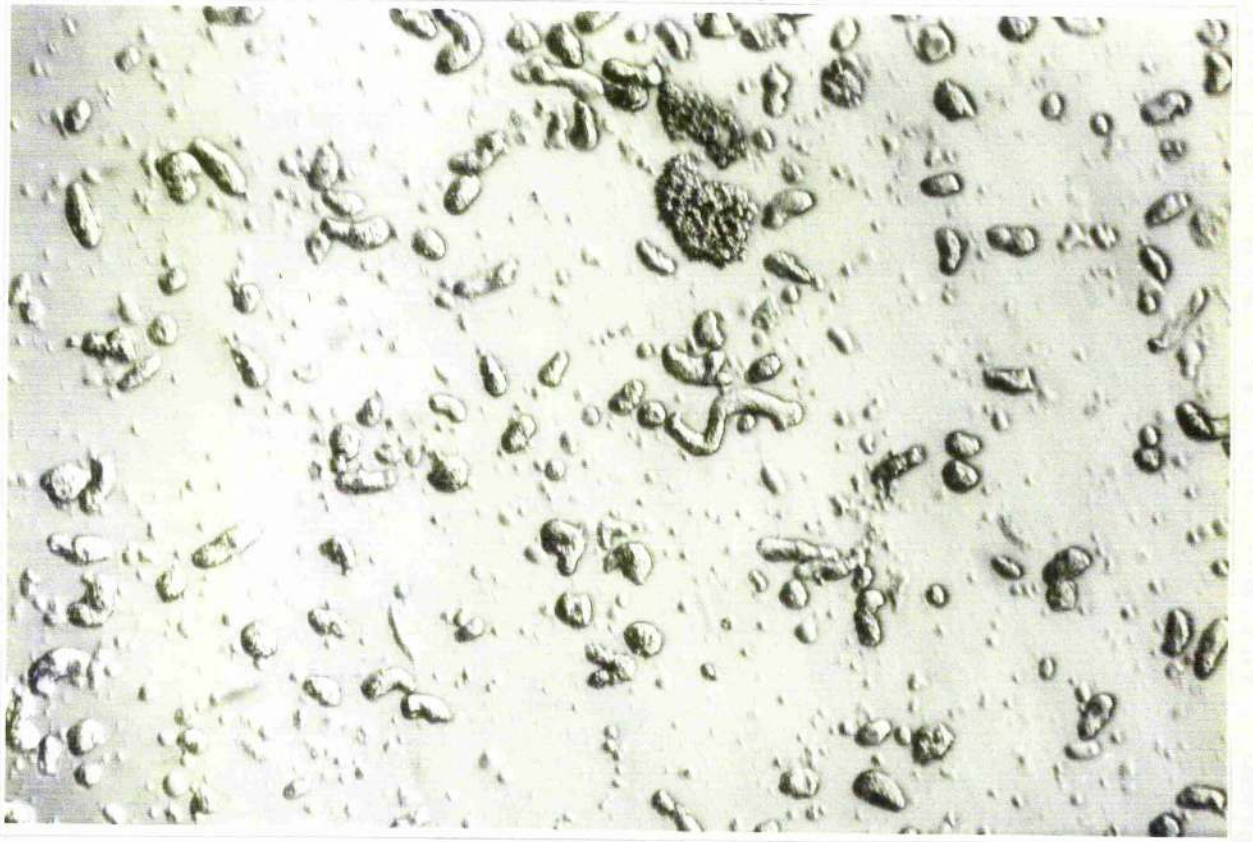
Photograph of *Geukensia demissa* myocytes approximately 12 hours after dissociation. These myocytes were smaller and rounder than the myocytes dissociated from either *Mya arenaria* or *Mercenaria mercenaria*. The different enzyme used in the dissociation was not responsible for the change in appearance of the myocytes. This was confirmed by the dissociations done using both enzymes on *Mercenaria mercenaria* hearts.

Patched *Mercenaria mercenaria* myocyte



100µ

***Geukensia demissa* Myocytes**



RESULTS - Part II

The channel characteristics and most of the results described here were recorded from several hundred patched myocytes from *Mya arenaria, Mercenaria mercenaria, Geukensia demissa*. Although a few myocytes from two *Trachycardiae* species were patched, the channel characteristics were not fully investigated and 5-HT was only tested on 4 myocytes in these species.

With normal ASW (10 mM K^+) in the patch pipette, channel currents were not seen when the pipette potential was 0 mV, i.e. close presumably to resting potential. When the electrode potential was held between -20 to -120 mV channel unitary currents were detected, the amplitude increased with the depolarisation of the membrane. The predominant current observed in every patch from the three major species was outward and characterised by a conductance of 38(+/-5)ps. The channel openings often appeared as bursts with current amplitude increasing steadily with increased depolarisation. The unitary current activity was consistently greater during the first 5 minutes following the seal formation. Therefore, I allowed the cell to stabilise during this period. The initial high rate of channel openings is likely to have been brought on by stretching the membrane by the applied suction during seal formation, Vandorpe and Morris(1992) and Morris (personal communication 1993). There was however no apparent inactivation during sustained voltages used to clamp the patch potential.

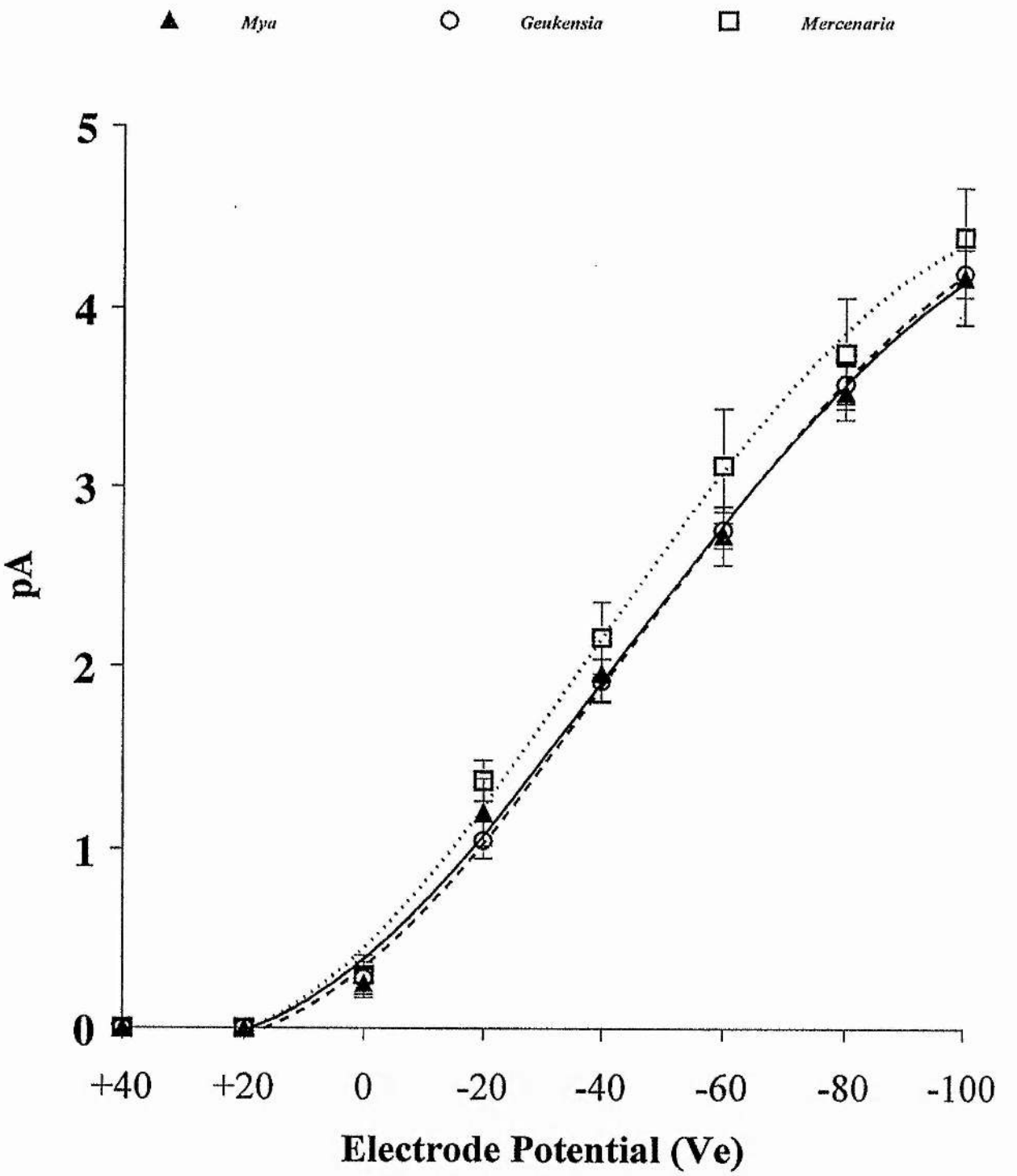


Figure II(E)

The relationship between the amplitude of unitary currents recorded from myocytes and electrode potential, with 10 mM K^+ ASW in the patch electrode. The myocytes from three species are identified. The mean unitary currents (+/- S.E.) are plotted vs. the electrode potential (V_e).

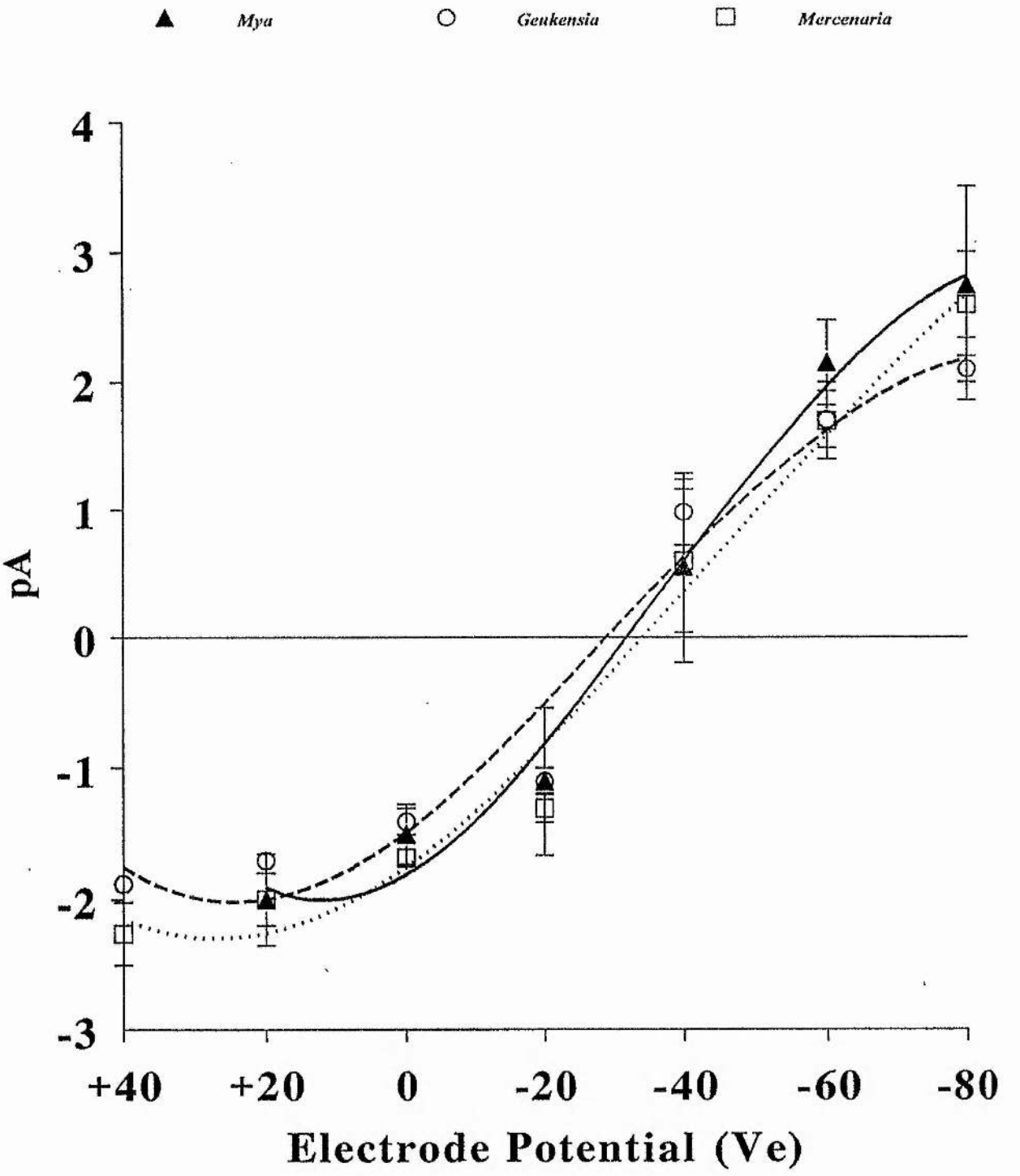


Figure II(F)

The relationship between the amplitude of unitary currents recorded from myocytes and electrode potential, with 100 mM K^+ ASW in the patch electrode. The myocytes from three species are identified. The mean unitary currents (+/- S.E.) are plotted vs. the electrode potential (V_e).

CHARACTERISTICS OF THE UNITARY CURRENT(S)

The predominant current activity observed was identified as an outward movement of K ions, verified by the observations shown in Figures II(E), and II(F), which plot the relationship between the size of the presumed potassium current and the electrode potential for three of the species (current-voltage plots). In Figure II(E) the conductance was found to be 38ps(+/-5), as calculated by the slope of the linear part of the curve, with 10 mM K^+ in the pipette electrode. The curves for all three species plotted in Figure II(F) shifted to the right, conductance was increased to 55 ps(+/-4) with 100 mM K^+ in the pipette. Comparison between these two figures, show the relationship between the size of unitary currents and electrode potentials with K^+ concentrations of 10 mM and 100 mM K^+.

With 100 mM K^+ ASW in the pipette inward currents were observed at electrode potentials more positive than -30mV. Whereas with 10 mM K^+ ASW no inward currents were observed. This is clearly illustrated in Traces II(G) and II(H), where I was successful in patching the same *Mya arenaria* myocyte twice; the first electrode was filled with 10 mM K^+ ASW; the second electrode contained 100 mM K^+ ASW. With 100 mM K^+ ASW channels were open even in the absence of membrane depolarisation implying that this K^+ channel contributes to the resting conductance of the cell.

In order to estimate the reversal potential (as determined from the interpolated zero-intersection point of the current-voltage relationship) I assumed the resting Vm to be -55mV. This was based on:

a) 3 experiments in *Mya arenaria* were I used "intracellular ASW" in the pipette, as used by Siegelbaum et al. (1982). The

membrane was ruptured and V_m was recorded in the range between -52 mV and -57 mV.

b) In *Lymnaea stagnalis* dissociated heart cells, Brezden et al. (1986), V_m for 30 cells were shown to range between -50 mV to -67 mV.

c) Sucrose gap experiments of Wilkens (1972) reported the V_m to be between -45 mV to -60 mV in *Mytilus edulis* and *Modiolus (Geukensia) demissus* heart preparations. They did comment that *Geukensia* constantly appeared to have a V_m in the lower end of this range.

The "reversal potential" based on an assumed V_m = -55 mV was usually -70mV to -75mV with 10 mM K^+ ASW in the electrode and about -25mV when the K^+ concentration was increased to 100 mM. The relationship between unitary current amplitude and the patch electrode potential is shown in Fig II(i):

Curve **(A)** $[K]_o$ = 10 mM and Curve **(B)** $[K]_o$ = 100 mM

The continuous line shows the relationship predicted by the Constant Field equation (Hodgkin and Katz 1949), assuming an internal $[K]_i$ = 240 mM. The value for the potassium permeability was calculated to be:

$P_K = 9.6 \times 10^{-13}$, and 8.5×10^{-14} cm^3/s respectively.

These data showed that the amplitude of the predominant unitary current was dependent on the concentration of K^+ ions in the patch pipette.

