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PHARMACOLOGICAL STUDIES ON ACETYLCHOLINE AND OTHER TRANSMITTER RECEPTORS FROM INVERTBRATE MUSCLE AND CENTRAL NEURONES

BY

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A Thesis presented for the degree of
Doctor of Philosophy.

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ABSTRACT

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Pharmacological studies on acetylcholine and other transmitter receptors from invertebrate muscle and central neurones.

by Abdul-Nabi Atya Hassoni

Muscle tension recordings were made from earthworm body wall muscle and the action of acetylcholine, cholinomimtics and cholinolytics examined. The effect of cholinergic agents on muscle twitches induced following field stimulation was also investigated. Carbachol was 8 times more active than acetylcholine in the presence of physostigmine.

α-Bungarotoxin, d-tubocurarine, gallamine, atropine, mecamylamine and hexamethonium reduced both electrical stimulation of the muscle and the acetylcholine response while β-bungarotoxin reduced the electrically induced twitch but enhanced the acetylcholine contraction. 0.4μM Hemicholinium abolished the electrically induced twitch while having no effect on the acetylcholine response, but at 4.0μM, did reduce the acetylcholine response. These results provide further evidence for cholinergic excitatory innervation of earthworm body wall muscle.

Intracellular recordings were made from identifiable central neurones of *Helix aspersa* and the action of anthelmintic compounds investigated. The anthelmintics pyrantel, morantel and deacylated amidantel mimicked acetylcholine induced excitation"D" and inhibition"H" had the same ionic mechanism and were blocked by d-tubocurarine. This suggests these compounds interact with acetylcholine receptors on *Helix* neurones. Levamisole only inhibited the activity.

A series of glutamate analogues was tested on *Helix* neurones which were either excited or inhibited by L-glutamate. The only analogue with clear glutamate-like activity was thio-glutamic acid. In normal saline L-glutamate hyperpolarises the membrane potential of cell F-1. This event is chloride mediated and is reversed to a depolarisation followed by hyperpolarisation in low external chloride. This afterhyperpolarisation is reduced in sodium or potassium free saline or following application of strophanthidin, 1.0-100μM.

The local anaesthetics procaine and tetracaine mimicked the "H" and "D" effects of acetylcholine on certain neurones. Tetracaine, 0.01μM, gradually and reversibly reduced both "H" and "D" responses of acetylcholine and the "H" response to dopamine. This provides evidence that local anaesthetics can interact with responses linked to chloride, sodium and potassium ion channels.

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Contents of the thesis

ABBREVIATIONS

A, nA	Amper, 10^{-9} amps
Ac	Acetate
Ach	acetylcholine
CNS	Central nervous system
C,nC	Coulomb, 10^{-9} Coulomb
DA	Dopamine
FET	field effect transistor
ECl	Equilibrium potential for chloride ions
EPSP	Excitatory post-synaptic junction
IPSP	Inhibitory post-synaptic juction
g,mg	Gram, 10^{-3} grams
GABA	Gamma amino butyric acid
Hz	Hertz
I,V	Current, voltage
ml	10^{-3} litres
cm, mm	10^{-2}, 10^{-3} metres
M, mM, µM	Molar, 10^{-3}, 10^{-6} molar
Mohm	10^{6} Ohms
MP	Membrane potential
mV	Millivolts
pH	$-\log_{10}$ (Hydrogen ion concentration)
Po_2	Partial pressure of oxygen
PTH	Post-tetanic hyperpolarisation
s,ms	Seconds, 10^{-3} seconds
S, mS, µS, nS	Siemen, 10^{-3}, 10^{-6}, 10^{-9} Siemens
S.E.	Standard error
Tris Hcl	Tris(hydroxymethyel) aminomethane hydrochloride
TEA	Tetraethylamonium
TTX	Tetrodotoxin
QX-314	Lidocaine derivatives
w/v	Weight/volume

CHAPTER 1

GENERAL INTRODUCTION

The molluscs and in particular the gastropods offer an ideal preparation for neurophysiological studies since they possess neurone systems with large cell bodies, with different shapes, colours and positions which can easily be identified from preparation to preparation. These neurones are arranged peripherally around a central neuropile region. In addition invertebrates often have giant neuronal components for example, soma measuring up to 500µM diameter in the gastropods and the giant axon in *Loligo* is up to 1000 µM diameter. Therefore, it is possible to insert one or more microelectrodes under visual control with relative ease. Gerschenfeld (1973) pointed out that most molluscan synapses are axo-axonal although some axo-somatic connections have been demonstrated.

Kerkut and Walker (1975) described the structure of the nervous system of *Helix aspersa* where the ganglia are fused together to form the brain which is contained in an envelope of connective tissue.

Neurones in the central ganglia of, for example, *Helix* and *Lymnaea* have been mapped and characterized according to their pharmacology and axonal projections through the main nerve trunks (Parmentier 1973; Kerkut *et al* 1975; Winlow and Benjamin,1976).

Molluscan neurones can easily be impaled by one or more microelectrodes in vivo or in vitro. When impaled they show resting potentials which vary between -40 to -60mV. After penetration with a microelectrode the membrane seems to form a seal around the glass, the membrane potential recovers quickly and there is little change in the cell cytoplasm (Nicaise and Meech, 1980). Normally these electrodes are

filled with 3M KCl to lower their resistance and reduce any diffusion potential. Electrodes with low resistances are less noisy and the membrane potentials are stable for longer. High resistance electrodes give recordings that tend to drift with time.

The molluscan nervous system contains neurones which can be isolated for single cell experiments, multiple electrodes can be placed inside these large neurones, so allowing types of experiment that are not possible on other preparations. Cephalopod giant nerve fibres have provided excellent experimental material for studies on the ionic bases for the resting and active membrane potential. Hodgkin and Huxley (1952 a,b) showed that the action potential in squid nerve is associated with sodium and potassium currents.

Later work from the mid 1950s till the mid 1970s using other preparations has largely confirmed the initial work using the squid giant axon. However, it is now clear that both calcium and second messengers play an important role in normal central nervous system function.

Electrophysiological techniques

These techniques have contributed greatly to our understanding of the electical properties of neurones. For example, the results obtained by recording the membrane potential, membrane resistance and transmembrane currents of signal neurones with reference to drug action are of greater value than those achieved by experiments on population of neurones. The following techniques are the mostly common used.

Voltage clamp

Studies on the action potential have established important concepts concerning the ionic basis of the resting potential and action potential. These ideas were confirmed and given a strong quantitative basis by a new type of experimental procedure, the voltage clamp, developed by Marmont (1949), Cole (1949), Hodgkin and Huxley and Katz (1952) and Hodgkin and Katz (1949).

The fundamental experiments of voltage clamp were performed by Hogkin and Huxley in the period from 1949 to 1952 with the participation of Katz in some of early work. The Hodgkin-Huxley model of the nerve action potential is based on electrical measurements of the flow of current across the membrane of an axon, using the voltage clamp technique.

Voltage clamp has been the ideal technique for the study of ionic channels (Figure 1). This technique measures the current across the membrane when the membrane is electrically depolarised or hyperpolarised and then experimentally maintained (clamped) at a given potential. Two electrodes are inserted into the nerve axon. One is used to measure the voltage across the membrane, and the second passes electrical current into or out of the axon to keep the membrane potential at the constant value set by the experimenter. The injecting current is from the output of the feedback amplifier. The circuits are examples of negative feedback since the injecting current has the required sign to reduce any signal error signal.

Let us suppose the experimenter depolarises the membrane to a particular value. The depolarisation causes an opening of sodium channels and an influx of sodium ions,

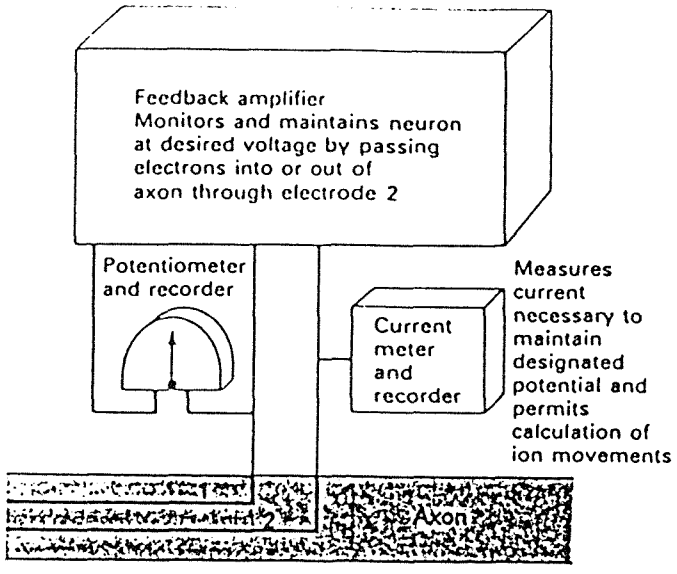


Figure Principle of the voltage clamp technique. Two electrodes, 1 and 2, are inserted into a large axon. The electrodes are thin wires that extend the length of the cell in order to ensure that the membrane voltage is the same at all regions of the membrane. Electrode 1 is used to monitor the potential across the plasma membrane. Electrode 2 is used to pass electric current (electrons) into or out of the cell. To depolarize or hyperpolarize the cell to a predetermined value, electrons are withdrawn or fed into the cell respectively, via electrode 2. The feedback amplifier adjusts the current flow into or out of the cell to maintain the membrane potential at this prearranged value. The electrons passed into or out of the cell by electrode 2 exactly neutralize positive ions, Na^+ or K^+, that pass out of or into the cell across the plasma membrane. Thus, the flow of current through electrode 2 is a measure of ion movements into or out of the cell.

which would normally lead to a further depolarisation of the membrane and an entry of more sodium ions. When the membrane potential is clamped at a specific depolarisation value no further depolarisation can occur. The inward and outward movement of ions can then be quantified from the amount of electrical current needed to maintaine the membrane potential at the designated value (Figure 2) under conditions when sodium ions removed from external saline, or TTX added to normal sodium saline. The current electrode balances the entry of each positive ion into the cell across the nerve membrane.

The early work of Hagiwara and Saito (1959) on the marine molluscan *Onchidium verruculatum* showed for the first time that two intracellular glass micropipettes could be used to voltage clamp a spherical central neurone. Inward and outward currents appeared upon depolarisation similar to those recorded from squid axon. When they perfused the cell with a solution in which sodium chloride had been replaced with sucrose it failed to abolish the inward current. Hagiwara and Saito described it as the "initial inward current". Later it became apparent that the neuronal action potential also depended on divalent ions (Oomura et al 1961), and the residual current in sodium free solution was a calcium current. Subsequently, the action potentials in many other molluscan neurones were found to depend on both sodium and calcium (Kerkut and Gardner, 1967) and voltage clamp revealed that the initial inward current was abolished by sodium and calcium free saline (Chamberlain and Kerkut 1969).

Figure 2 Current and ion movements across the membrane of the squid giant axon.

a The membrane potential is suddenly changed from the resting potential of -65mV to -9mV and clamped at that value.

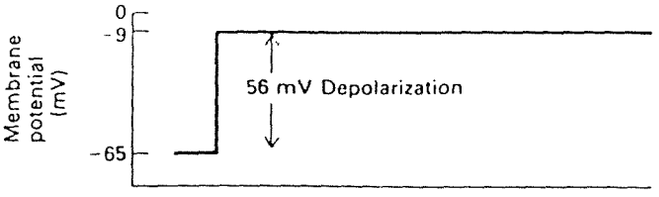
b This depolarisation induces a transient inward current followed by a delayed current.

c If the experiment is repeated with the axon bathed in solution that contains no sodium ions,there is no transient inward current but the delayed outward current does occur normally. This result establishes that the transient inward current is due to sodium ions.

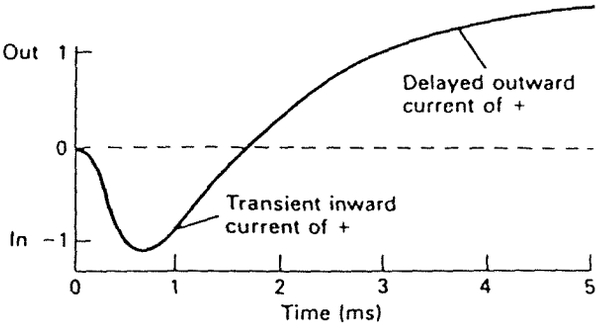
d By calculating the difference in the current due to ionic movements shown in (b) and (c), the ionic movements due to sodium ions can be deduced; upon depolarisation there is a transient increase in membrane permeability to sodium ions.

e That the late outward movement of current is due to outward movement of potassium ions is confirmed by specifically blocking the potassium channel with tetraethylammonium chloride (TEA). When TEA is added to the extracellular fluid, it does not alter the inward movement current but abolishes the later outward movement of current. The delayed outward movement of potassium ions continues as long as the membrane is depolarised, indicating that potassium channels remain open as long as is depolarised. In contrast, depolarisation induces only a transient opening of the voltage-dependent sodium channels. After it has opened and closed, the sodium channel becomes refractory (Hodgkin and Huxley 1952c).