Five experiments were carried out on *Mercenaria mercenaria* myocytes where the patch and bath solution contained 10 mM TEA and/or 10 mM 4-AP. No effect was observed on the predominant current activity.

Fig II(J), is a graph of the Index $NP_{(open)}$ against the electrode potential done in order to examine the voltage dependence of the channel openings. The channels showed weak

voltage dependence, with an increase in open time at depolarised potentials. However, this was usually only a two fold increase; this is consistent with the term "weak voltage dependence" as discussed by Siegelbaum et al. (1982) and Shuster et. al. (1991). Figure II(K) shows an example of recordings taken at different electrode potentials applied to a patch on a *Mya arenaria* myocyte. There was only a small increase in the probability of channel open-time observed throughout the range from Ve = -40 mV to -100 mV.

In experiments where the voltage was rapidly 'jumped' for 10 second intervals there was little evidence of more current activity in the first 5 seconds of the trace when compared to the final 5 seconds. This also supports the weak voltage dependent characteristic outlined above.

In several of the experiments on myocytes of two species (*Mercenaria mercenaria* and *Geukensia demissa)* smaller unitary currents were apparent when the patch was depolarised with electrode potentials between -80 mV to -120 mV. Since the larger currents described previously were always predominant, I restricted my study to the effects on these currents. It should be noted however that the infrequent, smaller, currents appeared to be more voltage dependent and responded to the elevated K^+ in the electrode in a similar manner. It is postulated that these unitary currents may be similar to the Ca^{++} activated K^+ current (which are voltage dependent) reported in *Lymnaea stagnalis* heart cells, Sigurdson et al. (1987) and in *Helix aspersa* and *Aplysia californica* neurones, Cottrell et al. (1984) and Vandorpe and Morris (1992).

Mya arenaria myocyte

Figures II(G) and II(H)

The same myocyte from *Mya arenaria* was patched twice successfully.

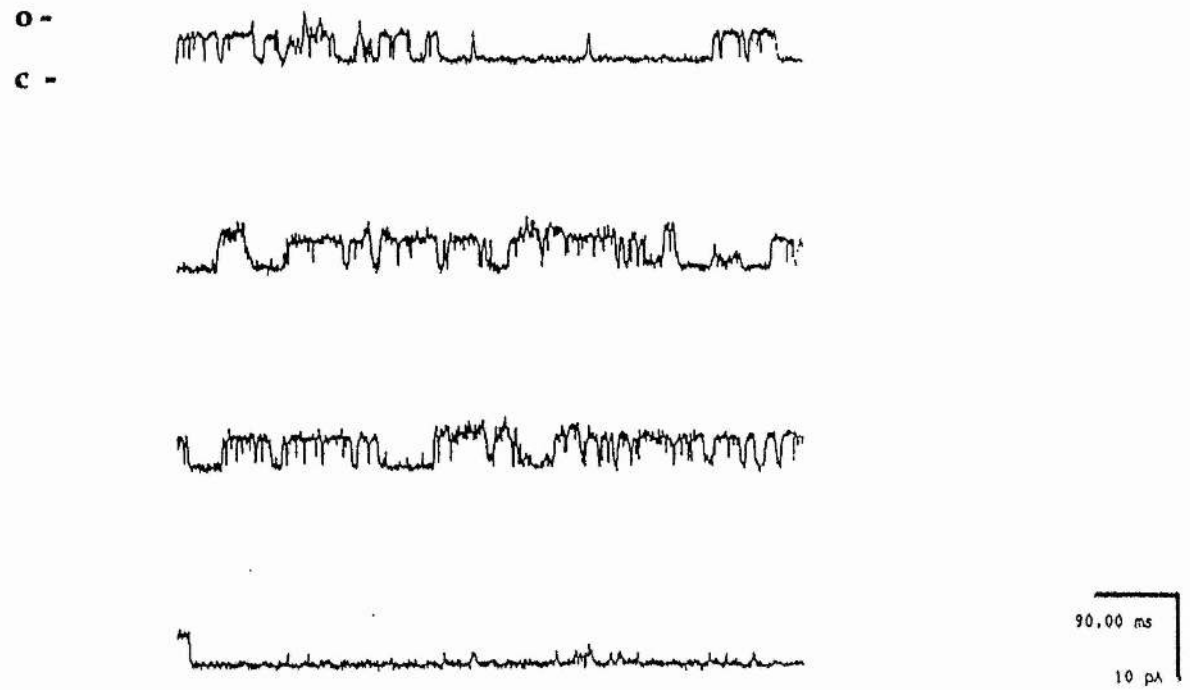
Patch 1. - The patch electrode contained 10 mM potassium.

Patch 2. - The patch electrode contained 100mM potassium.

The recordings in Figures II(G) and (H) show the difference in unitary current activity observed when the potassium content was increased tenfold in the patch electrode.

A single *Mya arenaria* myocyte patched twice

Patch 1: V_e = -40 mV (with 10 mM K in electrode)



Patch 2: V_e = -40 mV (with 100 mM K in electrode)

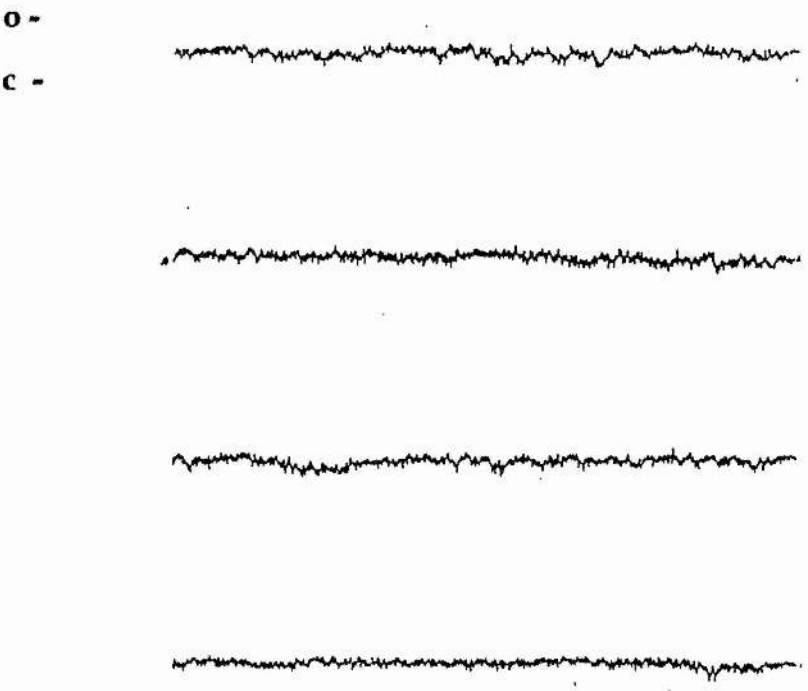


Figure II(G)

A single *Mya arenaria* myocyte patched twice

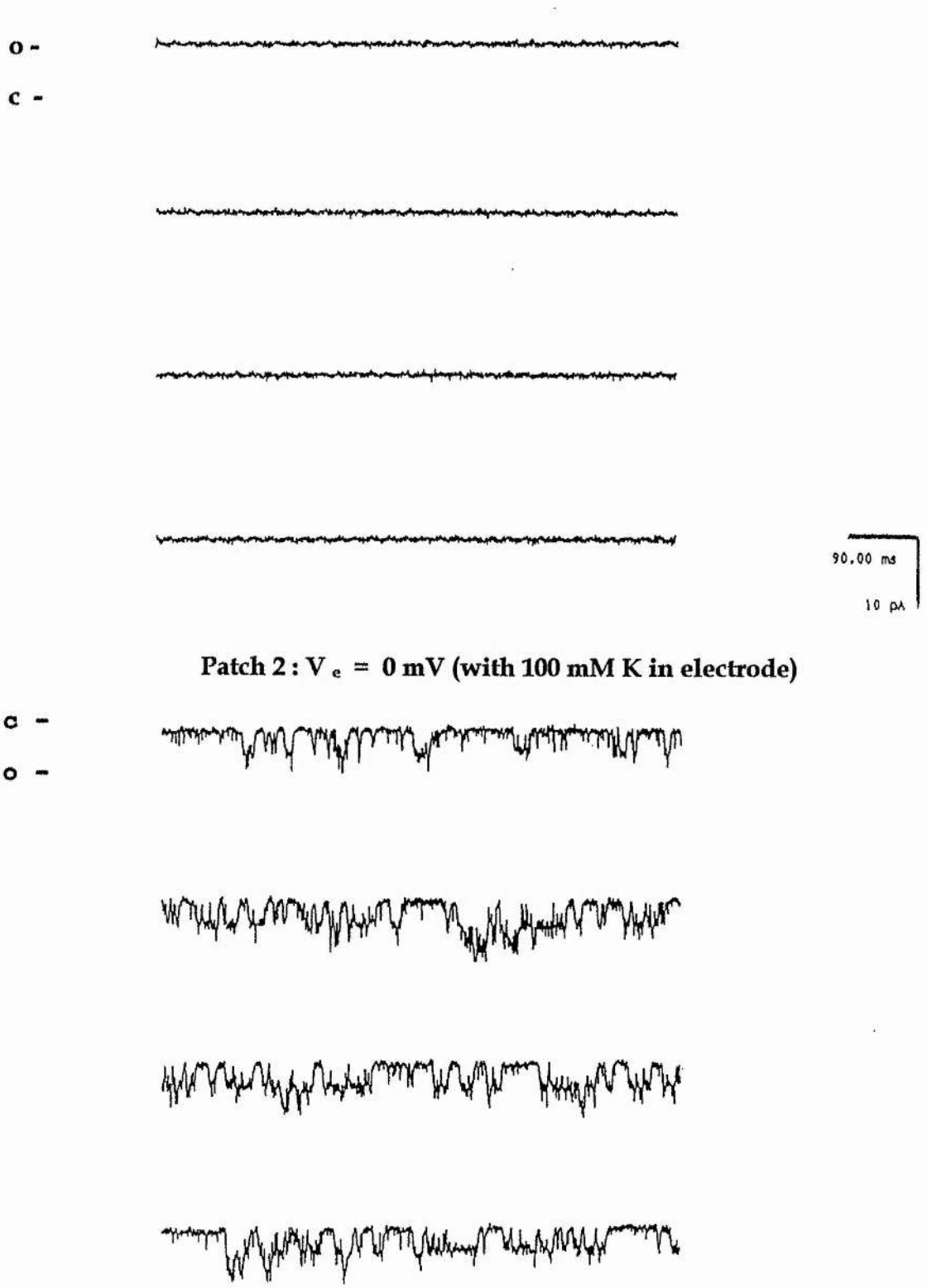


Figure II(H)

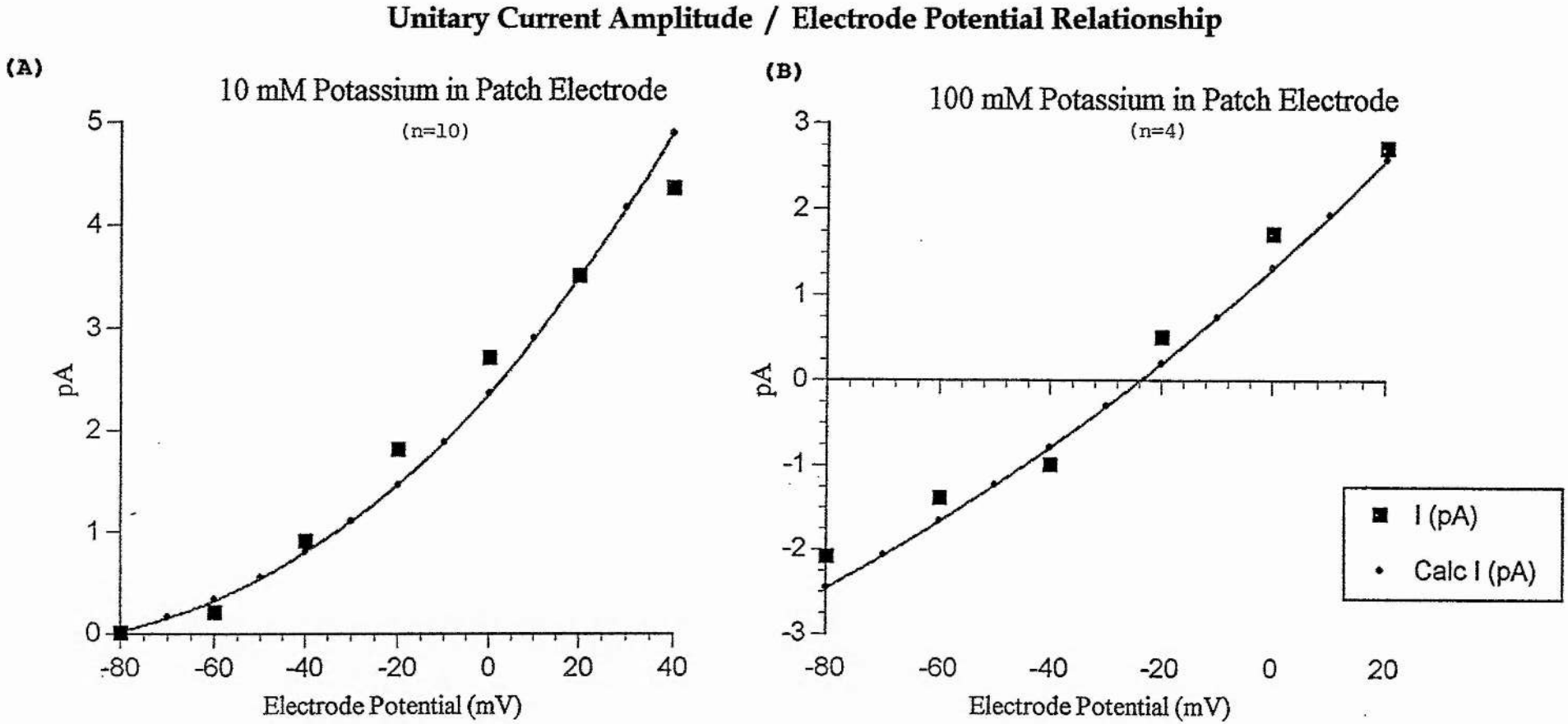


Figure II(i) Current-voltage relationship for unitary currents from *Mya arenaria* myocytes patched with either: (A) 10 mM K^+, or (B) 100 mM K^+ in the patch electrode.
The continuous line (Calc. i(pA)) is fitted by the Constant Field equation (Hodgkin and Katz 1949), assuming an internal potassium concentration of 240 mM. The calculated potassium permeability(P_K) was:
(A) $P_K = 9.6 \times 10^{-13}$ cm^3/s and (B) $P_K = 8.5 \times 10^{-14}$ cm^3/s

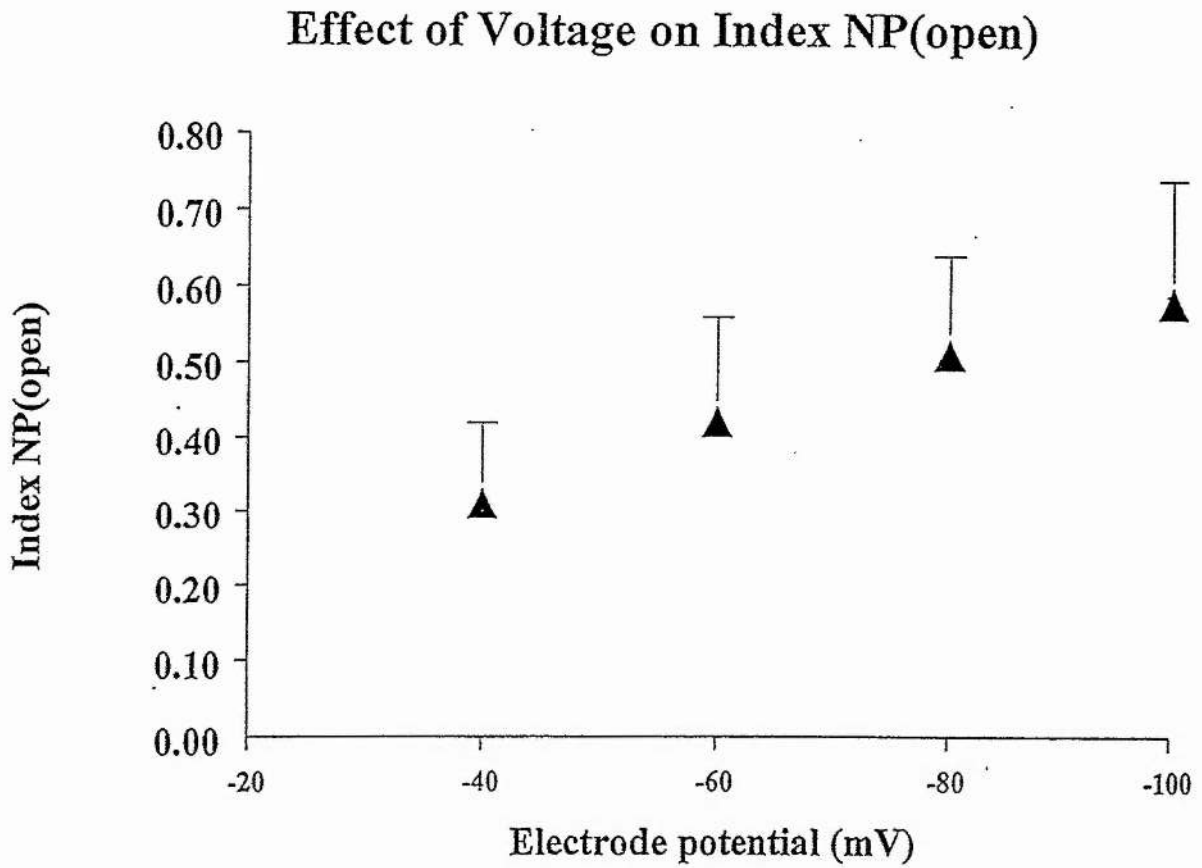


Figure II(J) The voltage dependence of the unitary current activity observed in myocytes was examined. The electrode potential was plotted against the mean Index NP_{open} (+/ S.E.), recorded from *Mercenaria mercenaria* myocytes. The depolarising voltage change produced between V_e = -40 mV to -100 mV, showed only a two fold increase in the probability that the channel would be open.

Example of Patch-clamp recordings made from a single *Mya arenaria* myocyte

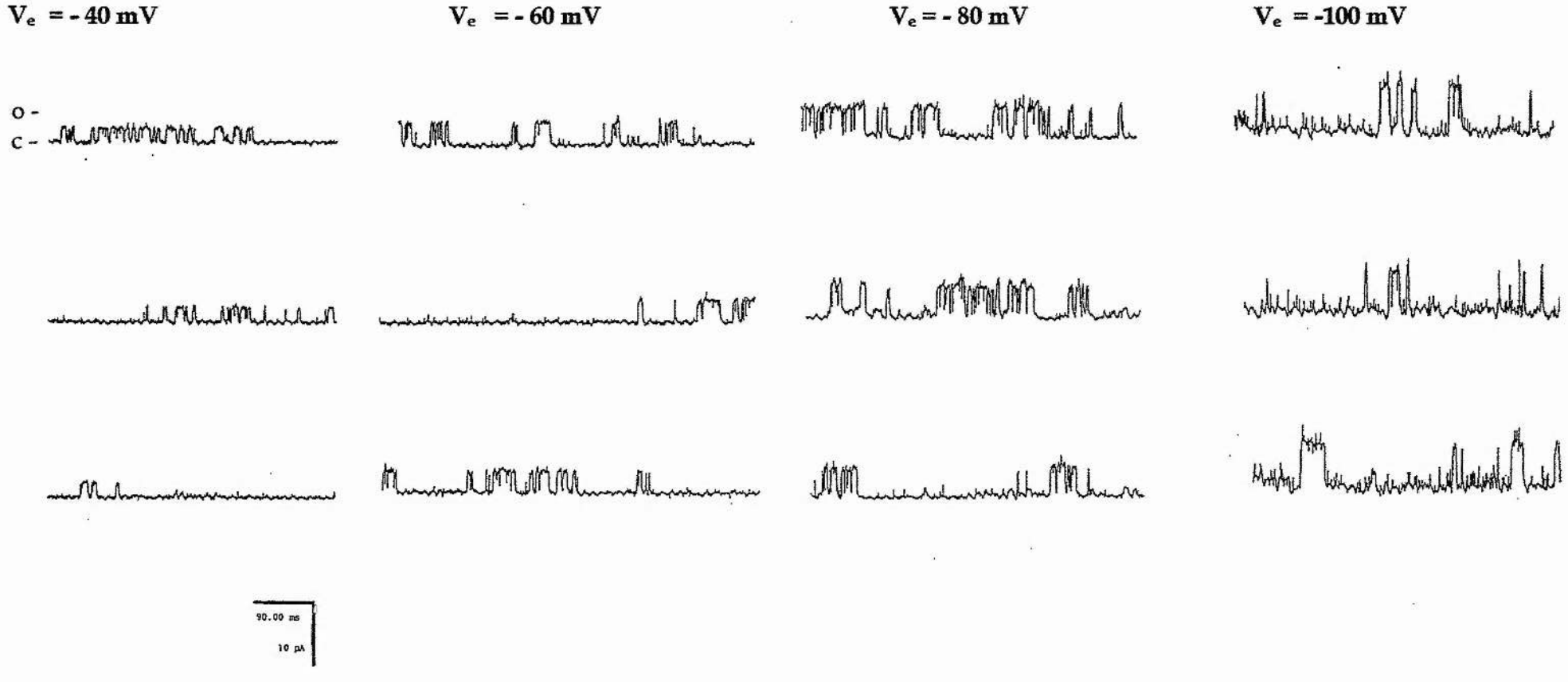


Figure II(K) This figure shows a recording from a *Mya arenaria* myocyte, that demonstrates weak voltage dependence with voltage changes applied to the patch. The unitary current activity does not increase very much over a relatively large electrode potential shift. C = closed state , O = open state

RESULTS - THE ACTION OF 5-HT

All traces shown were recorded at V_e = -80 mV (~ estimated V_m = 25 mV). The drugs were applied from pipettes containing a 10^{-4} M concentration of the drug to be tested and applied near the cell (see the method section).

Figure II(L) shows a graph of the effect of exposure to 5-HT, applied from a pipette containing 10^{-4} M 5-HT, on the unitary current activity of myocytes from 3 species. The current activity was significantly decreased within 3 minutes after 5-HT application to *Mercenaria mercenaria*, *Geukensia demissa* and *Mya arenaria* myocytes.

The recovery phase was recorded 10 minutes after exposure to the drug. In many cases it proved hard to hold a good seal for such extended periods, particularly after application of a drug that was excitatory causing depolarisation of the membrane. Only experiments which lasted to the 10 minute recovery phase were included.

Both *Mya arenaria* and *Mercenaria mercenaria* myocytes showed recovery trends in the unitary current activity by the 10 minute interval; but *Geukensia demissa* myocytes did not show the same recovery trend within this time frame. Traces II(M),(N) and (O) - show examples of individual experiments with 5-HT in the three species.

Figure II(P) shows a trace from one of 4 experiments done on *Trachycardiae* myocytes. 5-HT was seen to cause an increase in unitary current activity in three of the experiments. It was observed that the unitary channel activity appeared to be more voltage dependent. However, because the unitary current characteristics were not investigated in detail in this species, no comparison can be made to the other three species.

THE ACTION OF 5-CT AND 5-MEOT

On exposure to 5-CT or 5-MEOT (10^{-4} M in electrode) there was a marked decrease in unitary current activity, similar to that seen with 5-HT, in both *Mya arenaria* and *Mercenaria mercenaria* myocytes. Figure II(S) shows a recording from one such experiment. However, as seen in the Figures II(Q),(R),(T) and (U), *Geukensia demissa* unitary current activity increased after exposure to either 5-CT or 5-MEOT (at a pipette concentration of 10^{-4} M). Both agonists caused significant increases in unitary current activity.

THE ACTION OF OTHER ANALOGUES

The effect of 8-OH-DPAT was similar to 5-HT in those myocytes tested; interestingly in *Mercenaria mercenaria* the decrease in unitary current activity was noted to persist throughout the recovery period.

Figures in II(V),(W), and (X) show results from experiments in which myocytes from *Mya arenaria, Mercenaria mercenaria* and *Geukensia demissa* were exposed to either Forskolin or 8-Bromo-cAMP. Both Forskolin and 8-bromo-cAMP produced a similar decrease in unitary currents in both *Mercenaria mercenaria* and *Mya arenaria* myocytes. No such effect was evident when tested on myocytes from *Geukensia demissa*. There was a small decrease in unitary current activity seen with 8-bromo-cAMP, but this was not found to be statistically significant.

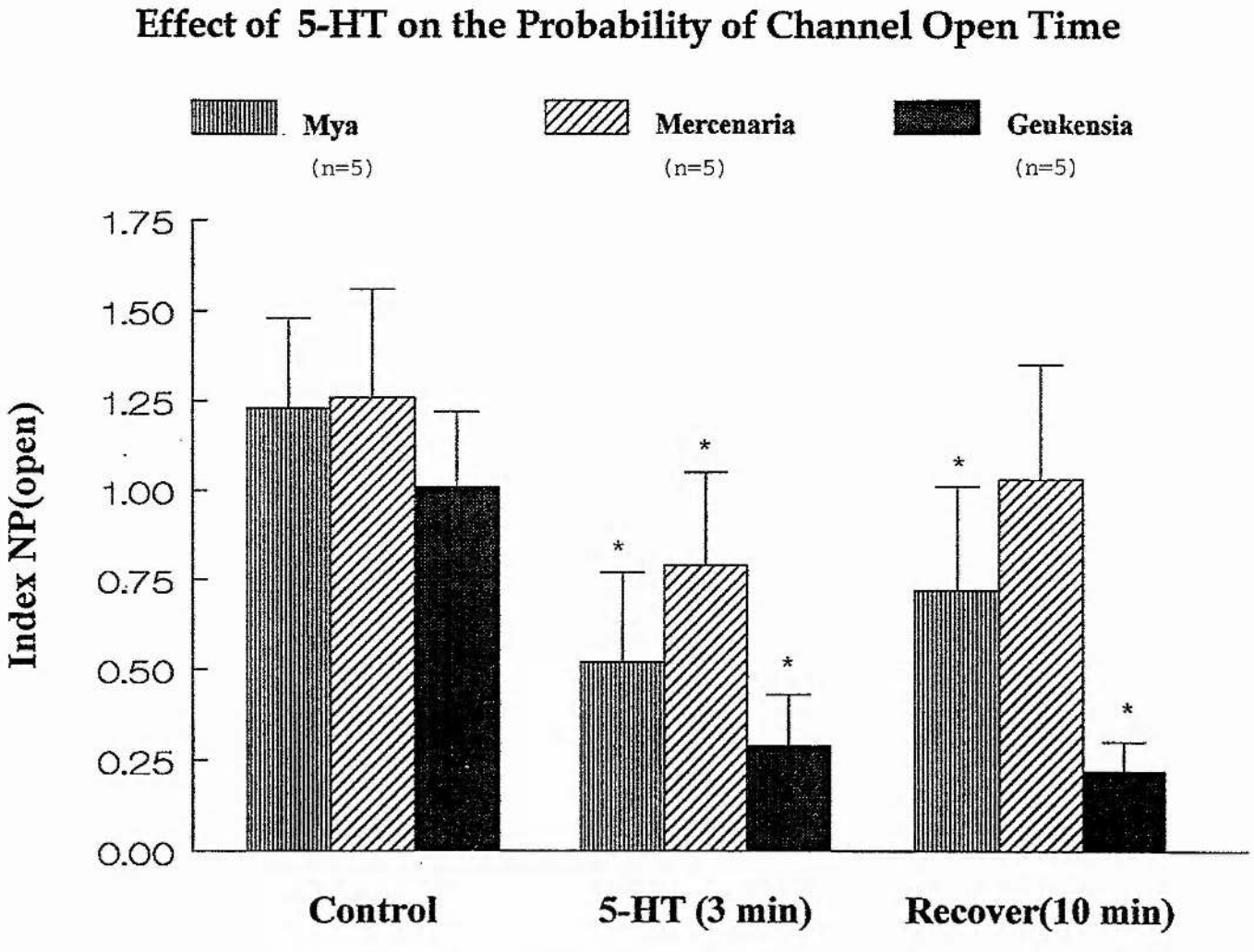


Figure II(L) The bar graph shows the mean Index $NP_{(open)}$ (+ S.E.) from myocytes of three species *Mya arenaria*, *Mercenaria mercenaria* and *Geukensia demissa* exposed to 5-HT. The **asterisks** identify where the difference was significant between the Control and the application of 5-HT (by paired T-Test $p=<0.05$)

Recording from a *Mya arenaria* Myocyte before and after application of 5-HT

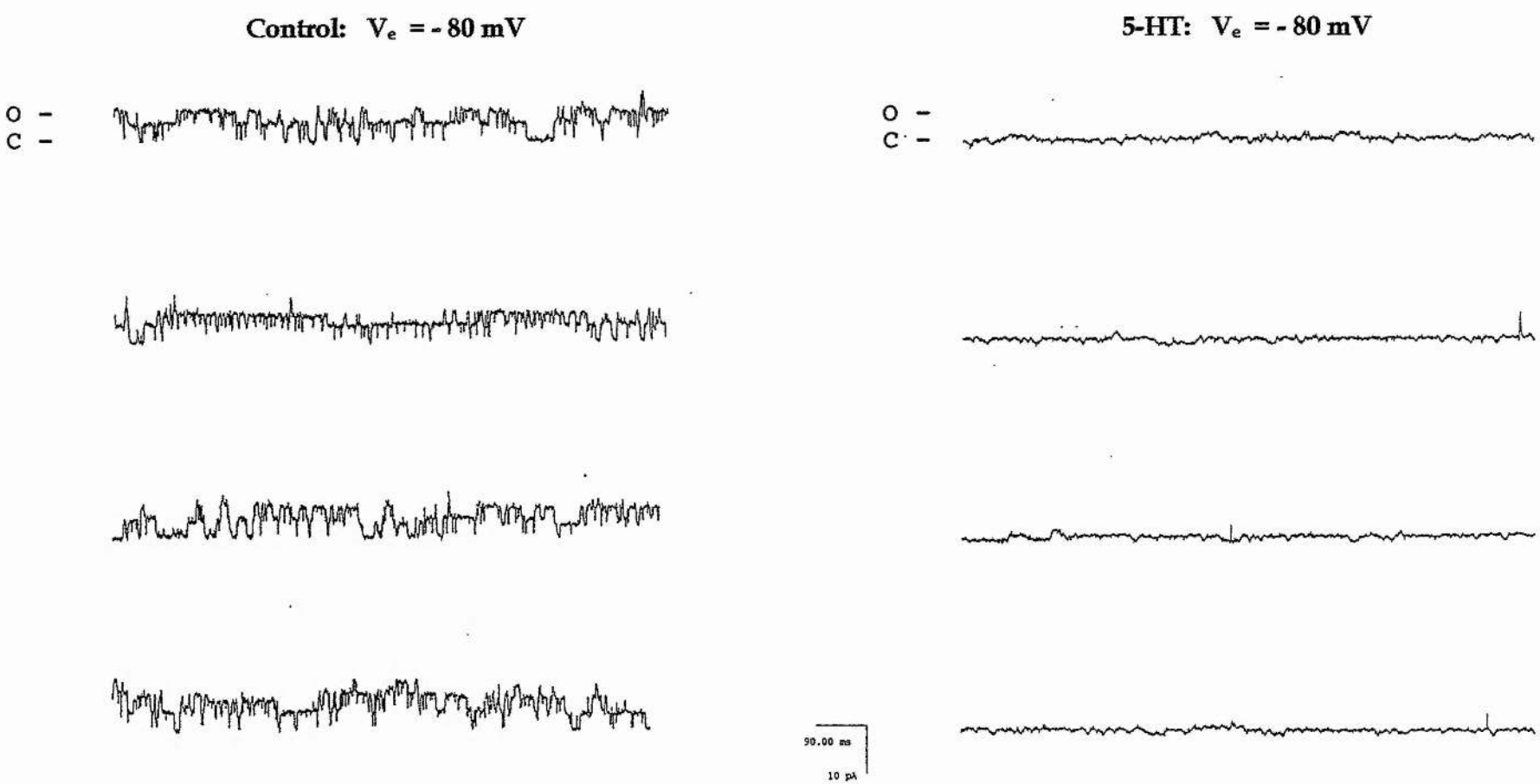


Figure II(M) This recording shows that 3 minutes after 5-HT was applied to this myocyte the unitary current activity was almost zero.