(a)

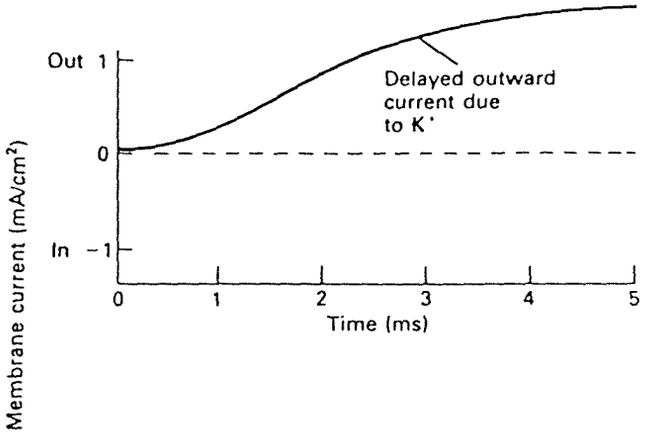


(b)



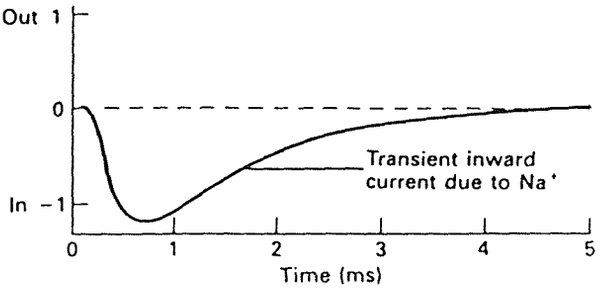
(c)

No sodium ions in external solution



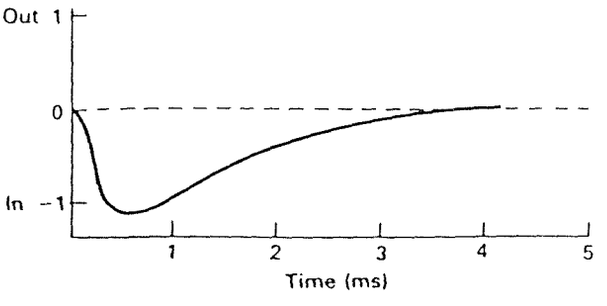
(d)

Calculated difference between b and c



(e)

Addition of TEA blocks K+ channel



Ionophoresis

Ionophoresis is an accurate way of applying very small quantities of charged drug molecule directly to a neurone receptor. An ionised drug can be ejected from a microelectrode by using a current of opposite charges. According to Faraday's law the rate of release from electrode is proportional to the intensity of current. Therefore the current can be used as a measure of dose applied.

Zieglgansberger et al, (1969) found that electrodes filled with glutamate solutions of low molarity (0.2-0.1M) showed some variation in their glutamate release. They concluded that a close relationship existed between current intensity and the release of L-glutamate, even up to very high currents.

The speed at which drug concentration develops at the cell surface depends on the distance between the cell and the tip of the microelectrode and also on the physical properties of the pipette. An ideal ionophoretic pipette would develop a steady state rate of release the instant when the current is turned on.

Ionophoretic application methods

A. Circuitry

An electronic current device is used for passing current through ionophoretic pipettes. The current source should be able to supply a steady retaining current, which is adjustable in a range of 0-5nA. as well as the ejecting current itself. It is rarely necessary for the ejecting current to exceed 100 nA. A high speed current is applied

for ultra-close application of drugs when rapid receptor responses are required (Dreyer and Peper 1974).

During the application of high currents the excitability of the neurone adjacent to the microelectrode tip may be altered as a result of extracellular potential changes as well as the pharmacological actions of the drug under test. The technique of "current balancing" can overcome this problem by ensuring that the algebraic sum of the currents flowing from a multi-barrelled assembly is zero.

B. Intracellular application

Intracellular injection is used to alter the concentrations of inorganic ions (Coombs et al 1955; Thomas 1977) or to mark the cell for subsequent histological examination (Kerkut and Walker, 1962a; Kerkut et al 1970; Kater and Nicholson, 1973).

The amplitude of the intracellular ionophoretic current injected may be restricted by the onset of membrane damage during large disturbance of the membrane potential. High currents can be injected through one microelectrode if an equal and opposite current is withdrawn from a second electrode. This technique is a variation of current balancing.

C. Miscellaneous Hints.

Cationic drugs are ejected more reliably than anionic drugs. In some cases it may be necessary to adjust the pH to produce optimal ionization (Curtis,1964) whereas other drugs

such as acetylcholine, carbachol, atropine and dopamine can be ejected directly as cations. Drug release from a pipette of 2µm tip diameter is far more diffcult to control than from 0.2µm tip.

Pipettes of very high resistance may require no retaining current, but in general the retaining current is adjusted until there are signs of spontaneous drug leakage. An initial estimate may be made by setting the retaining current to a given value. Excessive retain current has a great effect on the speed of ejection during a subsequent current pulse and bringing the pipette up to the cell. If there is an effect the retain current can be adjusted.

The alternative to ionophoresis is *pressure injection* through a micropipette. The advantages of pressure injection is that the quantities released are independent of the electrochemical properties of the active substance. Therefore it is possible to eject molecules which have small diffusion coefficients or a zero net charge or both. The rate of ejection is mainly dependent on the physical dimensions of the microelectrode tip which is proportional to the third power of the internal tip diameter. The disadvantage of using pressure injection is the blocking or shattering of microeletrode tip.

Transmitter gated and Voltage gated channels.

Synaptic excitation mechanisms differ in two important ways from the mechanism of generation of an action potential. These are due to the differences in the structure of the receptor ion channel proteins and in the mechanism by which channels are gated(Figure 3).

One difference is the movement of Na+ and K+ during the action potential is sequential whereas during the synaptic potential it is simultaneous. During the action potential membrane depolarisation leads to the opening of two independent channels, first one selective for Na+ and then one selective for K+. A transmitter opens special channels whose size and shape allow both Na+ and K+ to pass with nearly equal permeability. This channel is also so large that it allows large cations such as Ca^{+2}, NH^{+}_{4} and even certain organic cation to pass. Anions such as chloride are excluded. This cation selectivity suggests that the channel has a negative charge at its mouth that attracts a variety of cations of a certain size and repels anions because of their charge. Hille and his co-workers have estimated that the channel activated by acetylcholine is substantially larger in diameter than the sodium or potassium channel.

The second difference is the increase in sodium influx produced by the action potential is regenerative, whereas that produced by the synaptic event is not. The sodium and potassium channels responsible for action potential are voltage -sensitive. They are opened by depolarisation and closed by hyperpolarisation. The synaptic action is not controlled by voltage but depends on the concentration of a specific chemical transmitter such as acetylcholine. Therefore the depolarisation induced by the transmitter does not increase the total synaptic conductance. This observation explains why synaptic potentials tend to be relatively small and additive compared with large all-or-none action potentials.

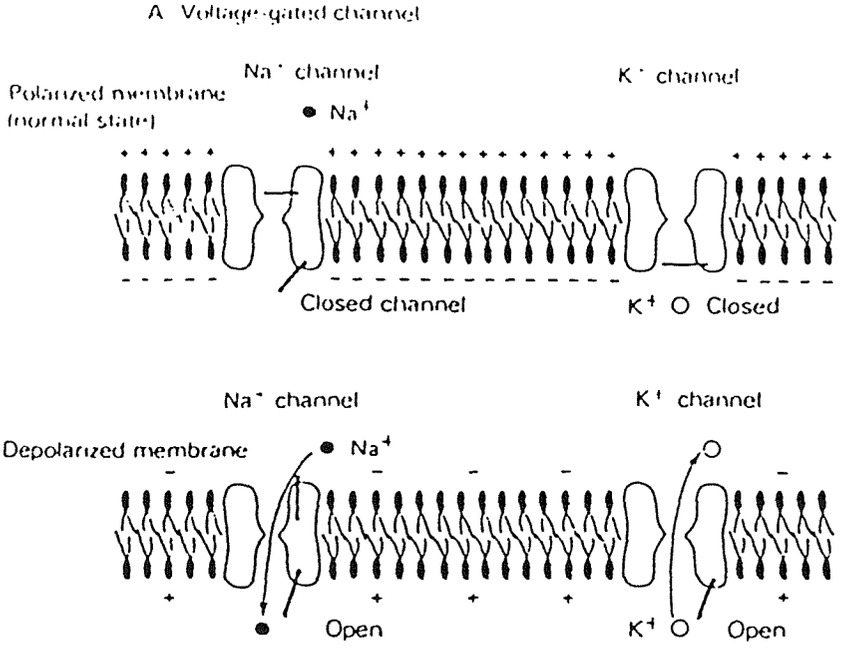
These two differences in molecular properties,suggest that the channels opened by transmitters differ pharmacologically from those opened by the action potential. The influx of sodium produced by excitatory transmission is

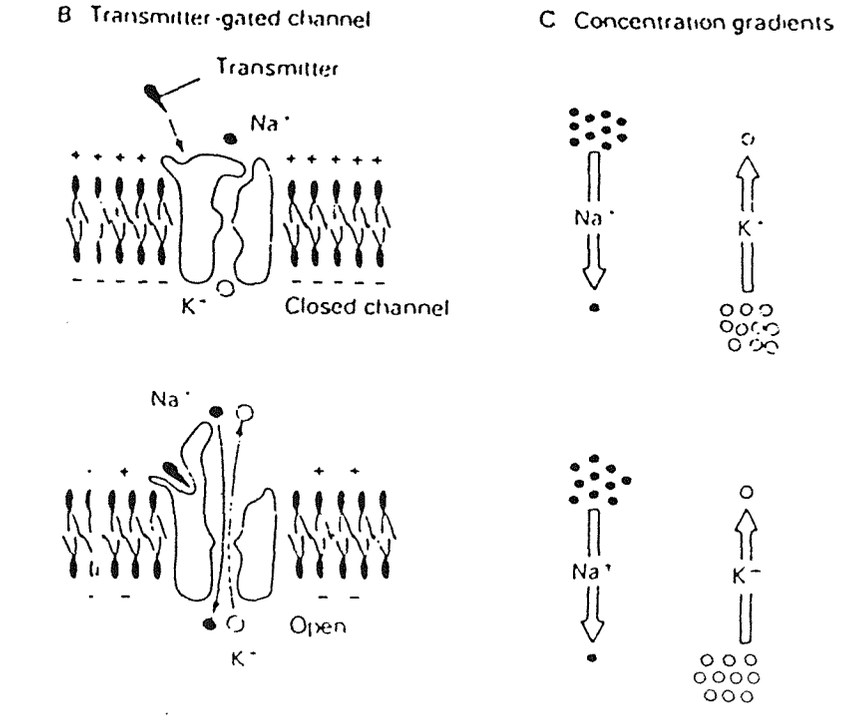
Figure 3 Voltage-gated and transmitter-gated channels operate by different mechanisms.

A Channels that contribute to the action potential are voltage-gated and selective for different cations. A separate channel exists for sodium ions (filled circle) and for potassium ions (open circle).

B In contrast, the transmitter-gated channel activated by excitatory transmitters (such as Ach in skeletal muscle are not gated by voltage and are permeable to both sodium and potassium.

C The ionic concentration gradient for the ions are the same for both classes of channels. (From Kandel and Siegelbaum 1985).


A Voltage-gated channel
Na⁺ channel
K⁺ channel
Polarized membrane
(normal state)
Na⁺
Closed channel
K⁺ O Closed
Na⁺ channel
K⁺ channel
Depolarized membrane
Na⁺
Open
K⁺ O Open


B Transmitter-gated channel
C Concentration gradients
Transmitter
Na⁺
K⁻
Closed channel
Na⁺
Open
K⁻
Na⁺
K⁺
Na⁺
K⁻

not blocked by tetrodotoxin (TTX), the drug that blocks the action potential. Similarly, α-bungarotoxin that blocks the action of acetylcholine does not interfere with voltage gated sodium or potassium channels. Other gates open in response to changes in the intracellular concentration of specific ions; for example, some potassium channels open when the concentration of free calcium ions in the cytosol increases.

The muscle cell membrane at synapses behaves as a transducer that converts a chemical signal in the form of a neurotransmitter into an electrical signal. This conversion is achieved by ligand-gated ion channel in the postsynaptic membrane. When the neurotransmitter binds to these ligands externally, they change their conformation by opening the channels to let ions cross the membrane and then alter the membrane potential. The ligand-gated channels are relatively insensitive to the membrane potential. They cannot by themselves produce an all-or-none self-amplifying excitation. Instead, they produce an electrical change that is graded according to their intensity and duration of the external chemical signal which depends on how much transmitter is released into the synaptic cleft and how long it stays there. This feature of ligand-gated ion channels is important in information processing at synapse.

Neurotransmitter

Acetylcholine is a neurotransmitter that is, a chemical which is involved in transfer of information from one nerve cell to another or from a nerve cell to a muscle cell. The theory of chemical transmission has been existence for about one hundred years though it was not fully accepted until the early 1930s. Prior to that it was assumed that information

passed from cell to cell by electrical transmission and this is so for a limited number of synapses. However the vast majority of synapses employ a chemical transmitter. There are now many chemicals which have been shown to act as neurotransmitters, for example, dopamine, noradrenaline, octopamine, 5-hydroxytryptamine (serotonin), histamine, various purines including adenosine and ATP, L-glutamic acid, gamma-aminobutyric acid (GABA) and glycine (Walker, 1986). In addition many, if not all, neurones contain neuroactive peptides and these can also be released following presynaptic stimulation and many have direct or indirect postsynaptic effects. While many of these have been first shown to occur in vertebrates there are an increasing number which have first been identified in invertebrates, including proctolin (first identified in insects by Brown and Starratt, 1975; Starratt and Brown 1975) and FMRFamide (first identified in a lamellibranch by Price and Greenberg 1977).

Acetylcholine was one of the first neurotransmitters to be studied(Loewi 1921). Acetylcholine together with its enzymes for synthesis, choline acetyltransferase, and breakdown, acetylcholinesterase occur widely in the invertebrates and are probably present in all phyla (Leake and Walker 1980). Frequently the invertebrate levels of acetylcholine are high, much higher than in vertebrates (Florey 1967). Kerkut and Cottrell (1963) found that *Helix* neurones contain 1-5µg Ach/g wet weight. Cholinesterase has been used as a marker for histochemical localization of cholinergic systems but this can be misleading as this enzyme occurs in places where acetylcholine is unlikely to be transmitter. For example, only a few neurones in the *Aplysia* abdominal ganglion contain choline acetyltransferase while cholinesterase occurs in all the cells examined (Giller and Schwartz 1971 a,b).

Criteria for a Compound to be a Neurotransmitter

Before a chemical can be identified as a neurotransmitter, a number of criteria have to be fulfilled (Werman 1966). These criteria are somewhat easier to satisfy in a peripheral preparation compared with the situation in the central nervous system. Firstly the neurotransmitter must be present in the nerve terminal and be stored in a way which protects it from enzymatic breakdown, usually in vesicles. There must be the necessary enzymes in the nerve terminal to synthesise the compound from its precursor(s). These precursors must be able to enter the cell and so there must be an uptake system whereby the precursor can be selectively taken into the cell. This is against a chemical gradient and so normally involves an energy process. Once taken up, synthesised and stored there must be a mechanism for release which is associated with calcium ions and so a calcium dependent release mechanism must be demonstrated. Once released the chemical rapidly diffuses across the synaptic cleft and then must have an effect on the postsynaptic membrane, normally on a specific component of the membrane, the receptor. This chemical receptor interaction then leads to a change in the conformation of the membrane and usually a change in permeability which in turn often causes a change in the membrane potential, that is, the response. There must be a method for inactivation since the neurotransmitter interact with a specific receptor. Chemicals have been synthesized which will occupy the receptor and prevent the action of the neurotransmitter, termed antagonists. Once identified the neurotransmitter can be chemically synthesized and applied to the postsynaptic membrane and its action should mimic that of stimulation of the presynaptic cell. Both events should be blocked or reduced by the selective antagonists (blockers).

The work presented in this thesis is concerned with acetylcholine receptors on earthworm somatic muscle and snail central neurones. In addition some experiments have been described using snail central neurones glutamate receptors.

CHAPTER 2

METHODS AND MATERIALS

In these studies two different preparations have been used: the body wall muscle of the earthworm *Lumbricus terrestris*, and the central nervous system of the *Helix aspersa*.

Experiments using earthworms were performed using an organ bath-muscle contraction system whereas experiments on *Helix* CNS required electrophysiological techniques.

1. Earthworm body wall muscle

Earthworms (*Lumbricus terrestris*) were collected locally and maintained in moist fresh soil at room temperature (20-23 °c) for at least 3 days before use. An anterior dorsolateral body wall section devoid of nerve cord was prepared by opening the worm ventrally and removing the intestines. A section of approximately 12 segments was suspended vertically in a 50 ml. organ bath.

The composition of the saline was as follows: NaCl, 130.5 mM; KCl, 2 mM; $MgCl_2$, 3 mM; $CaCl_2$, 5 mM, Tris, 5 mM; gassed continuously with 95 % O_2 and 5 % CO_2 (for some experiments). The pH of the solution was adjusted to a pH of 6.9-7.1 at room temperature. In the ion substitution experiments, sodium was replaced by Tris. In calcium free saline a partial substitution of calcium ions was made by cobalt.

Field stimulation with the following parameters was used: rectangular pulses of 1 msec width, 5-10 HZ frequency, 10-20 volts for a 4 second period every 30 seconds. Field stimulation was applied to the muscle using platinum ring electrodes connected to two Digitimer

stimulators.

These were connected to an isolated stimulator MK 1V and were set up to give trains of pulses. An audio monitor was connected to indicate when the stimulation period started. Muscle responses were recorded using an UF1 dynamometer strain (isometric transducer) gauge linked to a Vitatron (MSE) pen recorder. The contraction induced either by electrical stimulation or by chemical compounds was recorded with the Vitatron (MSE) pen recorder.

All the compounds were dissolved in saline to give a range of concentrations depending on the dose-response data required. 2 ml of experimental solution was applied near to the muscle in the organ bath using a 5ml. syringe.

An anticholinesterase agent, physostigmine sulphate, was allowed to equilibrate with the tissue for 5 min. prior the application of acetylcholine. Drugs (agonist) were added to the bath 30 seconds before electrical stimulation. After each treatment with an agonist, the muscle was washed for at least 15 minutes before the next dose was applied. The sensitivity of each muscle preparation differs therefore, the size of the response to a drug will differ as will the maximum response. The best condition of the muscle for a good drug response was obtained when the muscle was left in the organ bath for 30-45 minutes before applying drugs. The saline was changed three times during this period.

The dose-response data were plotted on a log-linear scale on semi-logarithmic graph paper and the effective dose that can induce half of the maximum contraction for acetylcholine in the presence of physostigmine was calculated. This value is called EC_{50}, i.e. the dose at

which muscle response was 50% of maximum.

In the case of the antagonists, the preparation was first exposed to physostigmine for 5 minutes. Then the antagonist was added and the muscle left for 5 min. Finally acetylcholine was added to the organ bath. Following the response, the muscle was washed and left in saline for 30 minutes in order to allow recovery. For atropine, gallamine and d-tubocurarine their relative potencies were assessed by calculation of pseudo PA values, using a method based on that of Schild (1947). Firstly a log dose-response curve was constructed to the application of the agonist (acetylcholine) then a second one was constructed in the presence of the antagonist.

$$PA = \log \left[\frac{(DR-1)}{[I]} \right]$$

where I is the concentration of antagonist and $DR=A_2/A_1$, A_1 is the dose of agonist required to produce 50% of the maximum response and A_2 is the dose of agonist needed to produce the same size response in the presence of the antagonist.

2. Central Nervous System of Snail

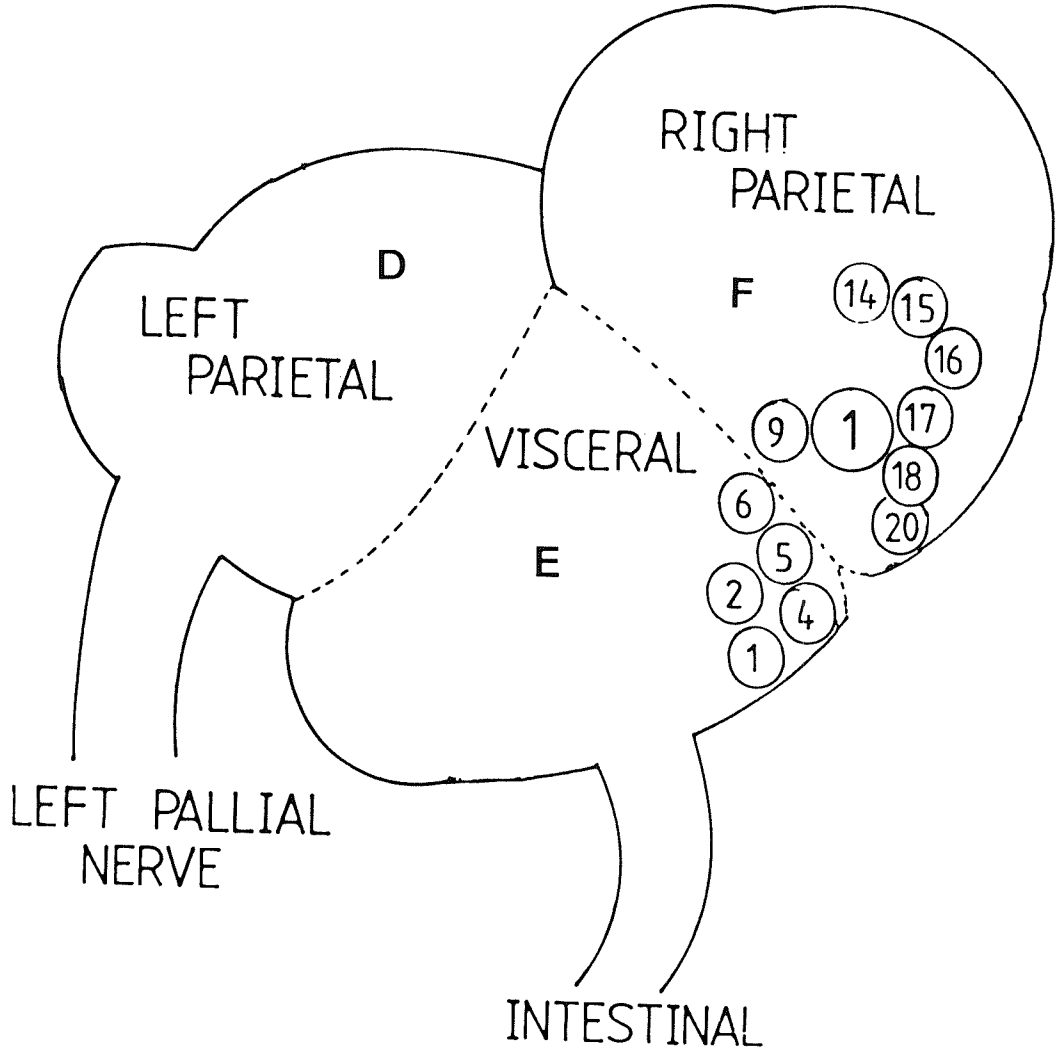
The Preparation

Experiments were performed on the common garden snail *Helix aspersa*. Specimens were collected locally and then kept under laboratory conditions. The snails used in this study were not less than six grams in weight. The brain was dissected from the animal following the procedure of Walker (1968). The shell was removed carefully, ensuring that the animal was not damaged. The whole animal was then pinned out, dorsal side uppermost, on a wax block. A longitudinal slit was made in the body wall, in the medial line.

The oesophagus and pharyngeal retractor muscle were cut, and the pharynx slipped through the circumoesophageal commissures.

The brain was then picked up by the cerebral ganglia and dissected out by snipping away the nerves on the ventral side. Following their removal from the animal, the ganglia were secured to a glass microscope slide, by means of elastic bands positioned below the cerebral ganglia and on the nerve trunks from the suboesophageal ganglia. The brain was lightly stained with the vital dye methylene blue and viewed under saline using a binocular microscope. Excess tissue was trimmed off and then the outer connective tissue carefully removed to expose the underlying neurones. A small tear was made in the inner connective, using two pairs of fine forceps. The desired cell body could then be stabbed by inserting a microelectrode through this hole in the inner connective tissue. The cells of the

Figure 4 Diagram of parietal and visceral ganglia of *Helix* to show the positions of the neurones used.


RIGHT
PARIETAL
D
F
LEFT
PARIETAL
14
15
16
9
1
17
VISCERAL
18
6
20
5
E
2
4
1
LEFT PALLIAL
NERVE
INTESTINAL

suboesophageal ganglia of *Helix aspersa* were mapped out by Kerkut et al. (1975). The neurones used in this study are situated in the right and left parietal ganglia and visceral ganglion. The positions of these neurones are shown in figure 4.

Saline Concentration

The composition of the various saline used in this study are given in table 1. Salines were made up from stock solutions. All of the stock solutions were 1M, except for the sodium chloride which was 4M. The volume was then made up to a litre using distilled water. The pH of the saline was 7.8. The pH was set by the pH of the Tris HCl.The pH of the Tris HCl was 4.7 when made up, but it was adjusted to 7.8 using NaOH.

In sodium free saline the sodium was substituted by Tris, which is an impermeant cation. In chloride free saline the chloride was substituted by acetate which is an impermeant anion. In sodium, chloride free saline, sodium was substituted by sucrose. Experiments where the saline was changed and in which the drugs were bath applied the drugs were made up in the modified saline.

Table 1. The composition of the various salines used in experiments, with the concentrations of the constituents in mM

Saline	NaCl/NaAc	KCl/KAc	$CaCl_2/CaAc_2$	$MgCl_2/MgAc$	Tris/Hcl	Sucrose
Normal	100/--	4/---	7/---	5/-	5	----
Na^+ free	----	4/---	7/---	5/-	10 5	--
Cl^- free	--/100	--/4	--/7	-/5	5	----
Mg^{++} free	100/--	4/---	7/--	-	5	----
K^+ free	100/--	----	7/---	5/-	5	----
NaCl free	---	---/4	--/7	-/5	-	180

Experimental Bath

The bath was made from Perspex and was 10ml. in volume. The bath and micromanipulators were situated inside a Faraday cage to isolate the experimental equipment from its electrical surroundings. Saline flowed from a reservoir situated above the bath, the flow being controlled by a two adjustable clips. The saline inlet was situated at the bottom of the bath whereas the outlet was situated at the top. The microscope slide to which the brain was attached, was positioned at an angle of 45 degrees to the horizontal in the bath. The bath earth, an earthed chlorided silver wire, was in a 7ml vial of 1M KCl behind the experimental bath. This vial was linked to the experimental bath via a 2% w/v Agar/KCl bridge. This bridge consisted of an U shaped piece of glass which was filled with agar dissolved in 1M KCl. The 1M KCl in agar jelly can be used to link pairs of solutions irrespective of their composition without introducing any junction potentials. The preparation was illuminated from the front using a Schott fibre optic system and viewed with an Olympus zoom binocular microscope.