Recording from a *Geukensia demissa* Myocyte before and after application of 5-HT

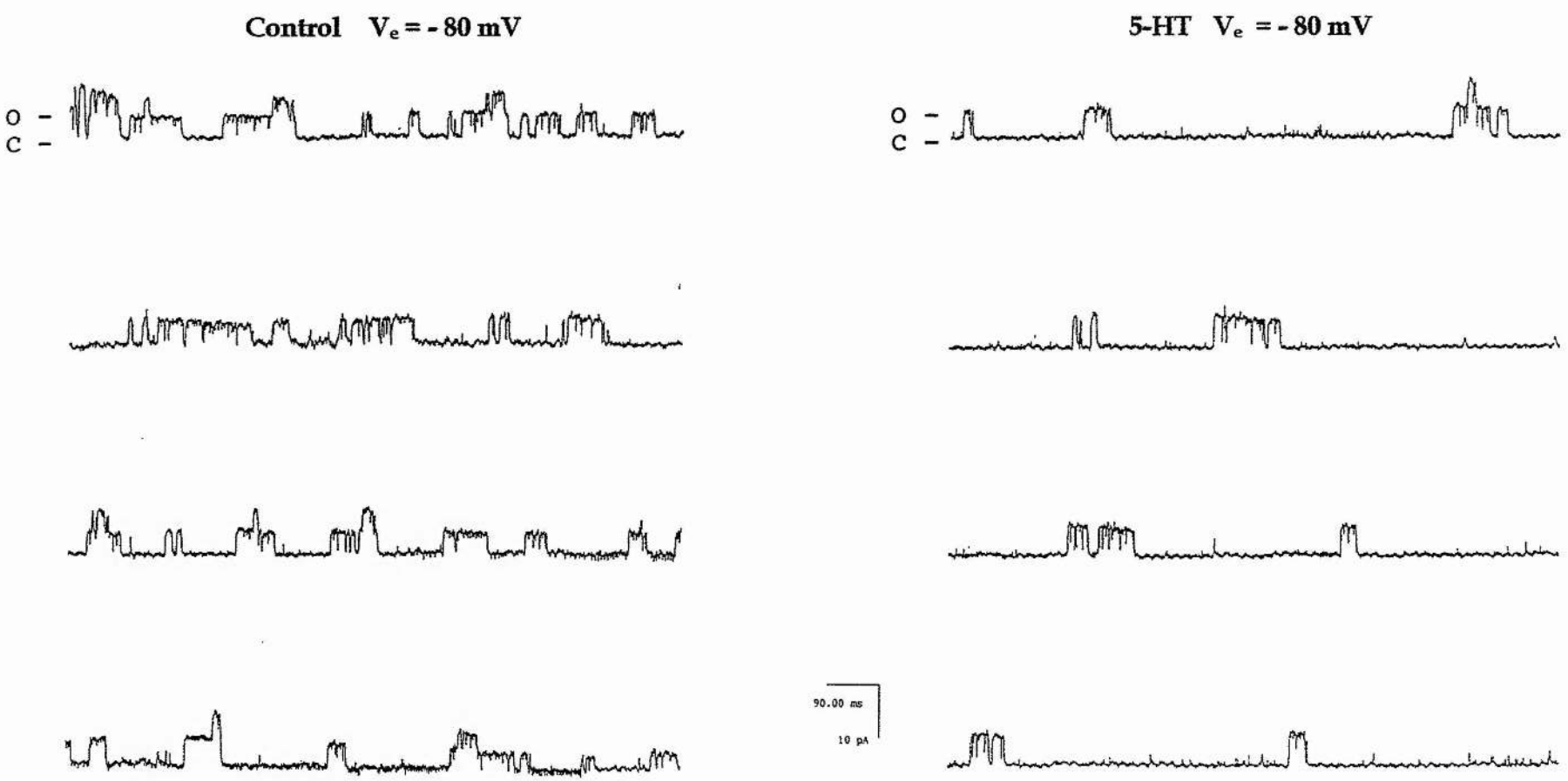


Figure II(N) These recordings show that 3 minutes after 5-HT was applied to this myocyte the unitary current activity was greatly decreased.

Recording from a *Mercenaria mercenaria* Myocyte before and after application of 5-HT

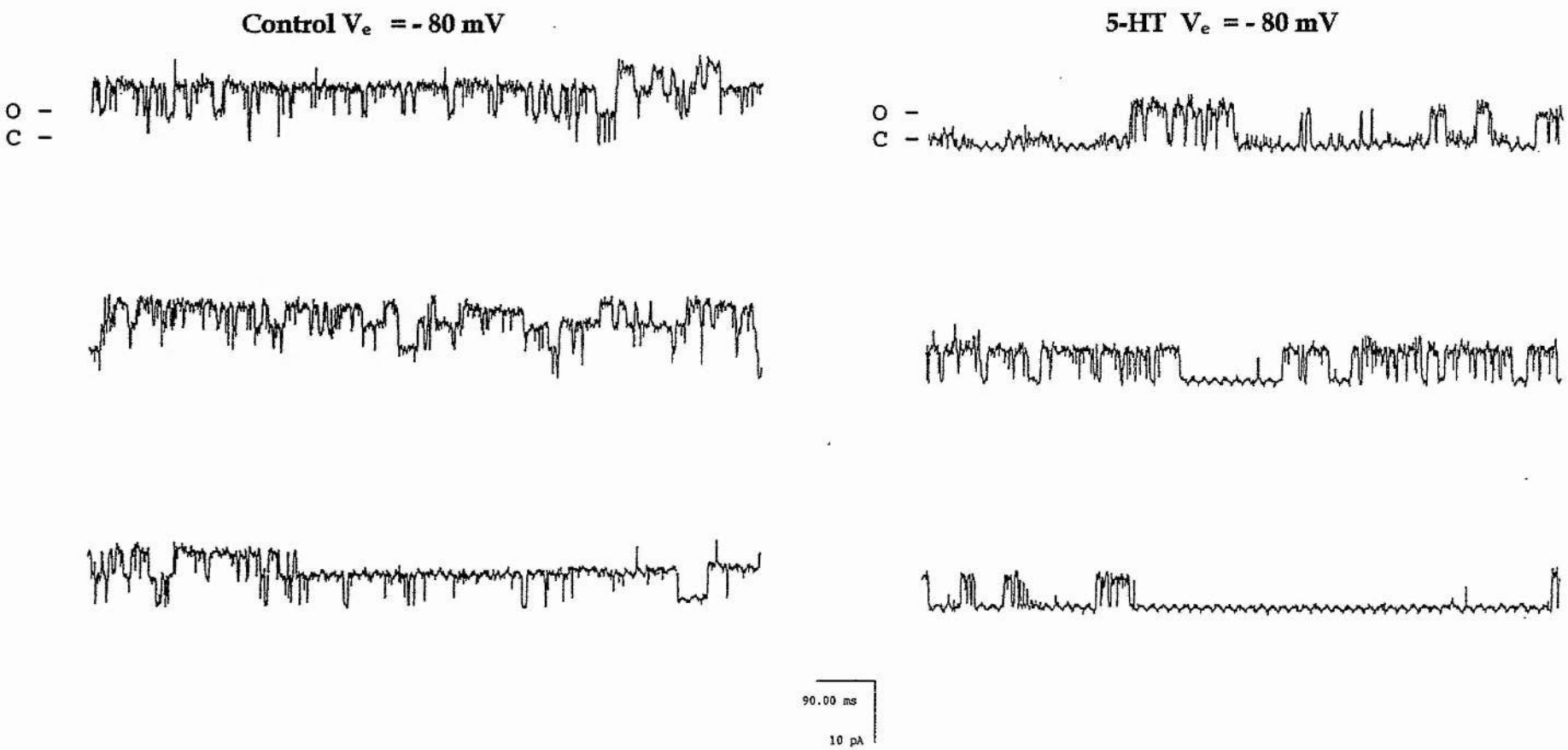


Figure II(O) These recordings show that 3 minutes after 5-HT was applied to this myocyte the unitary current activity was greatly decreased.

Recording from a *Trachycardium egmontianum* Myocyte before and after application of 5-HT

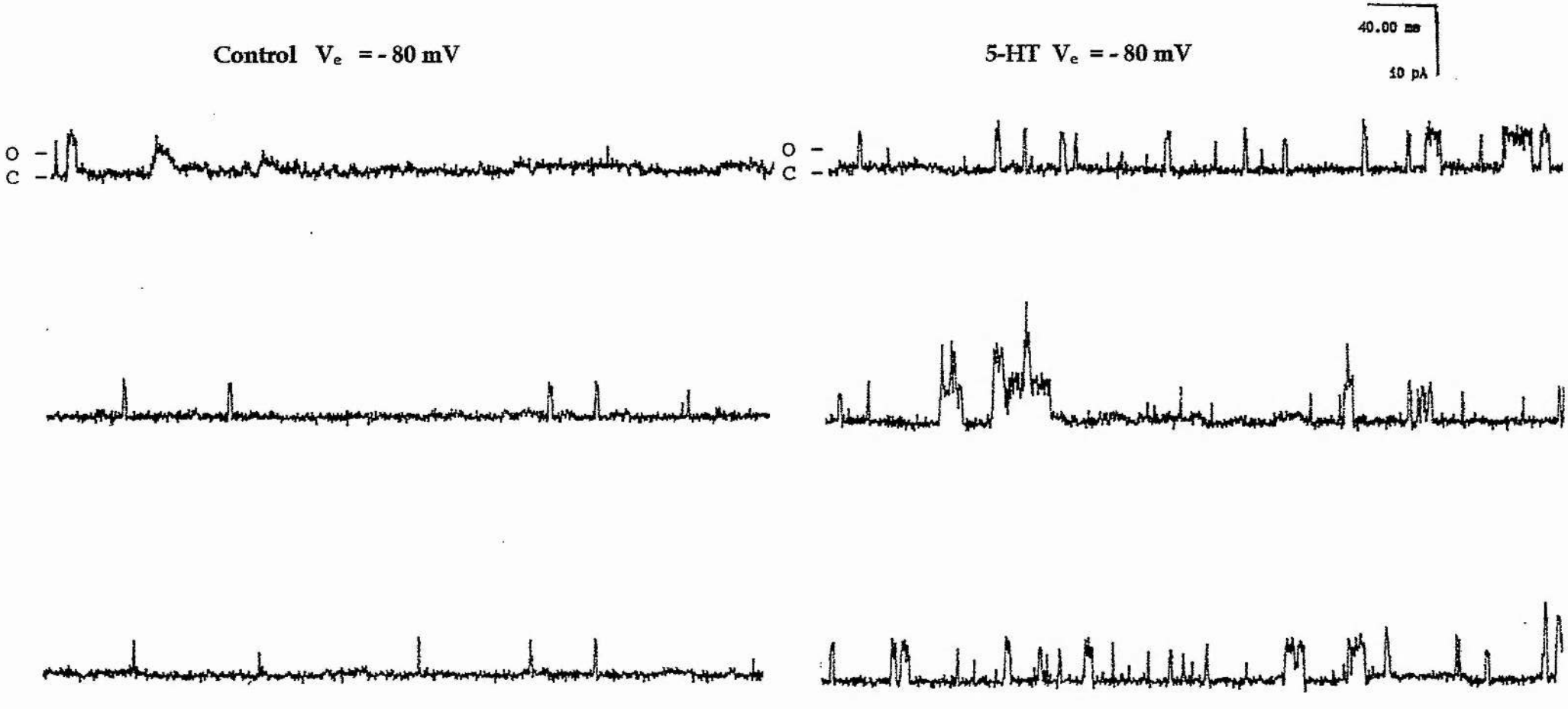


Figure II(P) These recordings show that 3 minutes after 5-HT was applied to this myocyte the unitary current activity was increased. The unitary current activity appeared to be more voltage dependent.

Effect of 5-CT and 5-MEOT on the Channel Open Time in *Geukensia demissa* myocytes

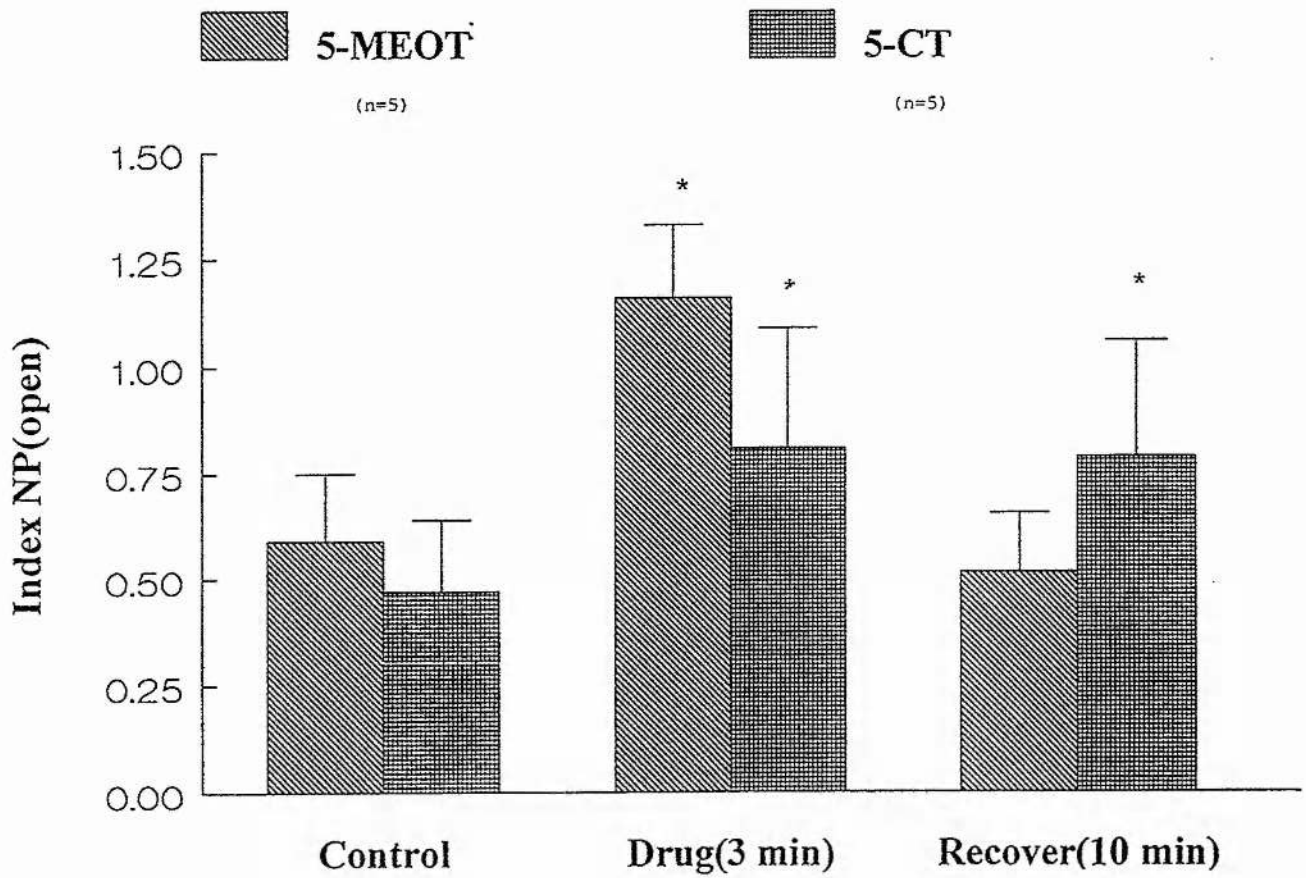


Figure II(Q) The bar graph(+ S.E.) shows data from *Geukensia demissa* myocytes exposed to either 5-CT or 5-MEOT. The **asterisks** identify where the effect was statistically significant (by paired T-test p=<0.05)