Microelectrodes

Two and half millimeter bore hard glass was cut into 8 centimeter lengths. The glass was then cleaned by boiling in dilute hydrochloric acid for 30 minutes. It was rinsed well in distilled water, then acetone and finally dried in an oven. Glass fibres were pulled from the same glass and glued using epoxy resin into the glass lengths. Electrodes were pulled using a vertical Narishige puller. The electrodes were filled with 1M potassium acetate and those with resistances in the range 5-15 Mohms used. The tips of

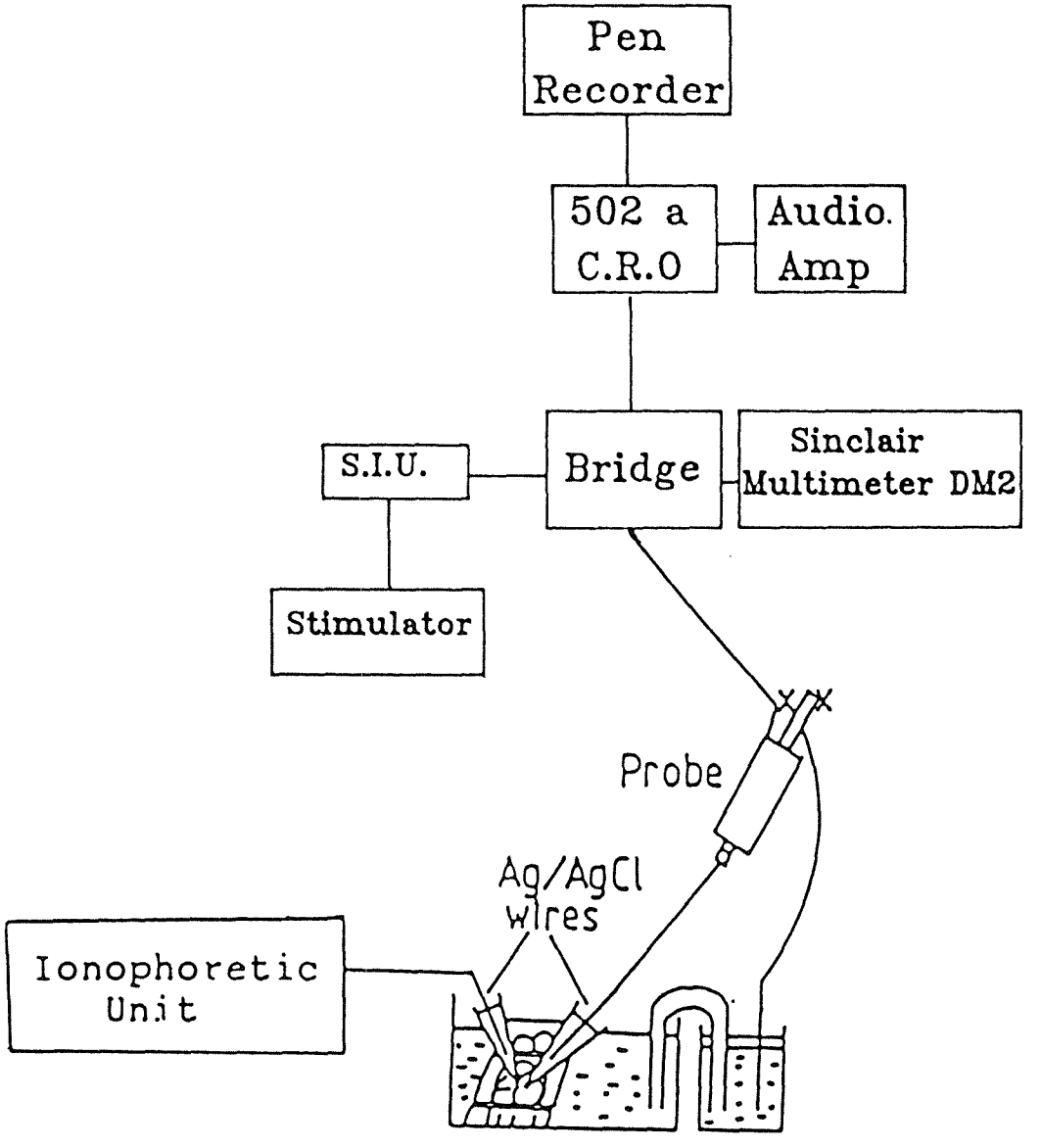
the electrodes were coloured with black ink to increase visibility. When filled, the microelectrode was placed in a Perspex holder connected to a Prior micromanipulator. The electrode could then be precisely positioned and the desired cell impaled.

Recording and Display of Potentials

Figure 5 shows a block diagram of the equipment used for recording and displaying intracellular neural activity. Silver wires were coated with a thin layer of silver chloride by electrolysis. The silver wire from the recording electrode was connected to the input of an FET (field-effect transistor) operational amplifier, contained in a probe mounted near to the preparation. The output of the probe was to a modified Wheatstone bridge circuit. The Wheatstone bridge was incorporated in the recording circuit to permit hyperpolaristion or depolarisation of a cell by passing current through the recording electrode. To balance the electrode the variable resistance was altered until there was no change in the potential measured at the electrode when a current was applied. It is necessary to balance the bridge because otherwise when the current was passed into the cell a false reading of membrane resistance and potential could result, in other words, the balancing effectively cancels the voltage drop across the electrode so that only the response of the cell is recorded.

The output from the bridge was displayed on a Tektronix 502A dual beam oscilloscope and monitored on a Sinclair multimeter DM2 (Digital Voltmeter) for accurate measurement of membrane potential.

Figure 5 A block diagram illustrating the arrangment of the equipment used for intracelluar recordings. Ionophoretic current and duration were preset and automatically controlled.


Pen Recorder
502 a C.R.O
Audio. Amp
S.I.U.
Bridge
Sinclair Multimeter DM2
Stimulator
Probe
Ag/AgCl wires
Ionophoretic Unit

The resistance of the recording electrode in Mohm can be read directly from the digital Voltmeter. A Grass S8 stimulater was connected to an auxiliary input of the bridge via a stimulus isolation unit and this allowed current pulses to be given. The recording electrode was used to pass rectangular hyperpolarising current pulses of 500 ms. duration, 0.2 HZ frequency and 1-2 nA amplitude in order to calculate the conductance. An audio amplifier was connected to the output of the 502A so that the firing pattern of the cell could be heard and as an instant guide to when penetration had occurred. Permanent records were made using a Watanabe WTR 211 pen recorder which received its input from the 502A output.

The Application of the Drugs

Drugs were added directly to the bathing solution in the bath. The drug was made up as 10mM stock solution in saline and this was then diluted down 1 part with a parts of saline until the required concentration was reached. Stock solutions were kept for two to three days in the fridge at 4C°, but dilutions were made up freshly each day. Agonists were applied as 0.3 ml. volume directly over the brain. The concentrations given in the figures is the concentration of the 0.3 ml. not the final concentration. With antagonists the volume added to the bath was 0.3-0.5 ml. having previously drained out 0.3-0.5 ml. of the bathing solution so that the final volume was still 10 ml. Antagonists were added away from the preparation and allowed to equilibrate for at least 3-5 minutes prior to testing. The dose was expressed as a final bath concentration in molarity.

For ionophoresis the micropipettes were made as previously described and filled as for recording electrodes but with a drug solution. The ionophoretic electrodes had a DC resistance of 5-10Mohms measured in the standard saline. The eject current was set to values up to 1000 nA. and the duration of the pulse was automatically controlled by a variable timer circuit. Single electrode ionophoretic doses of acetylcholine and dopamine were applied 1-3 seconds rectangular pulses with an interval of 4-8 minutes in order to prevent desensitisation. A retaining current of appropriate polarity was applied to hold the drug in the pipette and this automatically cut out when the eject current was activated and came back in on termination of the eject pulse. This retaining current was up to 5-10nA which eliminted any remaining background diffusion of acetylcholine and dopamine from the electrode tip.

Acetylcholine and dopamine used for ionophoresis were made up in distilled water. They were ejected as cations and were used at the following concentrations, acetylcholine chloride 0.5 M, and dopamine hydrochloride 0.5 M.

Calculation of input conductance

To demonstrate a change in membrane conductance, constant current pulses from the stimulator were applied to the external stimulus input of the "bridge" through the probe and recording electrode to the cell soma. These pulses were observed on the CRO beam as a negative deflection and their size directly measured. A change in the size of the pulses indicated a change in membrane resistance.

The change in input conductance is normally obtained by

subtracting the conductance calculated at the resting membrane potential from the value measured after the addition of the drug.

Calculation of "resting" and drug evoked increase in membrane conductance is as follows

$$G = \frac{I}{V} \qquad \text{Ohm's law}$$

$$G_1 = \frac{I}{V_1} \qquad (1)$$

$$G_2 = \frac{I}{V_2} \qquad (2)$$

$$\delta G = G_2 - G_1 \qquad (3)$$

$$= \frac{I}{V_2} - \frac{I}{V_1} \qquad (4)$$

$$\delta G = I\frac{(V_1 - V_2)}{V_1 V_2} \qquad (5)$$

where

I: A constant current(nA).

V_1: The voltage of the membrane at resting potential.

G_2: The conductance of the membrane before the addition of a drug

G_2: The conductance of the membrane after the addition of a drug

V_2: The voltage of the membrane potential after the addition of a drug.

δG: Change in conductance.

Data Analysis

Determinations of drug potencies were made by constructing log dose-response curves. The dose-response data were plotted on log-linear scale on semi-logarithmic paper and from the graph the effective dose that induces half of maximum depolarisation or hyperpolarisation for the agonist was calculated. This value is called EC_{50}. The EC_{50} was calculated by getting a repeatable standard response to bath addition of acetylcholine and agonist. The potency ratio was calculated as follows:

$$\frac{EC_{50} \text{ of an agonist}}{EC_{50} \text{ of acetylcholine}}$$

Where the EC_{50} value is greater than one, the agonist is less active than acetylcholine. Conversely when the EC_{50} value is less than one, the agonist is more potent than acetylcholine. In some experiments the standard error of the mean values are quoted

Drugs and Sources

A list of the drugs used in this study and their sources is given below:

compound	*Source*
O-acetylcholine chloride	BDH
Amidantel	Bayer
N-Methyl-D-Aspartic acid	Sigma
Atropine sulphate	BDH
Bethanechol chloride	Merk Sharp and Dohme Research Lab
α-bungarotoxin	Sigma
β-bungarotoxin	Sigma
Carbachol chloride	BDH
Choline chloride	BDH
Deacylated amidantel	Bayer
Decamethonium iodide	Koch-Light Ltd
Dopamine HCl	Sigma
Etrenol	Winthrop
Gallamine triethiodide	Sigma
L-glutamate	B.D.H.
γ-Morpholinlamide-L-glutamate	Dr. Sharma
γ-N-Cyclohexylamide-L-glutamate	Dr. Sharma
γ-N-Cyclopentylamide-L-glutamate	Dr. Sharma
γ-N-Norbornylamide-L-glutamate	Dr. Sharma
γ-N-Tertiary butylamide-L-glutamate	Dr. Sharma
γ-Thio-L- glutamic acid	Dr. Sharma
γ-Thio-D-glutamic acid	Dr. Sharma
Hemicholinium-3	Sigma
Hexamethonium bromide	Sigma
Levamisole	Sigma
Mecamylamine hydrochloride	Aldrich

Morantel	Pfizer
Muscarine chloride	Sigma
Nicotine hydrogen tartrate	BDH
Physostigmine sulphate	Sigma
Procaine hydrochloride	Sigma
Pyrental	Pfizer
Strophanthidin	Sigma
Succinylcholine chloride	Koch Light Lab
Tetracaine	Sigma
Tetramethylammonium iodide	Sigma
d-Tubocurarine chloride	Sigma

CHAPTER 3

PHARMACOLOGY OF ACETYLCHOLINE RECEPTORS ON LUMBRICUS TERRESTRIS

INTRODUCTION

Muscle fibre membrane depolarisation normally starts at the motor end-plate, the action potential is transmitted along the muscle fibre and initiates the contractile response. A single action potential causes a brief contraction followed by relaxation. This response is called a muscle contraction and involves shortening of the contractile elements, but because muscles have elastic and viscous elements in series with the contractile mechanism, it is possible for contraction to occur without an appreciable decrease in the length of the whole muscle. Such a contraction is called isometric (same measure or length). Contraction against a constant load, with a decrease in the length of the muscle, is isotonic (same tension). Drugs may increase or decrease the contractions of striated muscle by affecting one or more of the processes in the excitation-contraction coupling sequence. The direct cause of the altered contractility is usually a change in the rate of Ca^{+2} release ,in the amount of Ca^{+2} released ,or in the rate of Ca^{+2} re-uptake by the sarcoplasmic reticulum.

The concept of specific acetylcholine receptors was proposed even before acetylcholine was accepted as a neurotransmitter. Dale (1914) showed that it had two different types of action in vertebrates, and postulated the existence of two different acetylcholine receptors. The action on smooth and cardiac muscle which could be mimicked by muscarine, an extract from a fungus, was termed muscarinic and the action on striated muscle and autonomic ganglia which could be mimicked by nicotine, a plant alkaloid, was called nicotinic. With few exceptions, nearly all peripheral vertebrate cholinoreceptors fit into these categories. In the vertebrate, therefore,there must be

at least two possible chemical conformations for acetylcholine to fit these two cholinoreceptors.

Since receptors of mixed character have been found in the central nervous system of vertebrates, the nicotinic/muscarinic classification is probably a gross over simplification for vertebrate, and may not apply to invertebrate at all (Sakharov,1970). However, since the concept helps one to visualize alternative cholinoreceptors, it is still of use and is therefore retained.

Acetylcholine is unique in that it is a weak agonist in the absence of anticholinesterase. The anticholinesterase physostigmine was shown many years ago to be an ester of carbamic acid(NH_2COOH). Physostigmine combines reversibly with cholinesterase so promoting accumulation of acetylcholine in the tissue enhancing both muscarinic and nicotinic effects. The primary function of acetylcholinesterase in animal tissue appears to be catalysis of the hydrolysis of acetylcholine to choline and acetic acid. Some anticholinesterase agents have the ability to potentiate the effect of acetylcholine and related agonists of leech muscle(Flacke and Yeoh, 1968a).

The simplest way to characterize and identify the physiological receptor of acetylcholine in an excitable membrane is to use compounds that are structurally related to acetylcholine and thus present a high affinity for the cholinergic receptor site. Interestingly, it has been shown recently that certain toxins from snake venoms, although completely unrelated structurally to acetylcholine, nevertheless act much like curarine. One of them is α-bungarotoxin, a basic polypeptide of molecular weight 800. α-Bungarotoxin is purified from the venom of an elapid snake from Taiwan (*Bungarus multicinctus*), and the purified toxin gives irreversible neuromuscular blocking effects, in

addition , d-tubocurarine protects against the action of α-bungarotoxin. From these findings Lee and Chang (1966) have concluded that α-bungarotoxin combines irreversibly with the cholinergic receptor at the motor end-plate.

The other snake neurotoxin is β-bungarotoxin whose effects appear to be mediated exclusively by an action on the pre-synaptic membrane, releasing system. It therefore is a useful agent for studying transmitter release mechanisms. β-bungarotoxin is a polypeptide which is present in the venom of *Bungarus multicinctus*. Its pre-synaptic mode of action has been established principally by electrophysiological studies. In the rat phrenic nerve diaphragm preparation, the toxin acts in two phases by initially increasing acetylcholine release as monitored by spontaneous miniature-endplate potential discharge rate, and causing a complete blockade of both spontaneous and evoked transmitter release (Bowman and Rand 1984). Unlike the situation with α-bungarotoxin which acts on the postsynaptic acetylcholine receptor (Chang and Lee 1973). Pre treatment with d-tubocurarine cannot protect skeletal muscle from the action of β-bungarotoxin.

d-Tubocurarine is a non-depolarising, competitive neuromuscular blocking drug. This drug combines with acetylcholine receptors on the postjunctional membrane of the motor end-plate without stimulating the receptors. d-Tubocurarine therefore blocks the action of acetylcholine The blocking action may be overcomed by any procedure that increases the local concentration of acetylcholine at the neuromuscular junction. Under certain conditions, d-tubocurarine and gallamine may block the open ion channels as well as blocking the receptor recognition site(Bowman and Rand 1984).

Hemicholinium produces a gradual failure of

transmission at cholinergic transmission sites, the onset,extent and duration of which are enhanced by increasing the frequency of nerve stimulation. The amount of acetylcholine released from cholinergic nerves in response to nerve stimulation is reduced by hemicholinium. The synthesis of acetylcholine by intact cholinergic neurones and synaptosomes is inhibited by hemicholinium but there is no direct inhibitory action on free choline acetyltransferase (Bowman and Rand 1984).

The findings with hemicholinium strongly suggest that its main action is to compete with choline for the choline-carrier in the nerve ending with the result that choline acetyltransferase is deprived of its substrate for acetylcholine synthesis. Transmission failure becomes evident when the preformed stores of acetylcholine have been partially exhausted by frequent nerve impulses and when synthesis is inhibited to the extent that it cannot keep up with the demand. Excess choline then overcomes the transmission failure by competing more favourably with hemicholinium for the carrier mechanism, and so restoring the substrate to the synthesizing enzyme.

There is evidence that hemicholinium may be transported by the choline carrier mechanism in place of choline, where it competes with acetylcholine for intracellular binding sites. Choline acetyltransferase can catalyse the acetylation of hemicholinium invitro, although with only about one quarter of the efficiency with which choline is acetylated. Nevertheless, it is possible that some of the hemicholinium is acetylated in the nerve endings and released by nerve impulses on to striated muscle.

There is considerable evidence for acetylcholine as a peripheral excitatory transmitter in the annelids, particulary in leeches and earthworms(Gardner and Walker,

1982).

In this study it was decided to investigate the nature of the acetylcholine receptor on the body wall muscle of the earthworm *Lumbricus terrestis*, therefore, a range of nicotinic and muscarinic agonists and antagonists were tested on this preparation.

RESULTS

Agonists

Acetylcholine and physostigmine

Physostigmine did not have any effect by itself when applied to the body wall muscle of the earthworm at a concentration of 0.6µM for 5 minutes. Acetylcholine is a weak agonist in the absence of an anticholinesterase while in the presence of physostigmine, its potency is increased (Figure 6). This potentiation of the acetylcholine effect is probably the result of inhibition of the earthworm body wall muscle cholinesterase. Physostigmine greatly sensitizes the earthworm body wall muscle to acetylcholine.

For a comparison between responses to acetylcholine 1mM in the presence of physostigmine 0.6µM and acetylcholine 10 mM alone, the response to 10 mM acetylcholine is about half the amplitude of the response to acetylcholine 1 mM in the presence of physostigmine (Figure 6E). This would suggest that physostigmine increases the potency to the acetylcholine by 20 times. On the other hand, acetylcholine 1mM in the presence of physostigmine 0.6µM, compared with acetylcholine 1mM alone (Figure 6A-D), indicates an increase in potency of five times.

Acetylcholine dose-response curves

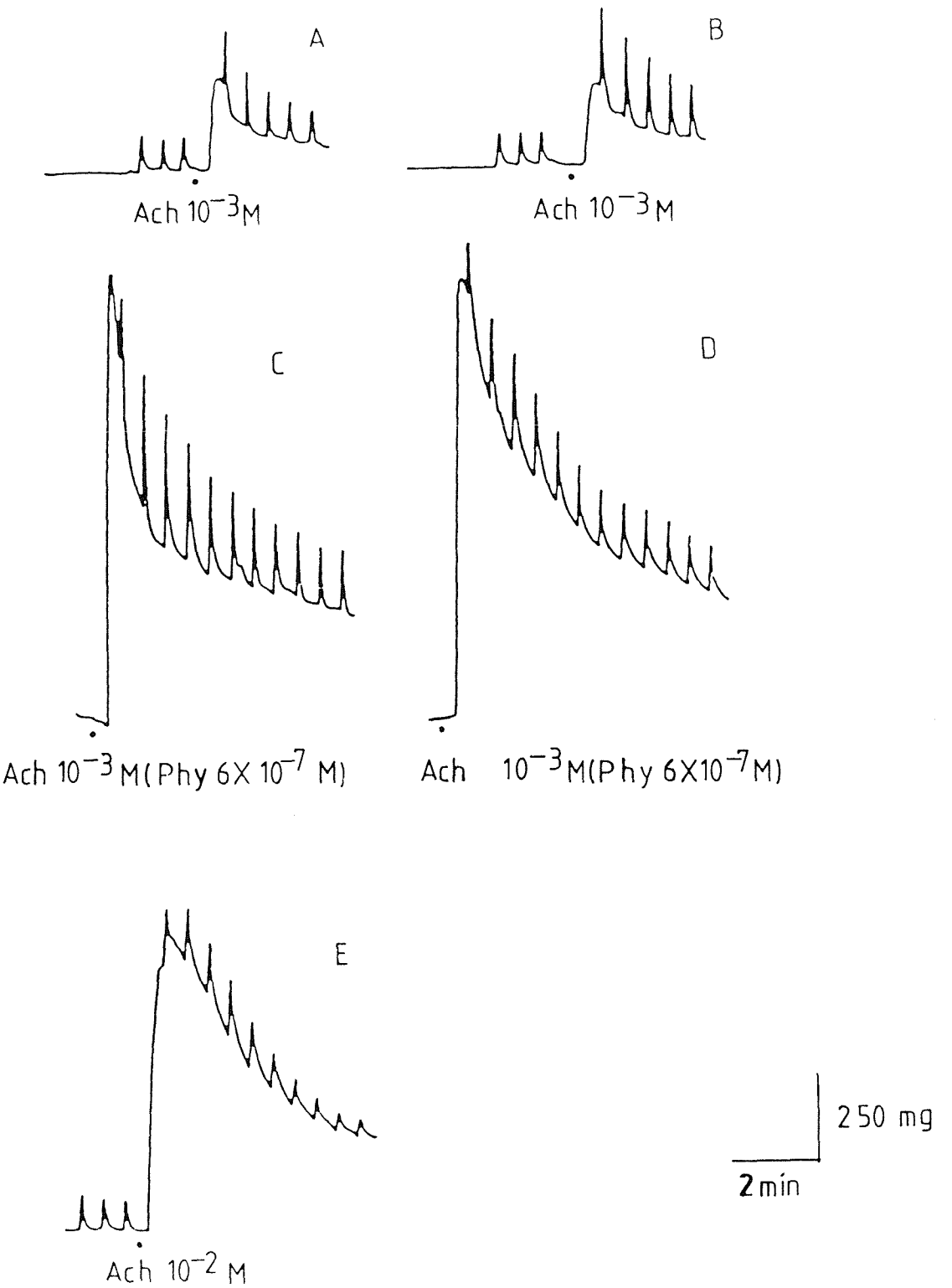
In most earthworm body wall muscle preparations, the concentration of acetylcholine required to produce

Figure 6 Shows the ability of anticholinesterase (physostigmine) agent to potentiate the acetylcholine responses in the earthworm body wall muscle.

A and B indicate the response following the application of 1mM acetylcholine (control).

C and D Acetylcholine 1mM in the presence of physostigmine 0.6µM.

E Acetylcholine 10mM alone.


A
Ach 10^{-3}M
B
Ach 10^{-3}M
C
Ach 10^{-3}M(Phy 6X 10^{-7} M)
D
Ach 10^{-3}M(Phy 6X10^{-7}M)
E
Ach 10^{-2} M
250 mg
2 min

a contraction in the absence of physostigmine is 10mM. Physostigmine was applied prior to the acetylcholine in all the experiments unless otherwise stated. For the dose-response curve, acetylcholine was applied in increasing concentrations: 10µM, 50µM, 100µM, 500µM, and 1mM (Figure 7). Dose response curves for four animals were obtained by measuring the maximum contraction for each dose and the EC_{50} the effective dose that caused half the maximum contraction was obtained for each preparation, the values were 0.25mM, 0.2mM, 0.1mM, and 0.2mM. The sensitivity of each preparation to acetylcholine was therefore similar with a mean EC_{50} of 0.18mM.

In a series of experiments, acetylcholine was applied in Na^+ free saline, Ca^{+2} free saline or Ca^{+2} free with Co^{+2} added to it. The result in Figure (8) shows acetylcholine 50µM in Na^+ free saline, the response of the muscle to acetylcholine was reduced by about 95% compared with the control, whereas the recovery was potentiated about 10% above control (Figure 8E).

Acetylcholine 50 µM was also tested in 50% Na^+ saline. The response to acetylcholine was reduced by about 75% and the recovery was potentiated about 33% above control. In general, Na free saline not only reduced the responses to acetylcholine but the muscle twitch was also depressed (Figure 8C,D). Acetylcholine at a concentration of 10µM, in Ca^{+2} free saline was tested. The response of acetylcholine was reduced by 90% and the recovery was about 55% of control. The muscle twitch was also abolished. The acetylcholine response was also tested in an experiment with Ca^{+2} free saline to which 1mM Co^{+2} had been added. In both solutions the response to acetylcholine 30 µM was reduced by 95% and the recovery was about 50%. The muscle twitch was also abolished reversibly.

Choline chloride

Choline chloride 10mM effects the body wall muscle of the earthworm in a similar manner to acetylcholine, except that it is less effective. When choline chloride was applied to the preparation, it was more than 500 times less potent than acetylcholine in the presence of physostigmine (Table 2).

Succinylcholine

On the earthworm body wall muscle, succinylcholine produced a slower contraction than acetylcholine. The potency of succinylcholine was 40 times less than acetylcholine (Table 2). Succinylcholine also evoked a prolong contraction (Figure 9B), but had a faster rise time than carbachol (Table 3).

Decamethonium

Decamethonium produced excitation but less potent than acetylcholine (Figure 9A). The potency of decamethonium was about 80 times less than acetylcholine (Table 2). There is only a slight difference in the potency between decamethonium and succinylcholine on the body muscle.

Compound	Experiments					Relative Potency
	1	2	3	4	5	
1. Acetylcholine + physostigmine	1	1	1	1	1	1
2. Succinylcholine	10	>10	30	100	-	40
3. Decamethonium	>100	100	50	>100	-	>80
4. Choline chloride	>500	>500	>500	>500	-	>500
5. Muscarine	>100	100	500	-	-	230
6. Carbachol	0.2	0.2	0.1	0.05	0.1	0.13
7. Nicotine	>100	100	100	100	100	100
8. Tetramethylammonium	250	100				175
9. Bethanechol	400	500				450

Table 2. The relative potencies of acetylcholine agonists. Where the value is greater than one, the agonist is less potent than acetylcholine. When the value is less than one, the agonist is more potent than acetylcholine.