Effect of 5-MEOT on the Channel Open Time on *Geukensia demissa* and *Mercenaria mercenaria* myocytes

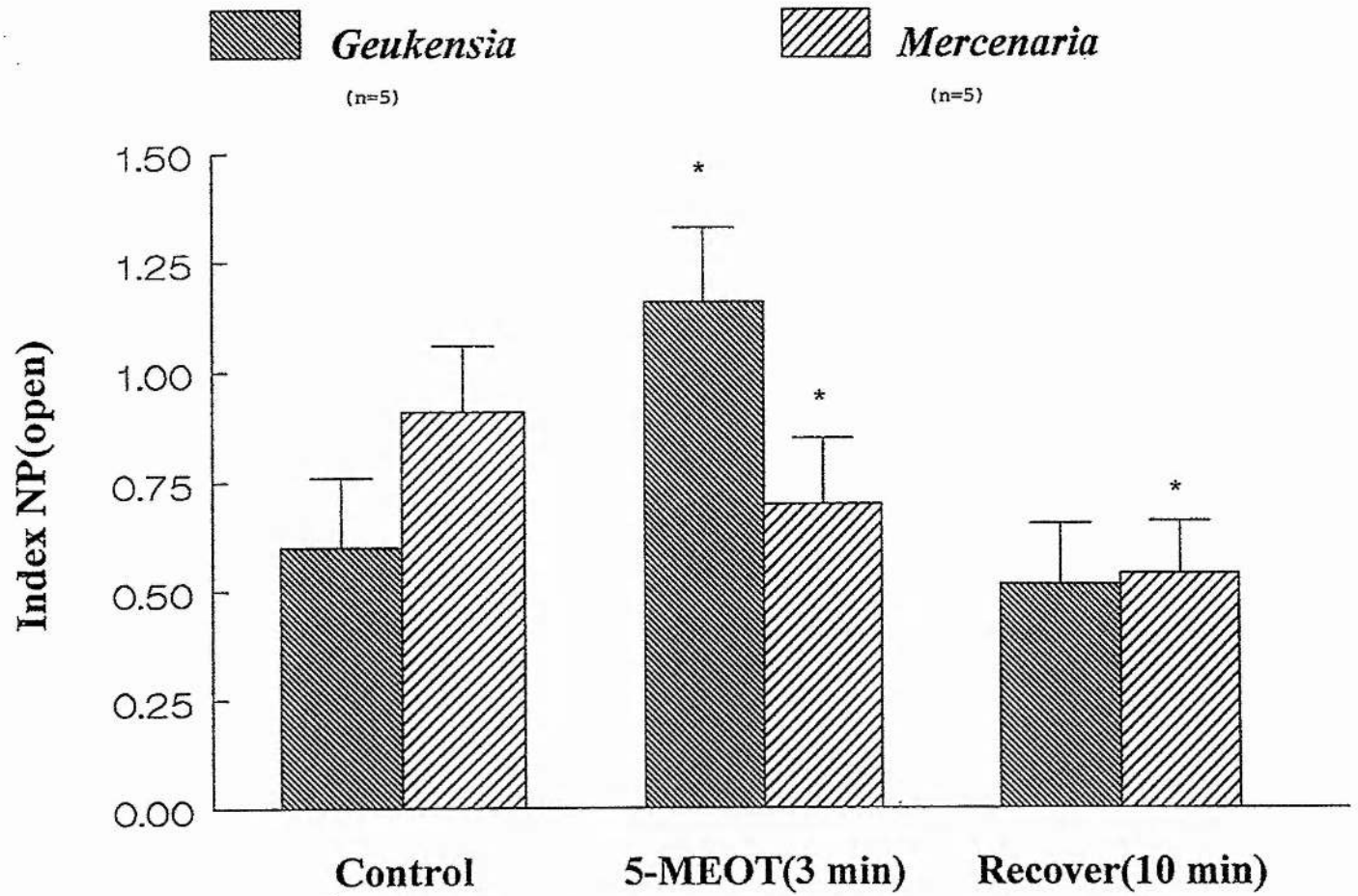


Figure II(R) The bar graph (+ S.E.) shows data from *Geukensia demissa* and *Mercenaria mercenaria* myocytes exposed to 5-MEOT. The **asterisks** identify where the effect was statistically significant(by paired T-test p=<0.05)

Recording from a *Mercenaria mercenaria* Myocyte before and after application of 5-MEOT

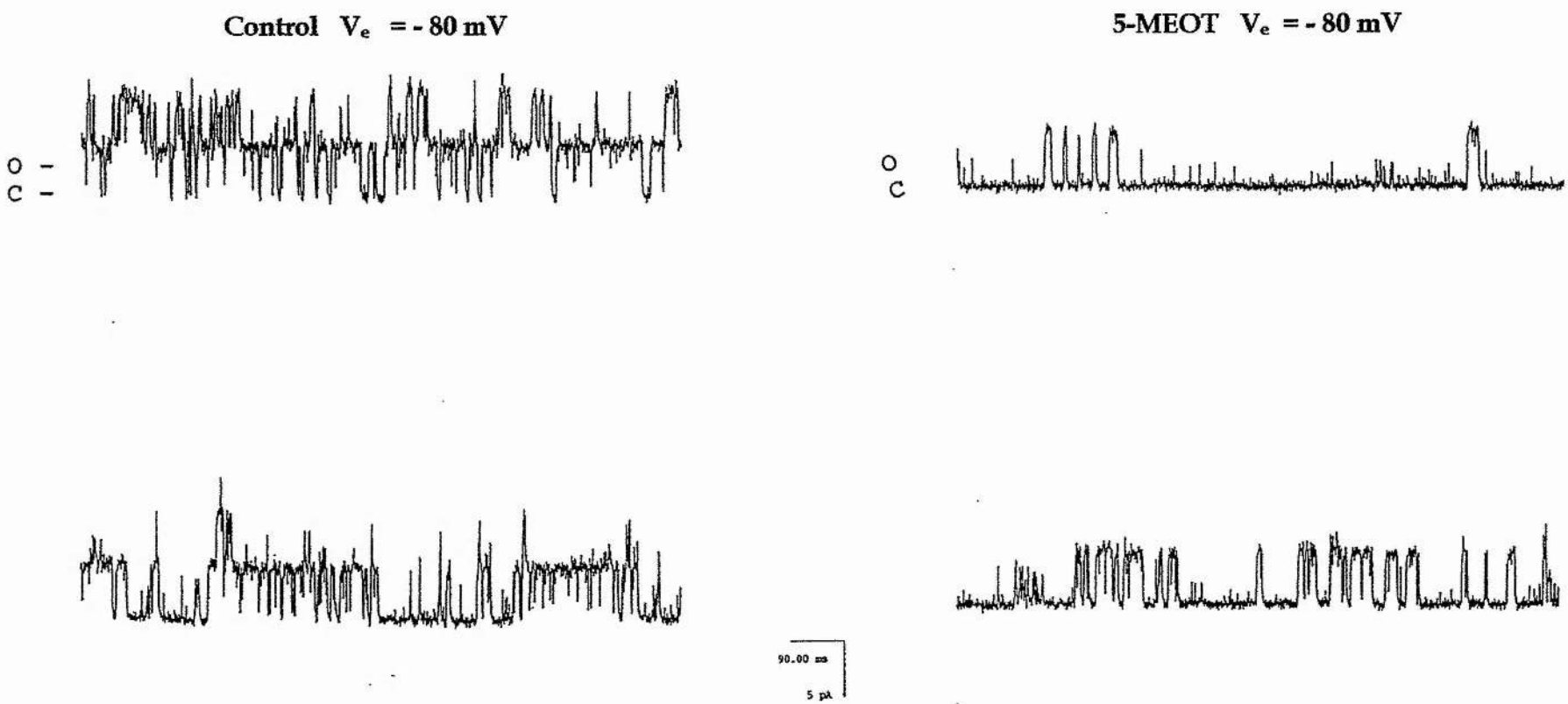


Figure II(S) These recordings show that 3 minutes after 5-MEOT was applied to this myocyte the unitary current activity was greatly decreased.

Recording from a *Geukensia demissa* Myocyte before and after application of 5-MEOT

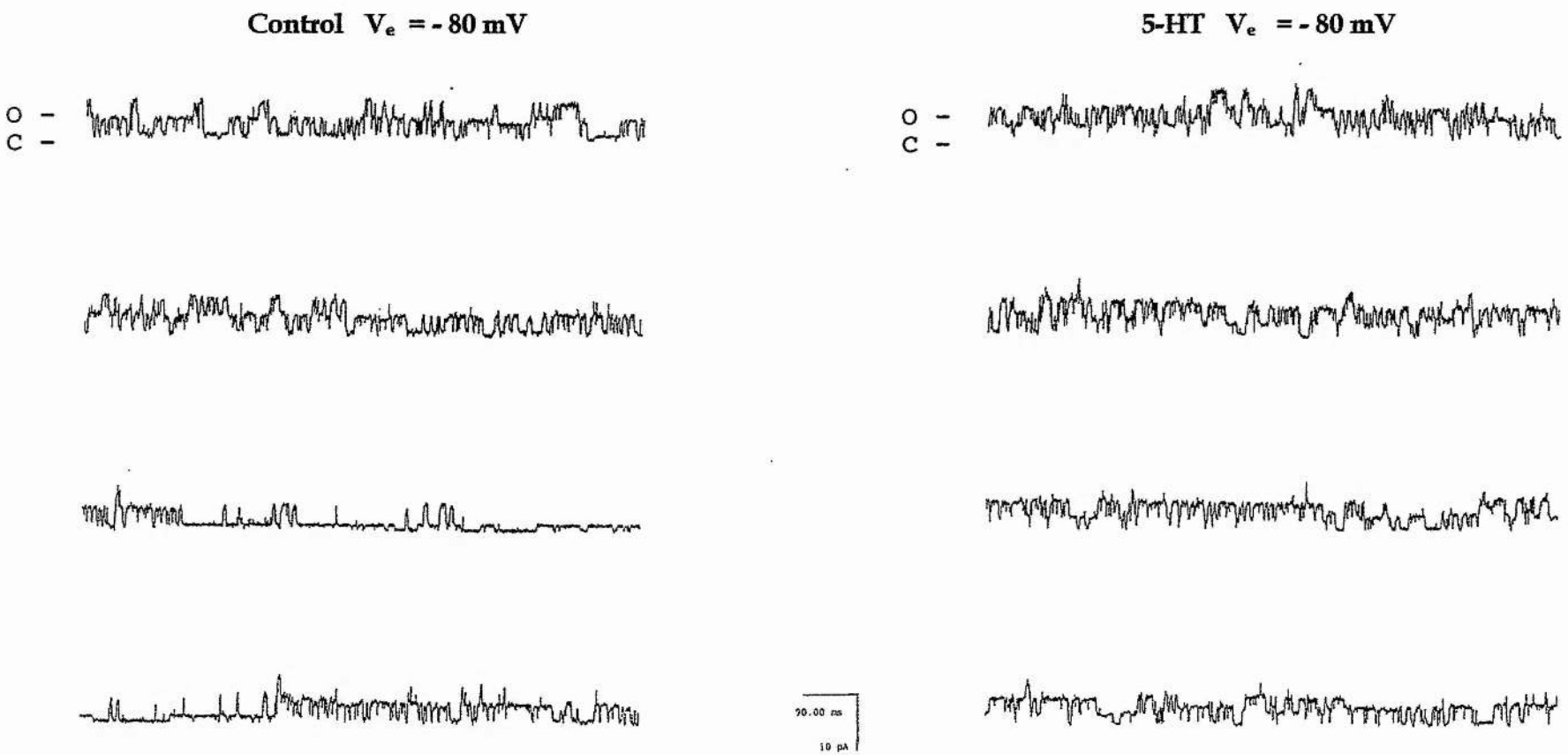


Figure II(T) These recordings show that 3 minutes after 5-MEOT was applied to this myocyte the unitary current activity had increased.

Recording from a *Geukensia demissa* Myocyte before and after application of 5-CT

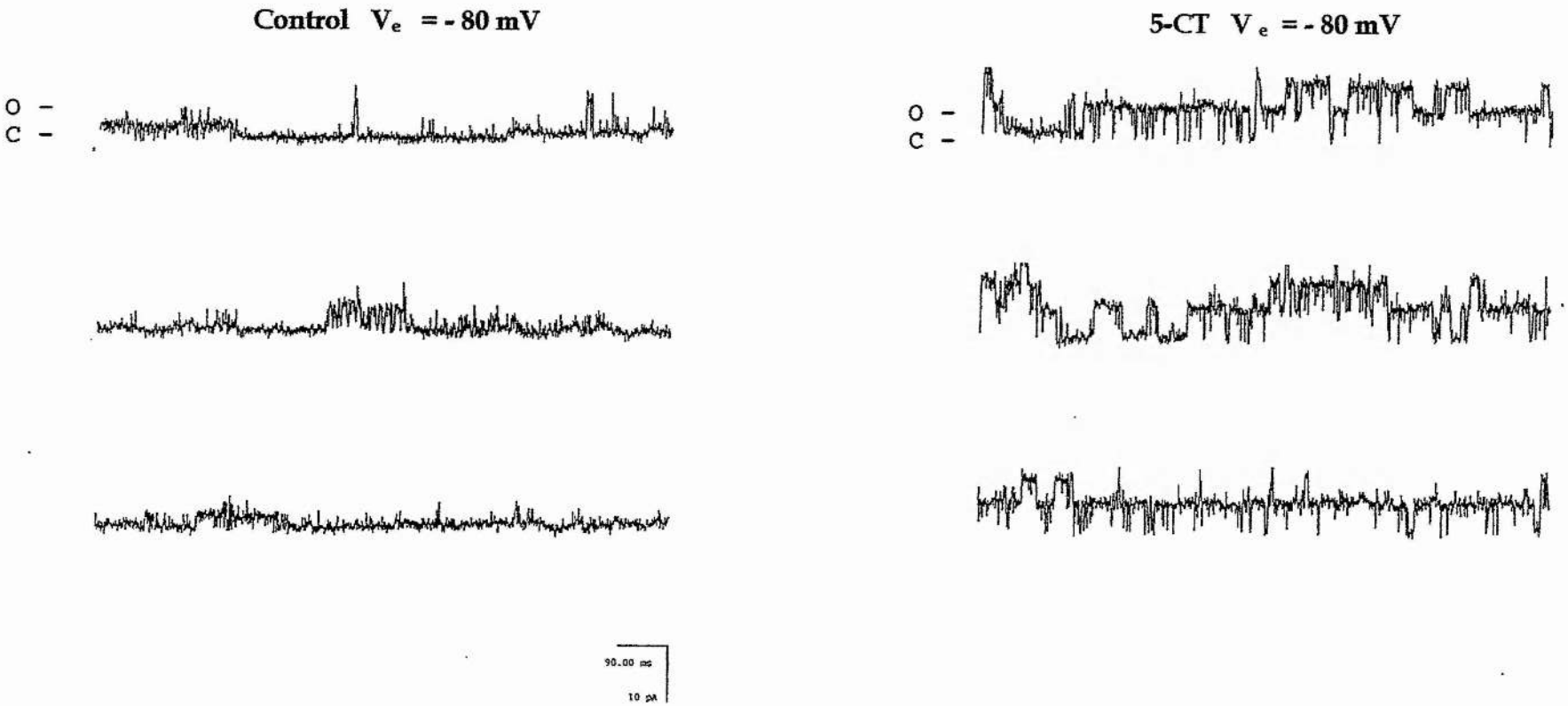


Figure II(U) These recordings show that 3 minutes after 5-CT was applied to this myocyte the unitary current activity had greatly increased.