Nicotine

Nicotine induced a contraction of the body wall muscle at a concentration of 10mM (Figure 9D). At this concentration there is a rapid contraction of the muscle following addition of nicotine and it is probably a full agonist. The period of contraction is often long lasting, the muscle remaining contracted even after prolonged washing. Repeated application of nicotine can therefore only be given with relatively long intervals, for example, 30 minutes. In some preparations, 10mM nicotine desensitised the preparation to subsequent doses of nicotine. Interestingly following the application of 46µM gallamine some preparations were more sensitive to nicotine. Nicotine exhibits the slowest decay time, for example (Table 3).

Carbachol

A comparison between acetylcholine and carbachol is of interest since their molecular structures are similar. Carbachol is resistant to enzymatic hydrolysis and was eight times more potent than acetylcholine in the presence of physostigmine (Table 2; Figure 9C,E). However, the time to peak of the carbachol response was much slower than acetylcholine (Table 3).

Carbachol also induced a contraction of the muscle at a concentration of 30µM. Repeated application of this concentration often produced a contraction of increasing amplitude up to maximum which was then constant (Figure 10). Carbachol was capable of producing a maximum contraction of a similar amplitude to acetylcholine in the presence of physostigmine and is therefore a full agonist.

Table 3. Summary of the time to peak and 50% decay times of typical responses of a range of cholinomimetics together with their maximum contraction amplitudes at the maximum concentrations tested.

	Compound	Concentration	Time to peak of response (sec)	Maximum contraction (mm)	height at 50% decay (mm) time	time to 50% decay (sec)
1.	Acetylcholine + physostigmine	10^{-4}M + (6×10^{-7}M)	12	50	12	108
2.	Decamethonium	5×10^{-3}M	75	30	18	110
3.	Nicotine	10^{-2}M	85	42	28	260
4.	Succinylcholine	5×10^{-3}M	180	43	28	170
5.	Carbachol	10^{-5}M	270	50	38	240
6.	Tetramethylammonium	10^{-2}M	12	44	26	66
7.	Bethanechol	10^{-2}M	180	8	-	-
8.	Choline	10^{-2}M	78	6	5	180
9.	Muscarine	5×10^{-3}M	300	12	-	-

Tetramethylammonium

Tetramethylammonium (TMA) also induced a contraction in the muscle but was around 100 times less effective than acetylcholine in the presence of physostigmine. At a concentration of 10mM TMA induced a rapid contraction of the muscle.

Bethanechol

Bethanechol 10mM caused the muscle to contract but produced a slower contraction than that induced by TMA. As in the case of TMA, bethanechol was about 100 times less potent than acetylcholine in the presence of physostigmine. Also bethanechol at 10 mM induced a prolonged contraction of the muscle with a long time to peak of the response, (Table 3).

Muscarine

Muscarine had a similar effect to acetylcholine. The potency of muscarine on the earthworm body wall muscle was about 230 times less than that of acetylcholine in the presence of physostigmine (Table 2). Muscarine at a concentration 10mM induced a prolonged contraction of the muscle with a long time course of the response. The size of the contraction would suggested that it was a partial agonist (Table 3).

In the case of acetylcholine and TMA, the contraction rapidly rises to a maximum and fairly quickly decays.The time to peak of the response is slower with nicotine, decamethonium, succinylcholine, bethanechol and choline, while it is very slow in the case of carbachol and

muscarine. The relaxation time is slow in the case of nicotine and carbachol. These differences are summarized in Table (3), where it can be seen that acetylcholine and TMA exhibit the quickest response while carbachol takes over 20 times longer and muscarine takes about 25 times longer. In the case of carbachol the maximum amplitude is similar to acetylcholine. In terms of relaxation, the differences are also great, although TMA and acetylcholine are again the most rapid and nicotine and carbachol the slowest.

Agonist potencies

In table (2) a summary is given of the relative potency of a number of compounds where the potency of acetylcholine taken as one. Acetylcholine potency is calculated in the presence of physostigmine. Each value shown for a given compound was obtained on a different preparation. With reference to table (2), these observations can be made. Firstly the potency of choline chloride is 500 times less potent than that of acetylcholine in the presence of physostigmine, therefore, choline chloride has the lowest relative potency compared with the others and appears to be a partial agonist. Secondly, the highest relative potency is shown by carbachol. Thirdly decamethonium and succinylcholine are similar in potency.

Figure 7 Dose response of the contraction of the earthworm body wall muscle following addition of acetylcholine in the presence of physostigmine 0.6µM. In this experiment the EC_{50} value for acetylcholine was 0.25 mM and the maximum contraction was taken as 100%.

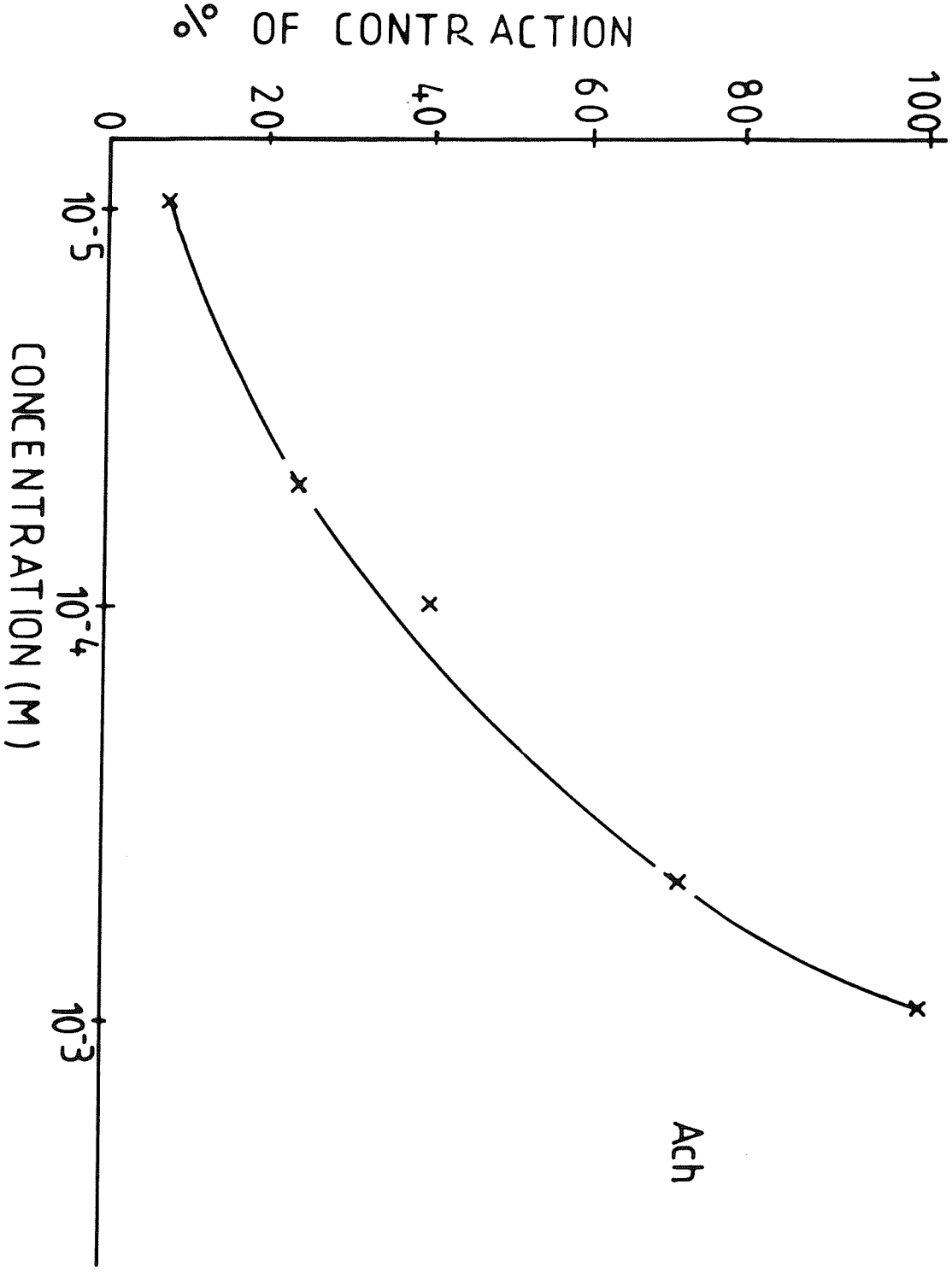

% OF CONTRACTION
0
20
40
60
80
100
CONCENTRATION (M)
10-5
10-4
10-3
Ach

Figure 8 shows the effect of sodium free saline on the acetylcholine response on the earthworm muscle. Acetylcholine was applied in the presence of physostigmine 0.6μM

A and B 0.05mM Acetylcholine as control.

C and D 0.05mM Acetylcholine in sodium free saline.

E Acetylcholine 0.05mM recovery in normal saline

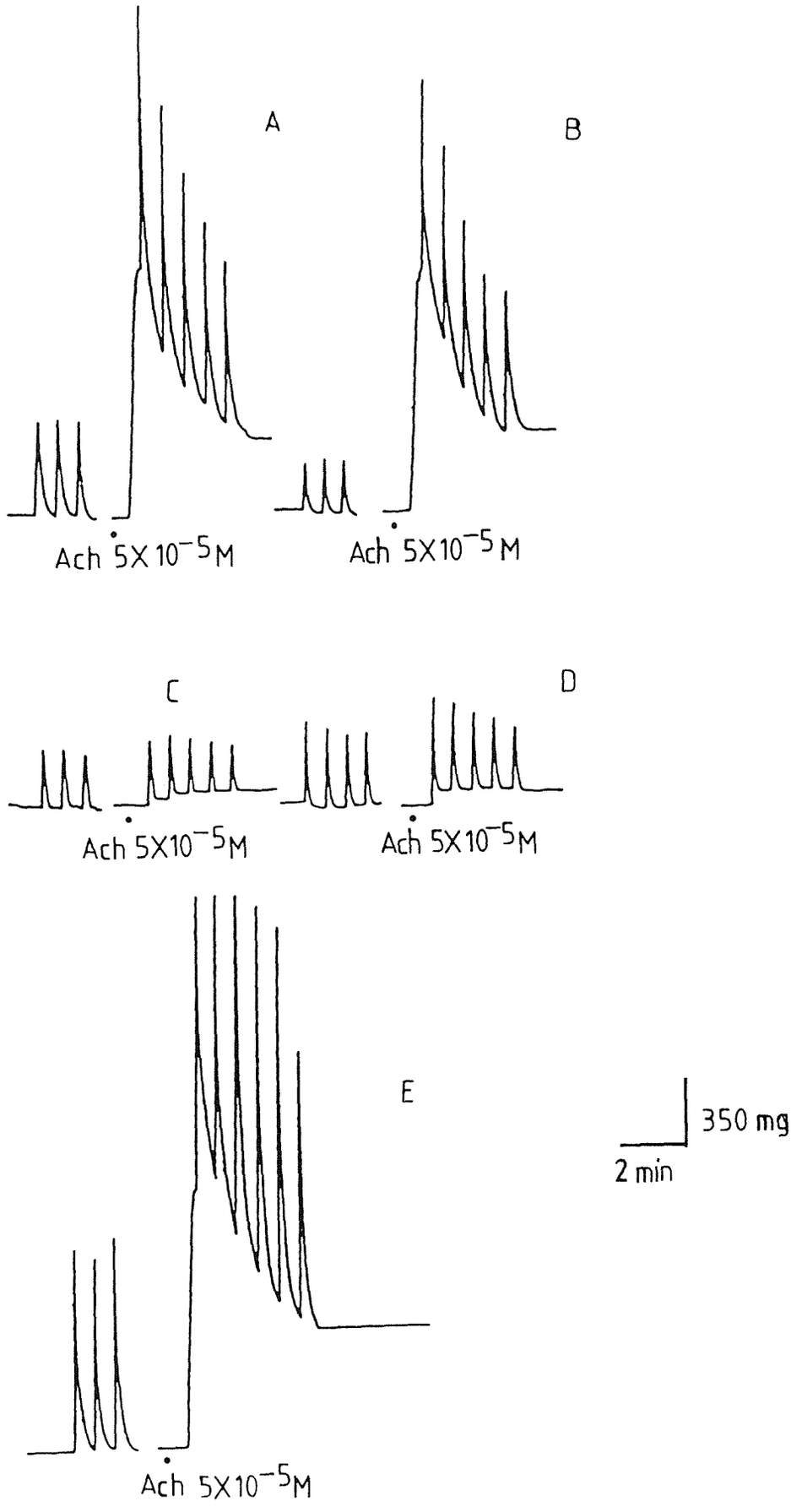

A
B
Ach 5X10⁻⁵M
Ach 5X10⁻⁵M
C
D
Ach 5X10⁻⁵M
Ach 5X10⁻⁵M
E
Ach 5X10⁻⁵M
350 mg
2 min

Figure 9 Shows a comparison between the responses of different compounds showing a variation in the muscle contraction and relaxation.

A Shows the response of the muscle to the application of 5mM decamethonium.

B Shows the response of the muscle to the application of 5mM succinylcholine.

C Shows the response of the muscle to the application of 0.01mM carbachol.

D Shows the response of the muscle to the application of 1mM nicotine.

E Shows the response of the muscle to the application of 0.1mM acetylcholine in the presence of 0.07µM physostigmine.

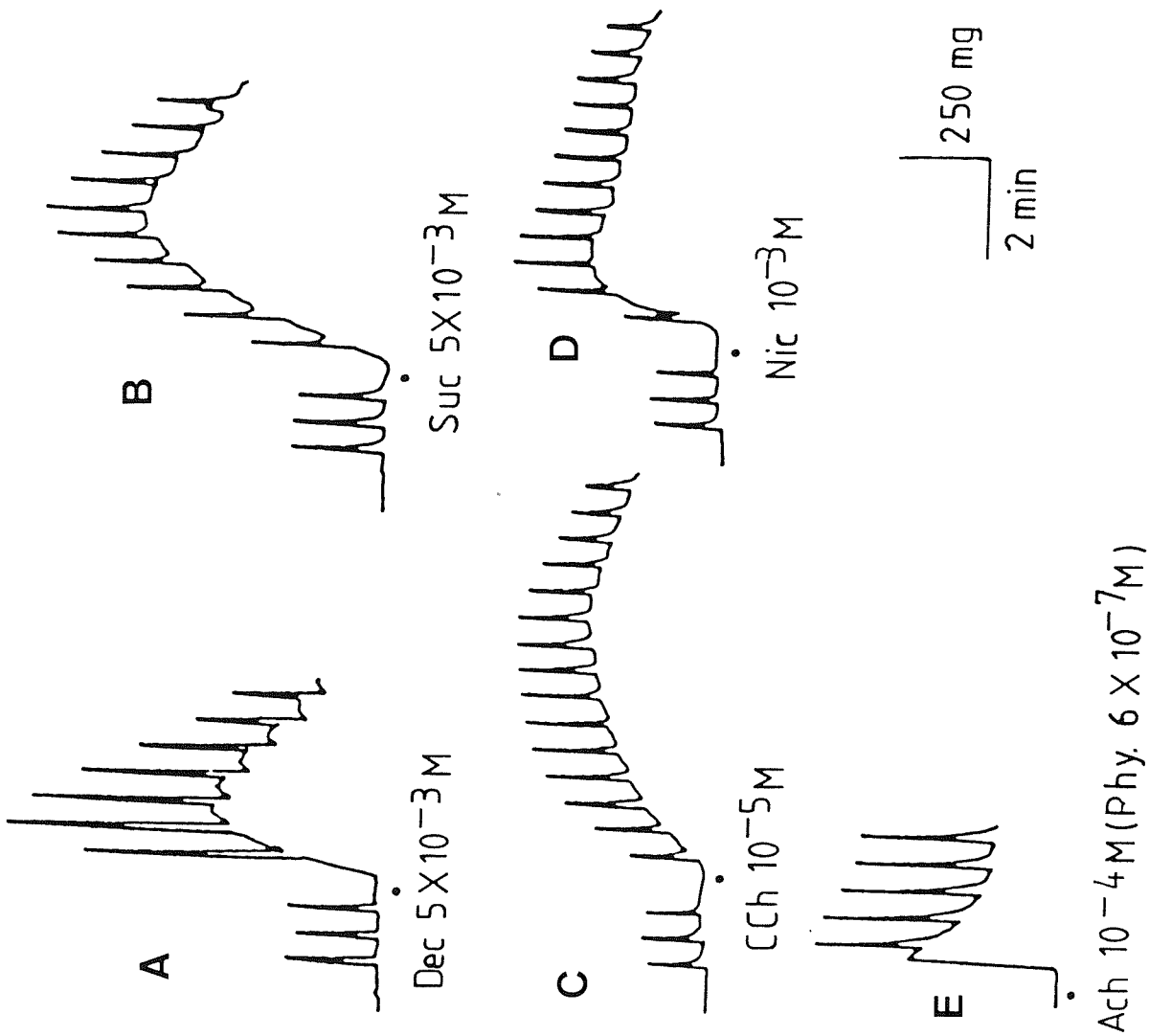
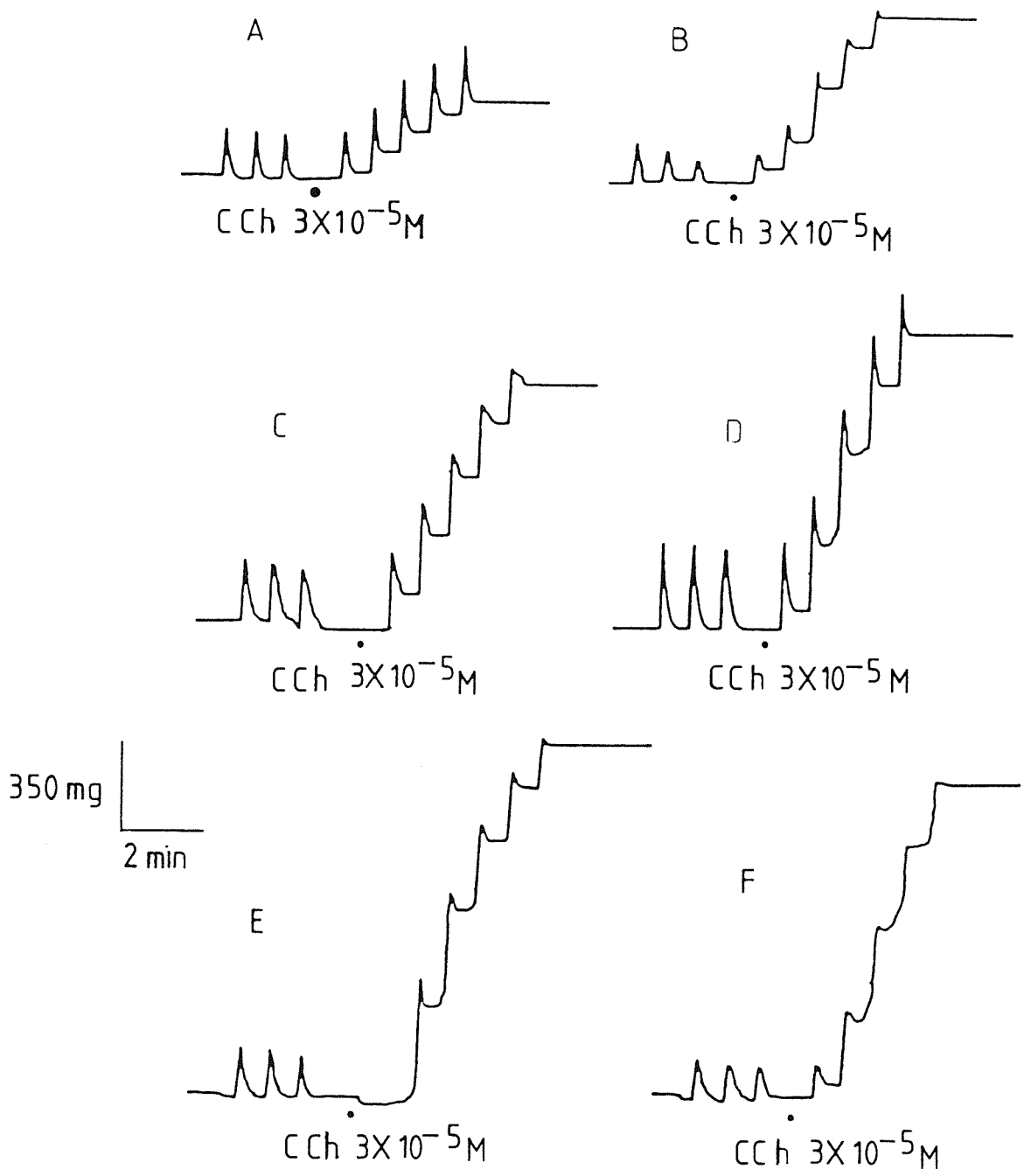

A
Dec 5X10⁻³M
B
Suc 5X10⁻³M
C
CCh 10⁻⁵M
D
Nic 10⁻³M
E
Ach 10⁻⁴M (Phy. 6 X 10⁻⁷M)
250 mg
2 min

Figure 10 Shows the increase in the sensitivity of the muscle to repeated doses of 0.03mM carbachol.


A
CCh 3X10⁻⁵M
B
CCh 3X10⁻⁵M
C
CCh 3X10⁻⁵M
D
CCh 3X10⁻⁵M
350 mg
2 min
E
CCh 3X10⁻⁵M
F
CCh 3X10⁻⁵M

Antagonists

Tubocurarine, atropine and gallamine displaced the dose-response curve of acetylcholine to the right, an example with gallamine is shown in Figure (11). In these experiments physostigmine 0.6µM was added to the saline. PA values were obtained for the three antagonists against acetylcholine and the values obtained were 4.42+\-0.36, 4.36 and 4.95+\-0.37 for tubocurarine, atropine, and gallamine, respectively. The effect of all three antagonists on the acetylcholine responses was similar.

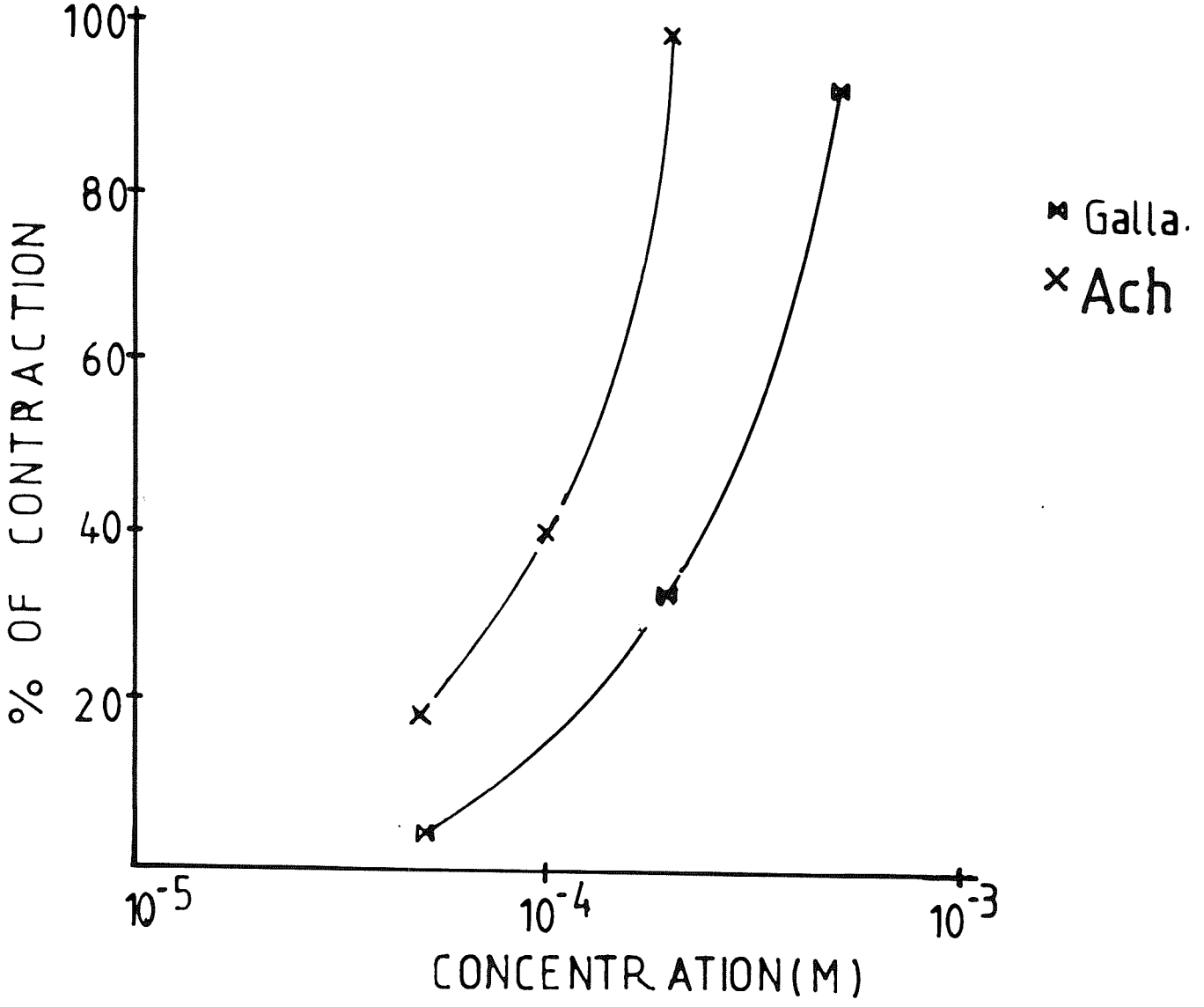
Tubocurarine

Tubocurarine 46µM abolished the response to acetylcholine 10µM completely and also reduced the electrically induced muscle twitch (Figure 12D). This concentration of tubocurarine also reduced the response to 20µM and 50µM acetylcholine (Figure 12 E and F). Figure 9 traces A, B and C are control responses to acetylcholine. Tubocurarine at lower concentrations, 4.6µM, reduced the acetylcholine response by 60% and normally this antagonism could be partially reversed following washing.

Gallamine

Gallamine, 46µM, abolished the response to acetylcholine 10 µM (Figure 13 D)and also reduced the response to 20µM and 50µM acetylcholine (Figure 13E and F). Figure 10 traces A, B and C are control responses to

Figure 11 The effect of gallamine on the contractile response to acetylcholine in the presence of 0.06μM physostigmine.