Recording from a *Geukensia demissa* Myocyte before and after application of Forskolin

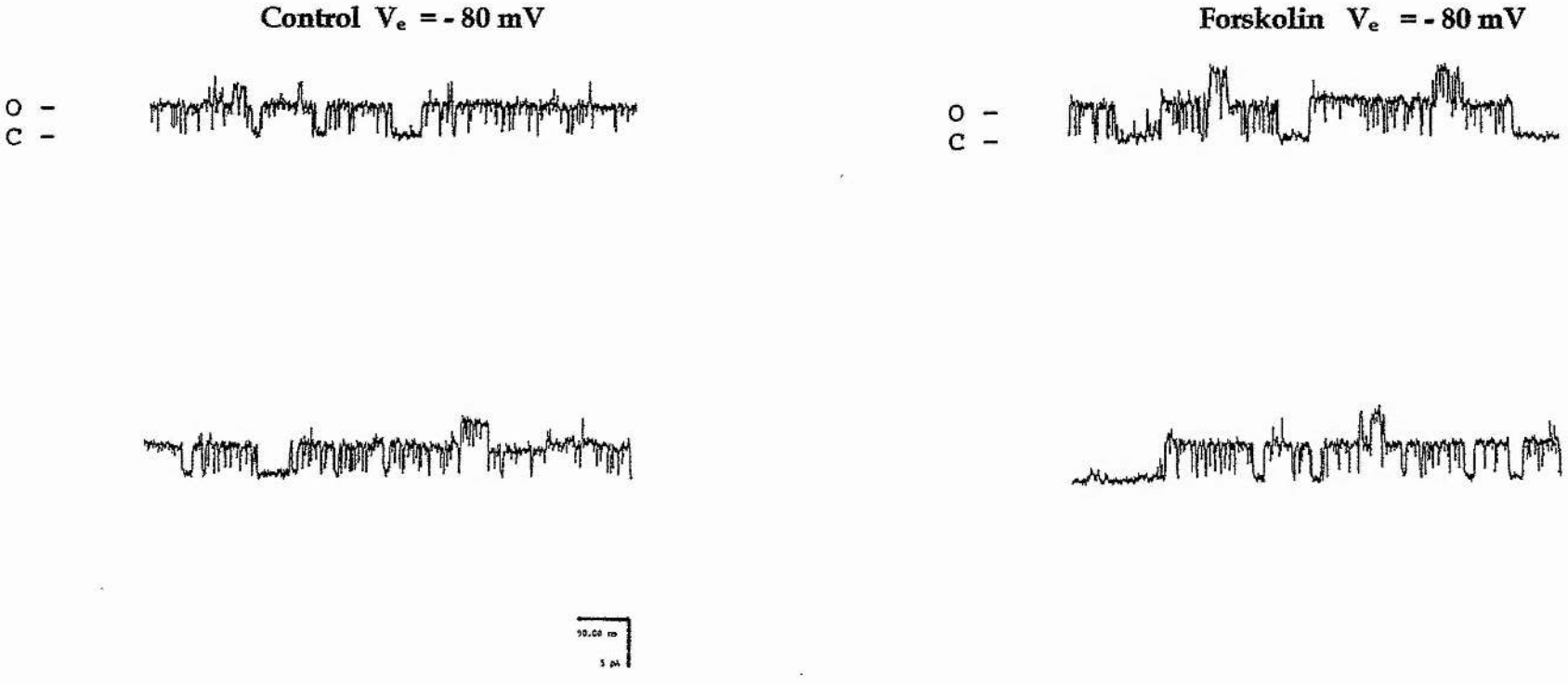


Figure II(V) These recordings show that 5 minutes after Forskolin was applied to this myocyte the unitary current activity had not been significantly effected.

Recording from a *Mercenaria mercenaria* Myocyte before and after application of Forskolin

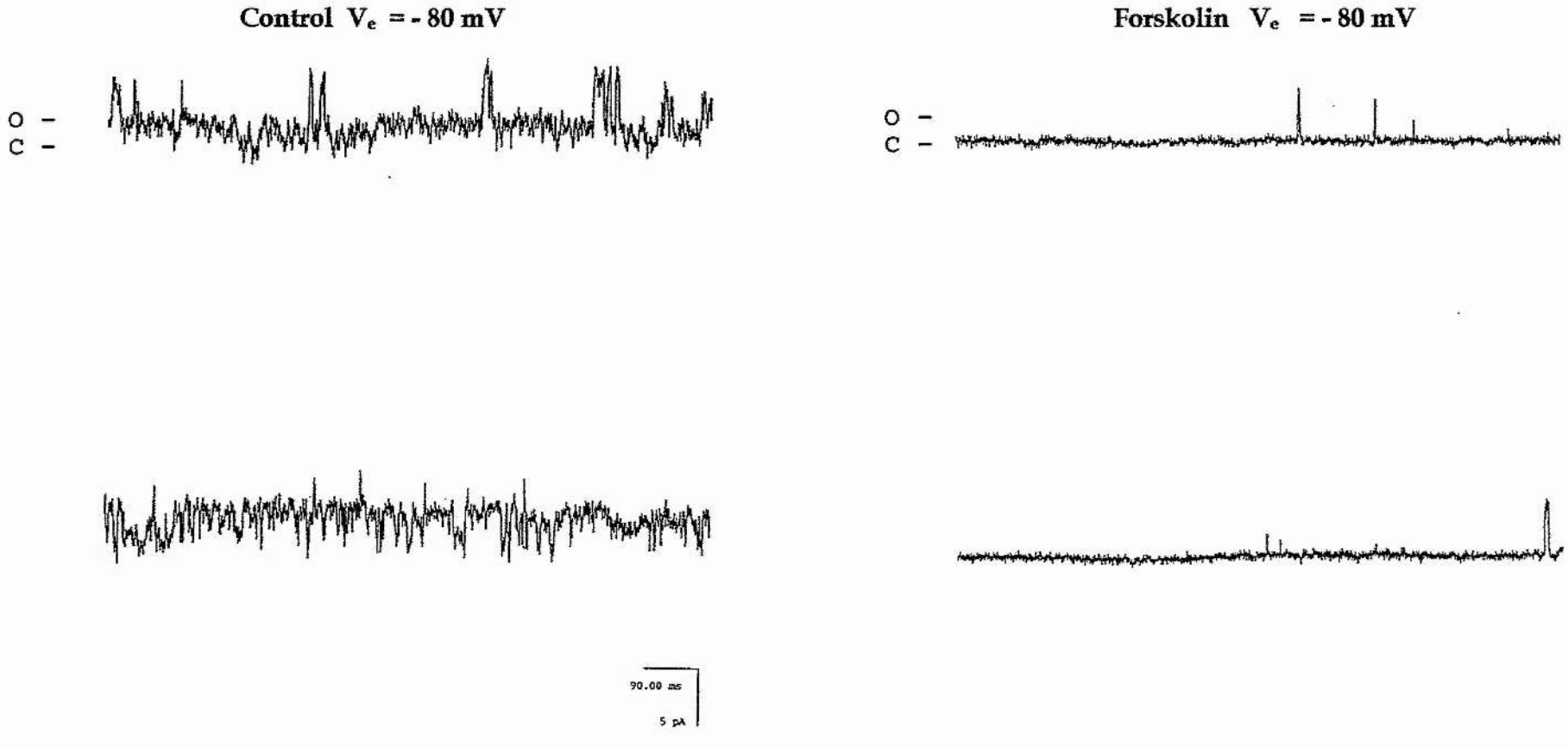


Figure II(W) These recordings show that 5 minutes after Forskolin was applied to this myocyte the unitary current activity was greatly decreased.

Figure II(X)

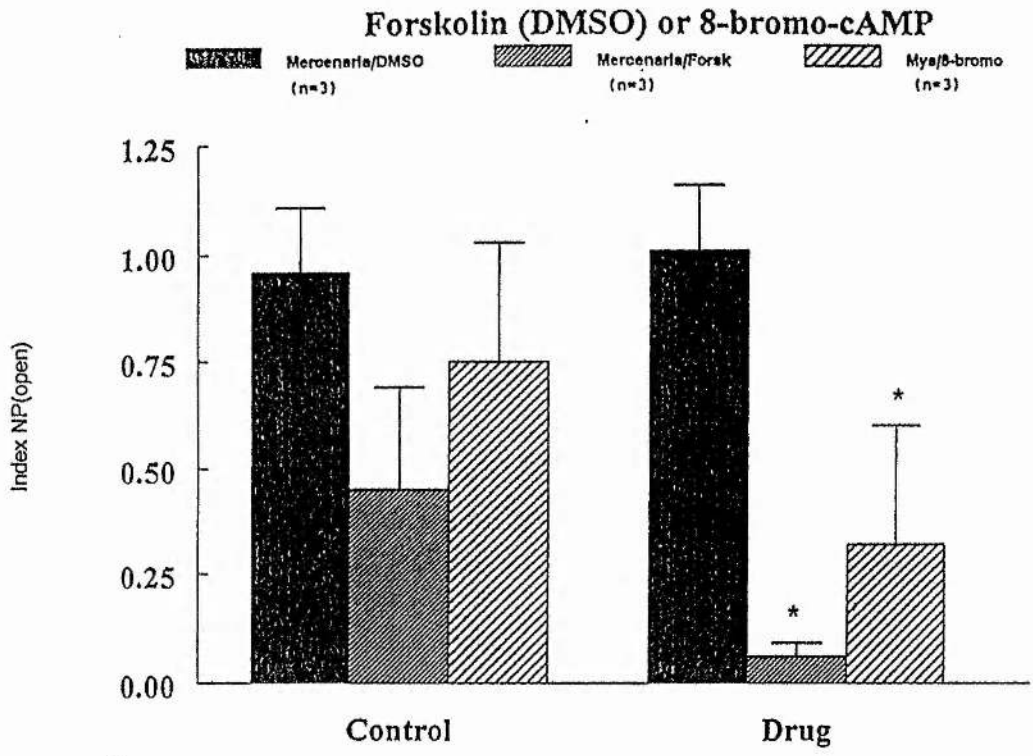
The two bar graphs (A) and (B) show the effect of elevating intracellular cAMP by applying two agents, Forskolin or 8-bromo-cAMP, on to the myocytes from three species.

(A) The effect of these agents on the probability of channel open-time recorded from *Mercenaria mercenaria* and *Mya arenaria* myocytes. The **asterisks** identify where the difference was statistically significant by paired T-test, between control and after the drug application ($p=<0.05$). Note: because Forskolin was initially dissolved in DMSO (before further dilution), several control experiments were run with only diluted DMSO.

(B) The effect on *Geukensia demissa* myocytes exposed to either Forskolin or 8-bromo-cAMP was not statistically significant, however there was a trend with 8-bromo-cAMP showing a slight decrease in unitary current activity.

Effect of Elevation of Intracellular cAMP on the Channel Open Time

Graph (A) **cAMP elevating Drugs on Index NP(open)**



Graph (B) **cAMP elevating Drugs on Index NP(open)**

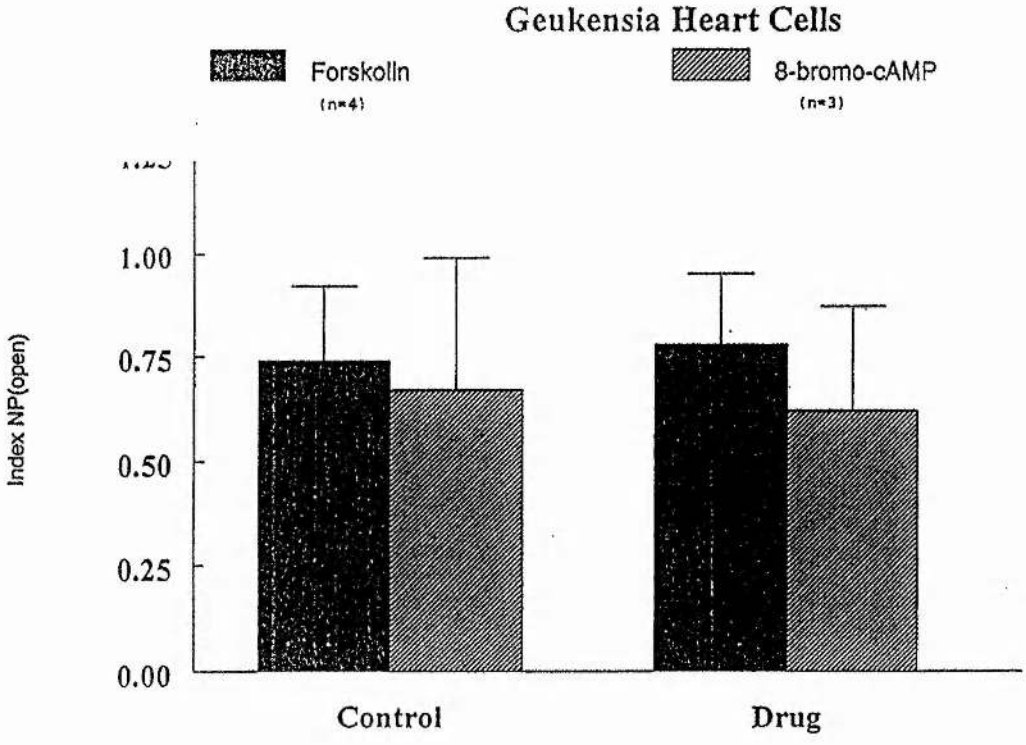


Table 11

Summary of Effects of 5-HT and Analogues on K^+ Channel Activity

Drug	*Mya arenaria*	*Mercenaria mercenaria*	*Geukensia demissa*	*[Trachycardiae]*
5-HT	closed	closed	closed	opened
5-CT	closed	closed	opened	not tested
5-MEOT	closed	closed	opened	not tested
Forskolin	closed	closed	no effect	not tested
8-bromo-cAMP	closed	closed	no effect	not tested

Table 11: Note the different effect of 5-HT and analogues on the *Geukensia demissa* myocytes, as well as the difference in the action of two agents which are known to elevate cAMP.

DISCUSSION - PART II

CHANNEL CHARACTERISTICS

I have summarised below the characteristics of the K^+ channel I identified in myocytes. The predominant current was demonstrated to be due to passage of K ions.

The reversal potential was estimated to be ~ 70-75 mV. This coincides closely to the cellular resting potential.

The K^+ channel activity contributed to the resting potential of the cell was demonstrated by the fact that K^+ current activity was recorded, with elevated K^+ in the pipette electrode (100 mM K^+) and Ve = 0 mV, therefore Vm = resting potential.

The K^+ channel was weakly voltage dependent and remained active throughout extended periods of depolarisation over a range of different voltage clamp potentials applied to the membrane patch.

The initial level of channel activity observed decreased and stabilised after approximately 5 minutes following the seal formation, suggesting the possibility of an initial response to the stretching of the membrane.

Neither 4AP or TEA added into the patch electrode and the bathing ASW appeared to affect the K^+ current activity.

This predominant K^+ current observed in the cell attached preparations of the myocytes has properties similar to the S-K^+ channel reported in *Aplysia californica* neurones, Siegelbaum et al. (1982, 1989), and the SAK^+ channel described in *Lymnaea stagnalis* myocytes and neurones by Brezden et al. (1986), Sigurdson et al. (1987) and Vandorpe and Morris (1992).

THE ACTION OF 5-HT ON THE K^+ CHANNEL ACTIVITY

The K^+ unitary current activity in the *Mya arenaria, Mercenaria mercenaria* and *Geukensia demissa* myocytes was consistently decreased by exposure of the cell to 5-HT. The decrease in activity caused by 5-HT was statistically significant in the three species. K^+ current activity returned within 10 minutes in myocytes from *Mercenaria mercenaria* and *Mya arenaria*, but this recovery was slower in *Geukensia demissa* myocytes.

Thus 5-HT was shown to modify the unitary current activity of a K^+ channel in the hearts of several species of *bivalvia*. These observations correlate with findings on a similar channel found in neurones from other molluscs, Siegelbaum et al.(1982).

Unitary current activity did increase with 5-HT in 3 out of 4 *Trachycardiae* patches but experiments were too few and the properties of the unitary currents were not investigated in this species. However, this observation, as will be discussed in the final section, fits with observations on these species which showed a predominantly inhibitory response to 5-HT in the screening study done by Painter and Greenberg (1982).

THE ACTION OF 5-CT AND 5-MEOT ON THE K^+ CHANNEL ACTIVITY

The K^+ current activity was significantly decreased by either 5-CT or 5-MEOT in both *Mya arenaria* and *Mercenaria mercenaria* myocytes similar to the effect of 5-HT. However, the K^+ current activity was increased significantly by 5-CT and 5-MEOT in *Geukensia demissa* myocytes, whereas the effect of 5-HT had decreased the same K^+ current activity. This suggests the possible presence of two 5-HT receptor subtypes.

EFFECT OF AGENTS THAT INFLUENCE cAMP LEVELS

A significant decrease in K^+ current activity, seen in response to 5-HT, was produced in both *Mercenaria mercenaria*

and *Mya arenaria* myocytes by exposure to Forskolin, or 8-Bromo-cAMP. Either agent is known to increase intracellular cAMP. As discussed in the Introduction, an elevation of cAMP levels in molluscan hearts by exposure to 5-HT has been reported in *Mercenaria mercenaria,* Higgins (1977) and *Aplysia californica,* Drummond et al. (1985). The closure of a K^+ channel by 5-HT in an isolated patch, which is mimicked by elevating intracellular cAMP levels, suggests that the mechanism of this action of 5-HT is likely via cAMP intracellular pathways.

The K^+ channel discussed above is an active component of the resting membrane potential of these molluscan cells and the gating of this channel appears to be modulated via second messenger pathways, one of which includes cAMP.

However, in *Geukensia demissa* the K^+ current activity appeared to be modulated by a pathway <u>not</u> connected to elevated cAMP. Neither cAMP elevating drugs caused an effect which mimicked the response to 5-HT. This correlates with the findings of Higgins (1977), who was unable to detect any alteration of cAMP levels in this species in response to 5-HT.

The predominant *Geukensia demissa* K^+ current activity which was decreased by 5-HT and was increased by two analogue agonists 5-MEOT and 5-CT appears to be effected by a different second messenger pathway.

5-HT ACTION ON MAMMALIAN HEART K^+ CHANNELS

Of interest to me was the work by Kaumann et al. (1990). They have reported that 5-HT caused the closure of a K^+ channel in isolated mammalian (guinea pig) heart cells and this effect was linked to the elevation of intracellular cAMP levels. The action of 5-HT on one of the cardiac K^+ channels appeared to

have similarities to the effect of 5-HT on the *bivalvia* myocytes I described, Kaumann (personal communication 1991).

One of the 5-HT receptors described in mammalian hearts has now been classified as a 5-HT_4 receptor. This group of receptors are reported to act by causing an increase in cAMP activity, which then modifies activity of one or more type of K^+ channel. The possibility that a 5-HT_4 receptor may also have a direct G-protein mediated coupling which could directly cause closure of potassium channels has been proposed by Ford and Clarke (1993).

SUMMARY

It will be important to try to define the different receptor characteristics further in the hearts of more species of bivalves. Newer analogues used with a combination of different patch clamp methodologies could identify many of the intracellular mechanisms. With this information and the protein sequencing data on the receptor(s) structure, the nature of the different receptors will be further defined.

However, it should be pointed out that there is still a great deal of work to be done in the future on the classification of the mammalian 5-HT receptors in the heart. Recently several new analogues and antagonists have become available as described by Hoyer et al. (1994), which should prove useful tools for the pharmacological definition of these receptors.

TABLE 12 - A Combination Table of all of the Results from Part I & II

Species Part 1&11	K^+ channel ps(10 mM)	K^+ channel ps(100mM)	5-HT hearts	5-HT Myocytes	5-CT hearts	5-CT Myocytes	5-MEOT hearts	5-MEOT Myocytes	Forskolin Myocytes	8-Bromo Myocytes
Mya arenaria	38 ps	55 ps	excite	channel ⇓	excite	channel ⇓	excite	channel ⇓	channel ⇓	channel ⇓
Mercenaria mercenaria	38 ps	55 ps	excite	channel ⇓	excite	channel ⇓	excite	channel ⇓	channel ⇓	channel ⇓
Geukensia demissa	38 ps	55 ps	inhibit/mix	channel ⇓	inhibit	channel ⇑	inhibit	channel ⇑	no effect	no effect
Saxidomus giganteus			inhib/excite		inhibit		excite			
Trachycardiae	30ps		inhib/excite	channel ⇑	inhibit					

FINAL DISCUSSION

EFFECT OF 5-HT ON A SINGLE MYOCYTE COMPARED TO THE WHOLE HEART

In Table 12, I have summarised the results obtained from the different species of *bivalvia* extensively studied in Part I and/or Part II. This discussion will focus on responses to 5-HT and analogues recorded from the whole heart, as compared to single cell data recorded after application of the same compounds directly to dissociated myocytes.

The correlation between the effect of 5-HT which was predominantly excitatory on both *Mya arenaria* and *Mercenaria mercenaria* hearts, to the closing of a K^+ channel in the cell attached patch preparation of a single myocyte was clearly demonstrated. This is the first time this 5-HT effect on the hearts of these two species has been linked by direct evidence to the closing of a specific K^+ channel.

SIMILARITY OF S-K^+ AND SAK^+ CHANNELS TO THE MYOCYTE K^+ CHANNEL

The characteristics of the S-K^+ channel in *Aplysia californica* neurones and the stretch SAK^+ channel in the hearts of *Lymnaea stagnalis* were outlined in the Introduction. The strong similarities between the S-K^+ and the SAK^+ channel has been reviewed by Vandorpe and Morris (1992).

The properties of the K^+ channel which I identified were discussed in the Results and Discussion sections of Part II. This K^+ channel demonstrated similar properties to both the S-K+ and the SAK^+ channels: similar conductance, sensitivity to membrane stretching, weakly voltage dependent and a unitary current activity which contributes to the resting potential of the cell. Of note was the finding that the K^+ channel activity was decreased by 5-HT in the myocytes, similar to the S-K+ channel in *Aplysia* neurones, Siegelbaum et al. (1992).

The effect of 5-HT observed on the myocytes must be initiated via intracellular transmitter(s) as the drugs were applied to the cell membrane outside the patch. Evidence linking the action of 5-HT to the closure of this channel by elevation of intracellular cAMP levels was shown in two of the species (see Results Part II). This is a similar finding to that described in the S-K^+ channel from *Aplysia californica* neurones, Siegelbaum et al.(1982).

ACTION OF 5-HT ON *Mya arenaria* AND *Mercenaria mercenaria*

In comparing data from the whole heart studies with that on single myocytes there is good correlation between both my findings and the literature. 5-HT and analogues were predominantly excitatory on the whole hearts which correlated to the closure of a K^+ channel by 5-HT on the myocytes from both species. The action of 5-HT on the molluscan heart has been linked to an elevation of intracellular cAMP in the literature, as discussed in the Introduction, Higgins (1977). I showed that agents which elevate cAMP mimicked the effect of 5-HT on the K^+ channel in myocytes. This fits with the literature and the S-K^+ channel properties discussed earlier.

ACTION OF 5-HT AND ANALOGUES ON *Geukensia demissa*

Data recorded from the *Geukensia demissa* whole hearts were complex; although the response to 5-HT was usually mixed with inhibition at higher doses, this was often accompanied by some increase of heart rate. But the analogues α-Me-5-HT, and 8-OH-DPAT were found to produce only excitation, without any inhibition. The effect of 5-HT on the myocytes from this species also showed closure of the K+ channel similar to *Mya arenaria* and *Mercenaria mercenaria*. However this channel closure was not mimicked in *Geukensia demissa* by Forskolin or

8-bromo-cAMP which presumably elevate intracellular cAMP. This observation also correlates with the findings of Higgins (1974,1977), who reported finding no elevation of the intracellular cAMP in response to 5-HT in this species.

The two analogues 5-CT and 5-MEOT actually increased the K^+ current activity quite markedly; this was not mimicked by elevation of intracellular cAMP either. Therefore, the responses to 5-HT, or it's analogues, is likely caused by the activation of different intracellular pathways independent of cAMP. Perhaps the mechanism by which this K^+ channel in *Geukensia demissa* is activated is more similar to the FMRFamide effect described in *Aplysia californica* neurones by Volterra (1990). FMRFamide was shown to cause an increase in the open time of a K^+ channel (with similar characteristics) by activation of cAMP independent intracellular pathways.

CLASSIFICATION OF 5-HT RECEPTOR SUBTYPES

In Diagram 3 I have modified a figure first presented by Hen (1992), which was further refined by Saudou and Hen in 1994. This diagram identifies some of the known and proposed 5-HT receptor subtypes and their intracellular (transduction) pathways taken from vertebrate and invertebrate literature. I have incorporated the data from my observations on the different *bivalvia* species studied. The diagram identifies by question marks where there are unknown pathways or receptor types not clearly understood, or fully defined.

The diagram also illustrates the importance of knowing all aspects of the membrane structure and the intracellular cellular pathways involved in receptor activation. This is essential in order to have an accurate classification as well

as a full understanding of the function of the different receptors.

Perhaps, in the examination of the known G-protein coupled 5-HT receptors and as new ones are identified, a variety of methods could be employed to study these receptors. Along with the different patch-clamp methods available, certain of the new G-protein toxins would provide useful selective blocking tools to further examine the different G-protein couplings. It is hoped that these methods may help to provide further insight into the different mechanisms, either intracellular pathways or the direct action on ion channels, activated by G-protein coupled 5-HT receptors.

The importance of the correlation of pharmacological data, receptor structures, functional characteristics and intracellular pathways has recently been reviewed from the perspective of an ''evolutionary view of drug-receptor interactions of the bioamine receptor family'' by Vernier et al. (1995). This group used the example of bioamine G-protein coupled receptors to show how knowing the evolutionary history of receptors can help to understand some of the inconsistencies seen when we attempt to characterise receptors using only pharmacological or molecular methods.

5-HT RECEPTORS IN THE HEARTS OF BIVALVES

In reviewing my observations reported in Part I and Part II, I will try to define some of the common characteristics of the 5-HT receptors found in the hearts of *bivalvia*. I have separated the responses of the hearts to 5-HT and other analogues in *Mya arenaria* and *Mercenaria mercenaria,* into one group, calling this **Group 1.**

Diagram 3.

This diagram was modified from Saudou and Hen (1994)and shows the fully identified or proposed 5-HT receptors* and their known effector systems.

* - I incorporated the $5\text{-HT}_{biv1,2,?}$ receptors in order to try to summarise possible similarities to already identified receptors.

* - In Hoyer et al. (1994) the receptor terminology was slightly different, i.e. 5_{ht5}, 5_{ht6} and 5_{ht7}. Structural, or intracellular pathway data has still to be fully identified for these receptors.

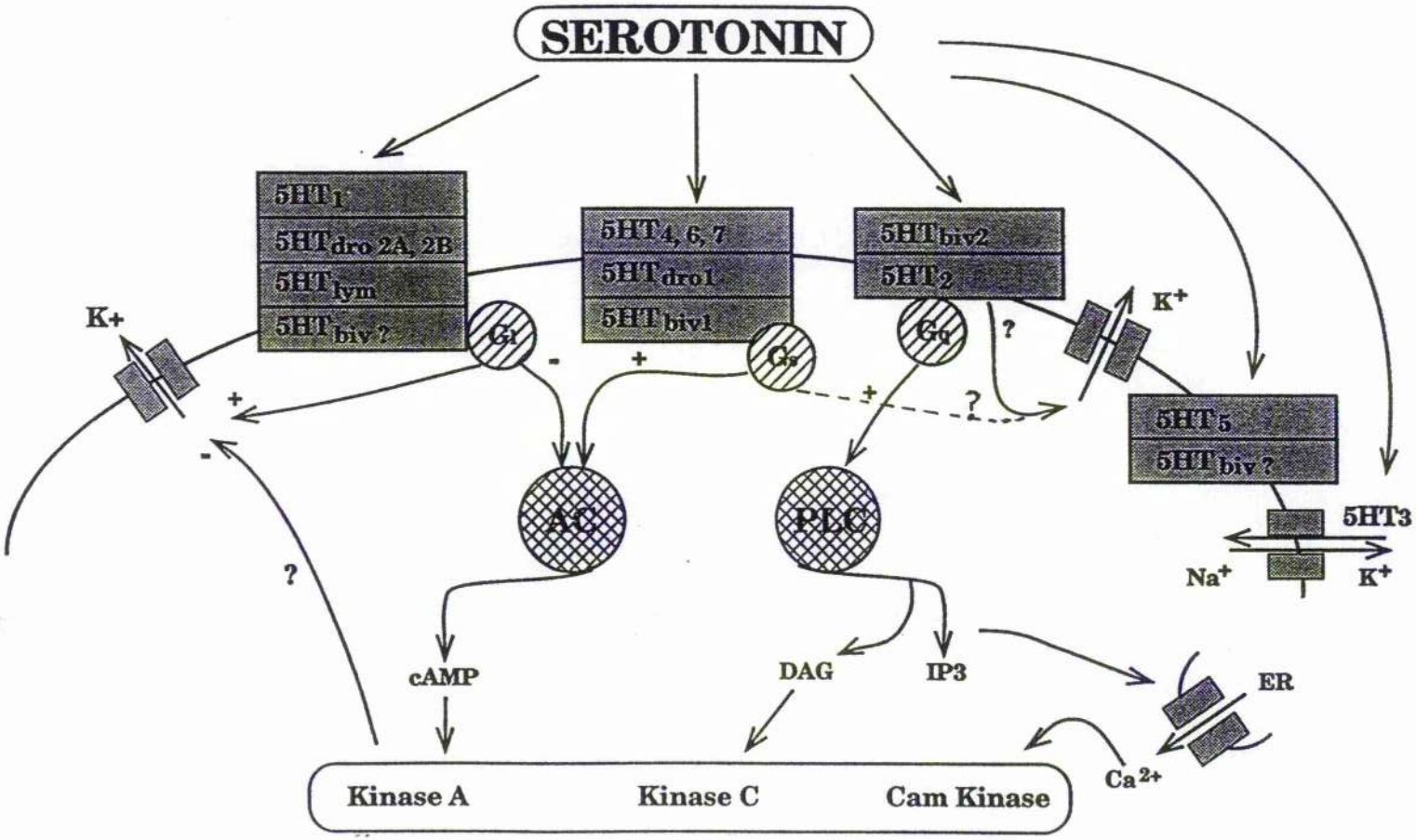
Effector systems:

G_i = pertussis toxin-sensitive G protein,
Gq = pertussis toxin-insensitive G protein.
AC = adenylate cyclase;
PC = phospholipase C;
DAG = 1,2,-diacylglycerol;
IP3 = inositol 1,4,5-trisphosphate; ER = endothelium reticulum.

5-HT RECEPTORS

5-HT_1, 5-HT_2, $5HT_4$, $5HT_5$, $5HT_6$, 5-HT_7	= mammalian 5-HT receptor classification, Saudou and Hen(1994)
5-HT_{dro1}, $5\text{-HT}_{dro2A,dro2B}$	= *Drosophila melanogaster* 5-HT receptors, Saudou et al.(1992) gene cloned for three distinct subtype of 5-HT receptors.
5-HT_{lym}	= Lymnaea stagnalis 5-HT receptor, gene cloned by Sugamori et al.(1993) similar to 5-HT_1-like
5-HT_{biv1}, 5-HT_{biv2}, $5\text{-HT}_{biv?}$	= possible similar 5-HT receptor(s) types in *bivalvia* hearts (proposed from observations outlined in this thesis)

Diagram of 5-HT Receptor Subtypes from Vertebrates and Invertebrates



The responses of the hearts from *Geukensia demissa*, *Saxidomus giganteus* and the *Carditidae* species (such as *Clinocardium nuttalli* and *Trachycardiae*) to 5-HT and analogues in are discussed as **Group 2.**

Group 1:

In this group 5-HT and all analogues tested caused similar responses on the hearts which can be summarised as follows:

- predominantly excitatory on the whole hearts,
- produced closure of a K^+ channel in single myocytes,
- possibly linked to elevation of intracellular cAMP.

I observed that α-Me-5-HT, 5-MEOT and 5-HT were potent agonists, while 5-CT was usually excitatory but somewhat less potent on the whole hearts.

On examination of the literature it becomes apparent that the 5-HT_4 family of receptors (also reported in the hearts of vertebrates) has many similar characteristics to this group, Hoyer et al. (1994). The agonists active at this receptor are listed in the literature as 5-HT, 5-MEOT, and α-Me-5-HT. The 5-HT_4 receptor has also been shown to cause elevation of intracellular cAMP; the response to 5-HT has also been linked to the closure of a K^+ channel in atrium cells, Kaumann et al. (1990), Ford and Clarke (1993) and Hoyer et al. (1994). However the prolonged excitation effect seen with LSD and 8-OH-DPAT is anomalous and difficult to incorporate easily into this classification scheme.

Group 2:

The species in this group did show excitation in response to α-Me-5-HT, but inhibitory or mixed responses to 5-HT, 5-MEOT

and 5-CT (5-CT was consistently the most potent inhibitory agent on the hearts of this group). In *Geukensia demissa* no evidence for the involvement of cAMP was observed or identified in the literature. The intracellular pathway by which 5-CT or 5-MEOT increased the unitary current activity of the K^+ channel was not identified. It should be noted that the effect of 5-HT or 5-CT on the intracellular levels of cAMP in *Saxidomus giganteus* hearts has never been examined to my knowledge and I did not investigate the myocyte preparation of this species.

5-CT has been linked as an agonist at both the $5\text{-HT}_{1A,B,D}$, and 5-ht_5 receptors, Hoyer et al. (1994), the former receptors are reported to cause reduction of cAMP levels as well as direct hyperpolarisation of neurones. Whether this agent actually caused a decrease of cAMP in the myocytes of *Geukensia demissa* was not investigated. Therefore a 5-HT1-like receptor similarity is a possibility.

However the proposed mammalian 5-ht_5 receptors, discussed by Hoyer et al. (1994) also have some similarities. This receptor group are reported to be potently activated by 5-CT and LSD, and the compound methiothepin acts as an antagonist, which makes it an attractive candidate. The intracellular messengers linked to activation of this receptor have not been identified.

LIGAND-GATED CHANNELS

Evidence for the presence of a 5-HT ligand-gated channel similar to the 5-HT_3 receptor described in mammals, was not found in either the whole heart or myocyte preparation. 2-Me-5-HT was not a potent agonist in most of the species tested. The responses seen in the whole hearts were slow in onset,

which is not usually a characteristic of ligand-gated receptor activation.

When either 2-Me-5-HT or 5-HT were added directly to the ASW in the patch electrode solution very little unitary current activity was observed, even shortly after seal formation to the myocyte surface. However as there was not a control before the drug was introduced into the patch media, it is impossible to draw any conclusions as to the possibilities of direct ligand-gated channels. Further studies on outside-out patches would be a useful method to investigate the possible presence of ligand-gated receptor populations.

ANOMALIES WHICH DEFY CLASSIFICATION

Perhaps one of the excitatory response to 5-HT and α-Me-5-HT (often found to be the most potent agonist tested), is due to activation of a receptor with some similarities to the 5-HT_2 family which are reportedly linked to cAMP independent pathways, Hoyer et al. (1994). It should be noted that methiothepin and UML (although often a partial agonist) were more effective antagonists than ketanserin on any of the whole heart preparations. A receptor described as a 5-HT_2 receptor reported in *Lymnaea stagnalis* neurones was blocked by high concentrations of ketanserin, Walcourt-Ambakederemo and Winlow (1994). However the doses they used, $1x10^{-4}$ - $1x10^{-3}$ M, were far higher than I had used. At such a high concentration I wonder whether the selectivity of ketanserin for the 5-HT_2 subtype of receptors is a valid assumption. For example, mammalian dopamine receptors are also blocked by high doses of ketanserin.

As I pointed out in Part I, mianserin has recently been shown to be a more potent antagonist of 5-HT in comparison to

ketanserin, in a gastropod embryonic preparation, by Goldberg et al.(1993). Perhaps this compound should be more extensively examined in other invertebrate preparations? Especially, in those tissues where 5HT and α-Me-5-HT have demonstrated a potent agonist action but where ketanserin has shown no significant selective antagonist action. This might help to further clarify 5-HT_2 receptor characteristics in some invertebrate preparations.

The whole issue is even more complex. As agonist activity by α-Me-5-HT at the 5-HT_4 receptor (in mammals) has been reported, illustrating just how difficult it is to try to classify 5-HT receptors simply by the use of so called ``selective analogues'' (Hoyer et al. 1994).

8-OH-DPAT was not as potent as 5-HT on the cell-attached myocyte preparations of *Mercenaria mercenaria*. However, the decrease in unitary current activity produced by this drug did persist much longer than seen with 5-HT. What are the intracellular cellular changes, which caused the prolonged effect produced by this agent (which persisted even after many washings)? These excitatory changes lasted for more than 30 minutes on both the whole hearts and the myocytes of *Mercenaria mercenaria*.

THE COMPLEX INTERACTION OF 5-HT ON THE HEARTS OF MOLLUSCS

It is important to remember that 5-HT is only one of several neurotransmitters and local hormones which are important to the complex physiological processes which govern the control of the molluscan heart. The role of other biogenic-amines such as dopamine and octopamine has been reported, whereas a role for adrenaline or noradrenaline appears lacking in the molluscs, Walker and Holden-Dye (1989).

This is probably because many of the dopamine and adrenoreceptors appear to have evolved after the initial divergence of the primordial 5-HT receptor. This theory discussed by Peroutka (1995) suggests that the current multiplicity of 5-HT receptors is a direct result of the evolutionary age of the 5-HT system.

Of importance to the biology of the molluscan heart are the effects of different neuropeptides such as FMRFamide. Many of these different peptides have been around since early in evolution, Cottrell (1993). Painter and Greenberg (1982), in their survey on the action of 5-HT on the hearts of *bivalvia*, also analysed the action of FMRFamide on the hearts of the same species. They showed that FMRFamide was a potent agonist on the hearts of bivalves, equally as potent as 5-HT. They also showed that this peptide could excite the hearts of some species while inhibiting others.

It has since been reported that FMRFamide can acts as a modulator of the same K^+ unitary current activity that was shown to decrease in response to 5-HT in *Aplysia* neurones, Volterra (1990). However, he found that the action of FMRFamide increased the channel activity through different intracellular pathways independent of cAMP. Volterra concluded that activation of antagonistic modulatory systems on competing second messenger systems may be the key to the activation of opposing functional or behavioural events in *Aplysia*.

I suggest that as we learn more about the biology of the 5-HT receptors in the hearts of molluscs, we will find evidence of more modulatory interactive second messenger systems similarly linked to other peptide receptors.

CONCLUSION

In reviewing the research observations in the literature, on the action of 5-HT on both the molluscan whole hearts preparations as well as on the K^+ channel identified in the myocytes and the neurones of *Lymnaea* or *Aplysia*. It can be seen how the different pieces of information taken from molecular, electrophysiological and pharmacological studies supports the existence of several subtypes of 5-HT receptors in a variety of molluscan species.

My observations as reported in this thesis also support the hypothesis that there are different subtypes of 5-HT receptors in the hearts of *bivalvia*. Even within the same species there appear to be more than one subtype of 5-HT receptor present in heart. Differences in the 5-HT receptor types were observed between species of bivalves. Perhaps, in the future, these different 5-HT receptors will be fully characterised. It will be interesting to see if there is any correlation between the different 5-HT receptors and the evolutionary separation of molluscs and in particular the families of B*ivalvia*.

In conclusion, I observed the following key results which supported my hypothesis:

- Whole hearts from different species respond differently to 5-HT and other selective agonists.
- Activation of 5-HT receptor subtypes, which produced a similar effect on a K^+ channel in all three species, appear to act via different intracellular pathways which were either dependent or independent of cAMP involvement.
- 5-HT was observed to cause depolarisation of the myocyte by decreasing unitary current activity of an identified K^+ channel in response to 5-HT.

- 5-CT or 5-MEOT caused an opposite effect on myocytes from one species by increasing unitary current activity of the same K^+ channel, suggesting the presence of different subtypes of receptors in the same myocyte.
- The *bivalvia* 5-HT receptors appear to have some similarities in common with either the mammalian 5-HT_1 or $5ht_5$ receptors; or the 5-HT_4 and possibly the 5-HT_2 families of receptors.

Other researchers have already confirmed the existence of multiple 5-HT receptors in a single species of invertebrate. Three different 5-HT receptor subtypes have been described in the insect *Drosophila melanogaster*, each with distinct molecular structures and transduction pathway characteristics, Saudou et al. (1992). Since then the molluscan 5-HT_{1ym} receptor has been cloned by Sugamori et al. (1993), several researchers have proposed other 5-HT receptors types in this species using 5-HT analogues, Walcourt-Ambakederemo et al. (1994).

Thus my observations, supported by these recent molecular biology and pharmacological studies on other invertebrate species, confirm the presence of different types of 5-HT receptors in the hearts of *bivalvia*.

With more molecular biology studies in future I am sure that the hearts of bivalves will show a diversity at least as complex as the salivary glands of *Drosophila melanogaster*. The correlation between the structural findings (amino acid sequence) with transduction pathways and electrophysiological data should help to fully define all of the 5-HT receptor types.

When all the studies are completed, it will be interesting to see how much of the structure and/or function has been retained throughout the evolutionary process of the 5-HT receptors. What will be the key similarities conserved by some of the 5-HT receptors found in molluscs, flies, mice or men?

Thesis Summary

1) The actions of 5-HT and some analogues on the hearts of several species of bivalve molluscs were investigated in two preparations. The effects of these drugs were examined on isolated ventricle preparations and on unitary currents of patch-clamped myocytes from the same species.

2) The isolated whole hearts of *Mya arenaria* were excited by 5-HT and all of the analogues tested. α-Me-5HT was more potent than 5-HT. 5-MEOT was equipotent to 5-HT and 5-CT was less potent than 5-HT. The excitatory responses were blocked by UML and methiothepin. The hearts were excited by the addition of Bz to the bath, this did not affect the potency of the drugs.

3) The isolated hearts of *Mercenaria mercenaria* were similar in their responses to 5-HT, α-Me-5HT, 5-MEOT and 5-CT. UML and Methiothepin were the most effective antagonists tested. Bz although excitatory did not modify the potency of the drugs tested. 8-OH-DPAT, although less potent than 5-HT, had a prolonged excitatory effect, even after several washes over an extended time. After exposure to this drug the hearts remained unresponsive to 5-HT for a prolonged time.

4) The action of 5-HT on the hearts of *Saxidomus giganteus* was biphasic. Low doses initially caused some inhibition, higher concentrations caused excitation. α-Me-5HT was only excitatory and more potent than 5-HT. 5-CT had a potent inhibitory action, this effect was completely blocked by both Methiothepin and Bz. UML demonstrated partial agonist activity, producing inhibition similar to 5-CT. 5-MEOT was usually weakly excitatory. After 8-OH-DPAT the same prolonged "excitation" effect remained as seen with *Mercenaria mercenaria*. No

effective antagonists of the excitation by 5-HT or α-Me-5HT were identified. Weak antagonism was observed with methiothepin.

5) The effect of 5-HT on the isolated hearts of *Geukensia demissa* was biphasic or inhibitory. Inhibition, excitation or a mixture of these responses was observed with increasing concentrations of 5-HT. α-Me-5HT was always found to be excitatory; 5-CT was found to be inhibitory and 5-MEOT was usually inhibitory. No effective antagonists were identified.

6) The potent inhibitory effect of 5-CT on several species was shown for the first time. Even the inhibition seen with 5-HT had been referred to only occasionally. The early studies of 5-HT and analogues were usually carried out with Bz added to the bath. This may have masked any inhibitory action of some of the analogues tested.

7) Myocyte unitary currents from different bivalves were studied in the cell attached mode using patch-clamp techniques. The effects of 5-HT, 5-CT, 5-MEOT, forskolin and 8-bromo-cAMP were studied on the myocytes. The latter two compounds are believed to either increase or mimic cAMP.

8) The major current activity was identified as the opening of a K^+ channel with the following characteristics:

a) a conductance of 38 ps (when 10 mM K^+ was present in the electrode ASW),

b) a high rate of unitary current activity was seen in the first 5 minutes after patching, probably due to the stretching of the membrane as the seal was formed,

c) a 10 fold increase in the K^+ level in the electrode ASW caused a shift in the unitary current / electrode potential(I/V) plot to the right by 50 mV, (conductance changing to 55 ps),

d) the I/V plot fitted the continuous line relationship predicted by Hodgin and Katz for K^+ ion permeability,

e) the current activity was weakly voltage dependent, there was an average two fold increase observed between Ve = -40 mV to -100 mV,

f) the K^+ channel unitary current activity contributed to the resting potential of the cell, this was demonstrated by the K^+ current activity observed when the Ve = 0 mV (with 100mM K^+ in the patch electrode)

9) The effect of 5-HT applied close to myocytes from *Mya arenaria, Mercenaria mercenaria* and *Geukensia demissa,* was a decrease in unitary current activity, due to increased closure of the K^+ channel identified. The effect was due to the action of 5-HT causing second messenger activity modification of the K+ channel opening. This assumption was made since the drug had no direct access to the patch membrane isolated by the electrode; therefore the effect had to be caused by activation or inhibition of intracellular pathway(s).

10) The effect of 5-CT and 5-MEOT on the myocytes from *Mya arenaria* and *Mercenaria mercenaria* was the same as observed with 5-HT, i.e. decreased unitary current activity.

11) The effect of 5-CT and 5-MEOT on the myocytes from *Geukensia demissa* was the opposite. These drugs caused an increase in unitary current activity due to increased opening of the K^+ channel identified.

12) Forskolin and 8-bromo-cAMP were tested, these drugs are known to elevate cyclic AMP levels. Both drugs decreased the unitary current activity in the myocytes of *Mya arenaria* and *Mercenaria mercenaria,* similar to 5-HT. However neither drug caused any significant change in the unitary current activity in the myocytes from *Geukensia demissa*.

13) 5-CT and 5-MEOT increased the unitary current activity which correlated with the effect of these drugs in the isolated heart preparations of *Geukensia demissa*, where both drugs were predominantly inhibitory. In contrast these drugs were excitatory on the hearts of *Mya arenaria* and *Mercenaria mercenaria* and they decreased unitary current activity.

14) The results from the whole hearts and the myocytes experiments support the hypothesis that there are more than one conformation of 5-HT receptor in the hearts of bivalves.

15) The 5-HT receptor activated in *Mya* and *Mercenaria* myocytes produced a decreased in K^+ unitary current activity across the membrane, which correlated with the effect seen by elevation of cAMP intracellularly. This fits well with the previous literature on excitation of the whole hearts by 5-HT.

16) In *Geukensia* the presence of another 5-HT receptor(s) was observed which when activated also caused a decrease in K^+ unitary current activity. This was not mimicked by elevation of intracellular cAMP. This fits well with previous literature on the action of 5-HT on these hearts.

17) 5-CT and 5-MEOT caused an increase in the same K^+ unitary current activity (in *Geukensia*), suggesting the presence of a second 5-HT receptor type in the same myocyte. This fits with the observed excitation / inhibition of the whole hearts seen with 5-HT, 5-CT and 5-MEOT. These effects in *Geukensia* are probably linked to the activation of a different intracellular second messenger pathway, independent of cAMP.

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