100
80
60
40
20
% OF CONTRACTION
10-5
10-4
10-3
CONCENTRATION(M)
Galla.
Ach

acetylcholine. This concentration of gallamine also reduced the electrically induced twitches. Gallamine antagonism was easily reversed following washing. Gallamine,46μM, also abolished the response to 10μM nicotine. A lower concentration of gallamine, 4.6μM reduced the contraction to 10 mM nicotine by about 30%. Gallamine was added to the preparation 1.5 minutes following the addition of nicotine and two minutes later the nicotine response was reduced to 40% of its maximum value. Normally the contraction to nicotine would have lasted for much longer duration.

Atropine

Atropine, 46μM reduced the response to 0.1mM acetylcholine by around 70%. A higher dose of acetylcholine, for example 0.5mM, would overcome the block due to atropine. This suggests that the block was competitive. In some experiments following washing out the atropine the response was potentiated compared to the initial control. At this concentration, atropine also reduced the electrically induced twitch responses. Atropine, 46 μM, also reduced the response to 10 mM nicotine by about 50%. This antagonism was reversed following washing.

Mecamylamine

At concentrations of 2.4μM to 40μM, mecamylamine reduced the response to acetylcholine, 50μM, by 70%. This antagonism was partially reversed following washing. Mecamylamine also reduced electrically induced twitches.

Hexamethonium

Hexamethonium at a concentration of 4μM reduced the response to acetylcholine, 50 μM,by about 70%. Following washing the acetylcholine response in two out of three experiments was potentiated. Hexamethonium also reduced the electrically induced twitch contraction.

Hemicholinium-3

Hemicholinium 40μM was allowed to equilibrate with the tissue for 50 minutes. The muscle was stimulated electrically every ten minutes during this period. Finally physostigmine 0.6μM was added five minutes prior to the addition of acetylcholine 50μM in order to see the effect of acetylcholine following 50 minutes incubation with hemicholinium. At this concentration hemicholinium was found to reduce both the amplitude of the electrically induced twitches and the response to acetylcholine. Following repetitive stimulation of the muscle at a rate of 5-7 Hz for four second periods every thirty seconds, the amplitude of the twitch declined to a constant value (Fig 14 X-X). In the presence of hemicholinium 0.4 μM the twitch decline more rapidly and was finally abolished (Fig 14 O-O). The rate at which the twitch declined in the presence of hemicholinium was greater at higher rates of stimulation, for example 20 Hz (for 4 second periods every 30 seconds) compared to 5 Hz with a concentration of 0.4 μM hemicholinium. Figure (15) shows that following application of hemicholinium the muscle twitch declined to zero trace B. Trace A is the control following abolition of the twitch in the presence of hemicholinium, raising the voltage to 25V and then to 30 V restored the twitch briefly but then decline (Figure 15 traces C and D). Hemicholinium 0.4 μM had little effect on the acetylcholine response (Figure $16D_4$) but at 4μM the

acetylcholine response was reduced (Figure 16C_3). Following the addition of hemicholinium there was only a slight decline in the twitch amplitude for the first 8-10 minutes and then the amplitude declined more rapidly. Following the removal of the hemicholinium the preparation took at least 30 minutes to recover.

α and β-Bungarotoxin

Following the exposure of the muscle to 2μM α-bungarotoxin for 30 minutes the twitch contraction was abolished (Figure 17 C_1). Under these conditions the response to acetylcholine 10 μM was reduced by around 50% (Figure 17 C_2), compared to the control traces A and B. Following prolonged washing there was partial recovery in the response to acetylcholine (Figure 17 D and E). When the exposure time to α-bungarotoxin was increased to 45 minutes the response to 10 μM acetylcholine was abolished (Figure 18 C_2) and there was no sign of subsequent recovery after 30 minutes washing (Figure 18D). However after washing for 60 minutes there was some slight recovery, up to 35% in some instances (Figure 18E). As can be seen in figure 18C_1 the twitch response was abolished .

β-Bungarotoxin 2 μM was applied to the muscle which was continually stimulated. After about 15 minutes the twitch began to decline (Figure 19C). Under these conditions the response to acetylcholine was enhanced compared with the control (Figure 19D). Following, washing the response to acetylcholine was further enhanced compared to either the control or in the presence of β-bungarotoxin.

Figure 12 Effect of d-tubocurarine on the contractile response of the earthworm muscle to acetylcholine. The dose response curve was obtained in the presence of 0.07µM physostigmine

A 0.01mM Acetylcholine.

B 0.02mM Acetylcholine.

C 0.05mM Acetylcholine.

D The effect of 0.046mM d-tubocurarine on the muscle response to 0.1mM acetylcholine.

E The effect of 0.046 d-tubocurarine on the muscle response to 0.02mM acetylcholine.

F The effect of 0.046mM d-tubocurarine on the muscle response to 0.05mM acetylcholine

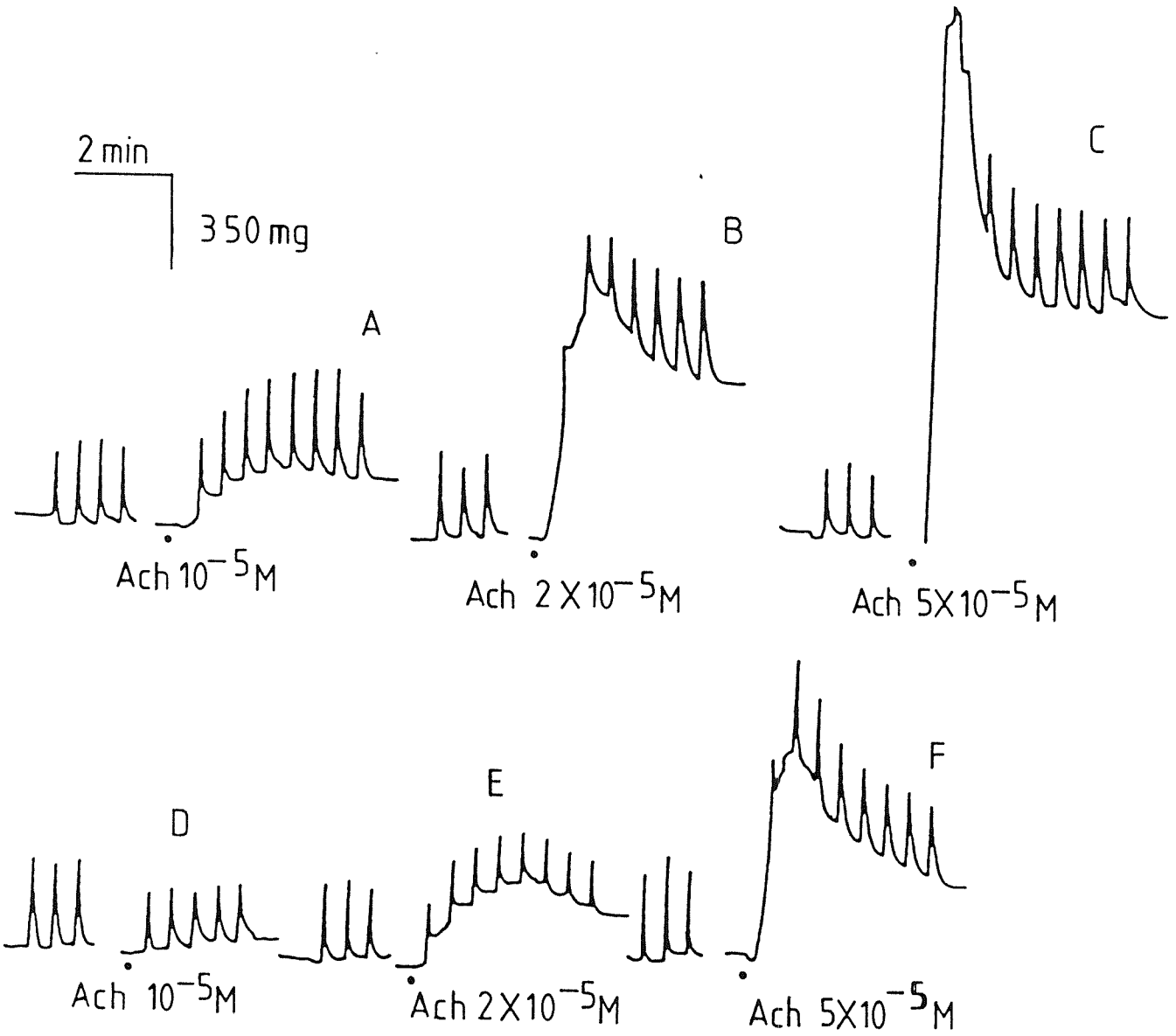

2 min
350 mg
A
B
C
Ach 10^{-5}M
Ach $2X10^{-5}$M
Ach $5X10^{-5}$M
D
E
F
Ach 10^{-5}M
Ach $2X10^{-5}$M
Ach $5X10^{-5}$M

Figure 13 The effect of gallamine on the contractile response of the earthworm muscle to acetylcholine.

A 0.01mM acetylcholine.

B 0.02mM acetylcholine.

C 0.05mM acetylcholine.

D The effect of 0.046mM gallamine on the muscle response to 0.01mM acetylcholine.

E The effect of 0.046mM gallamine on the muscle response to 0.02mM acetylcholine.

F The effect of 0.046mM gallamine on the muscle response to 0.05mM acetylcholine.

G The effect of 0.046mM gallamine on the muscle response to 0.1mM acetylcholine.

H The muscle recovery response to 0.02mM acetylcholine.

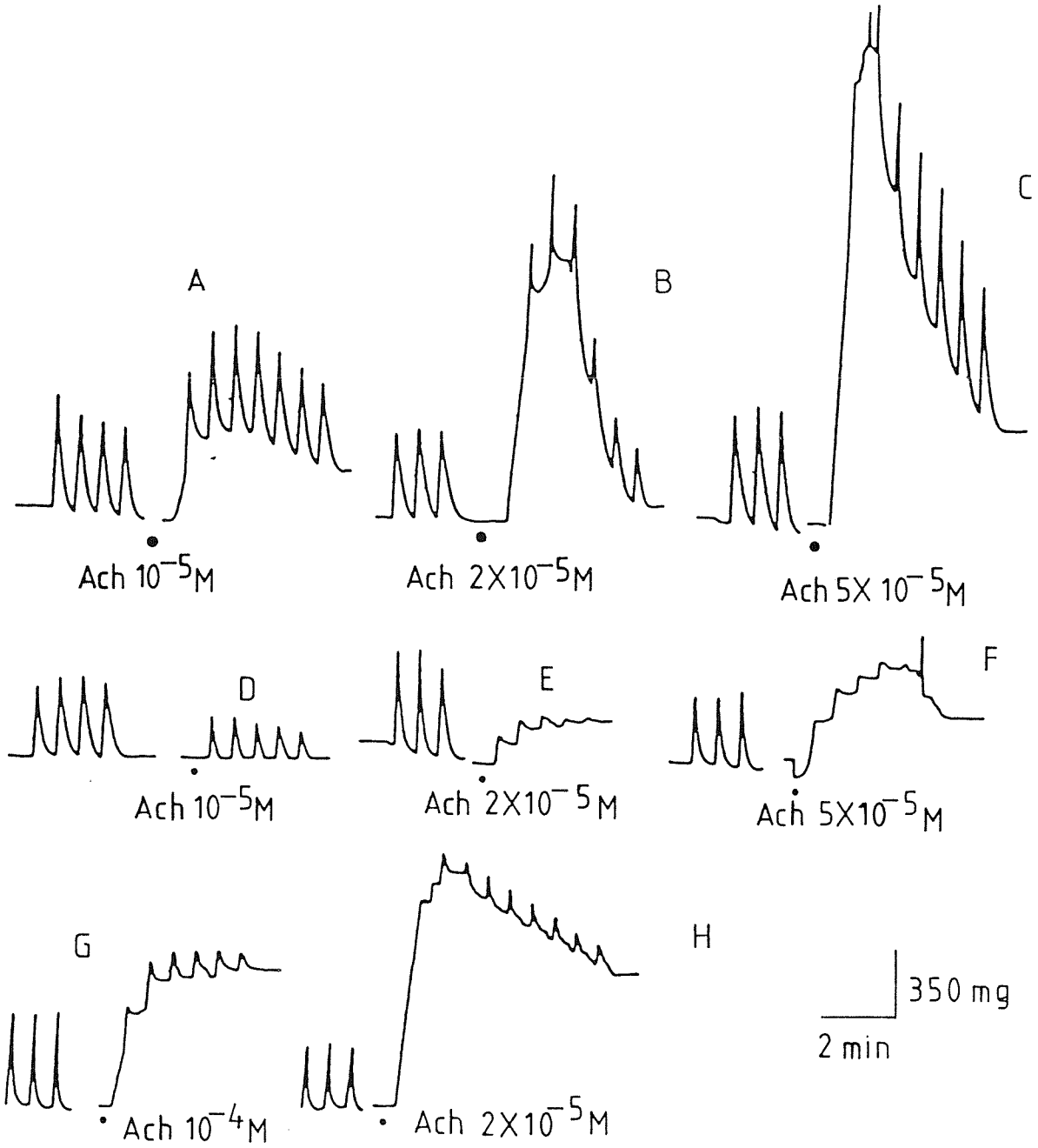

A
B
C
Ach 10^{-5}M
Ach $2X10^{-5}$M
Ach $5X10^{-5}$M
D
E
F
Ach 10^{-5}M
Ach $2X10^{-5}$M
Ach $5X10^{-5}$M
G
H
350 mg
2 min
Ach 10^{-4}M
Ach $2X10^{-5}$M

Figure 14 Shows the decline in amplitude of muscle twitch with continuous stimulation in the control(x-x) and in the presence of 0.04μM hemicholinium-3 (o-o).

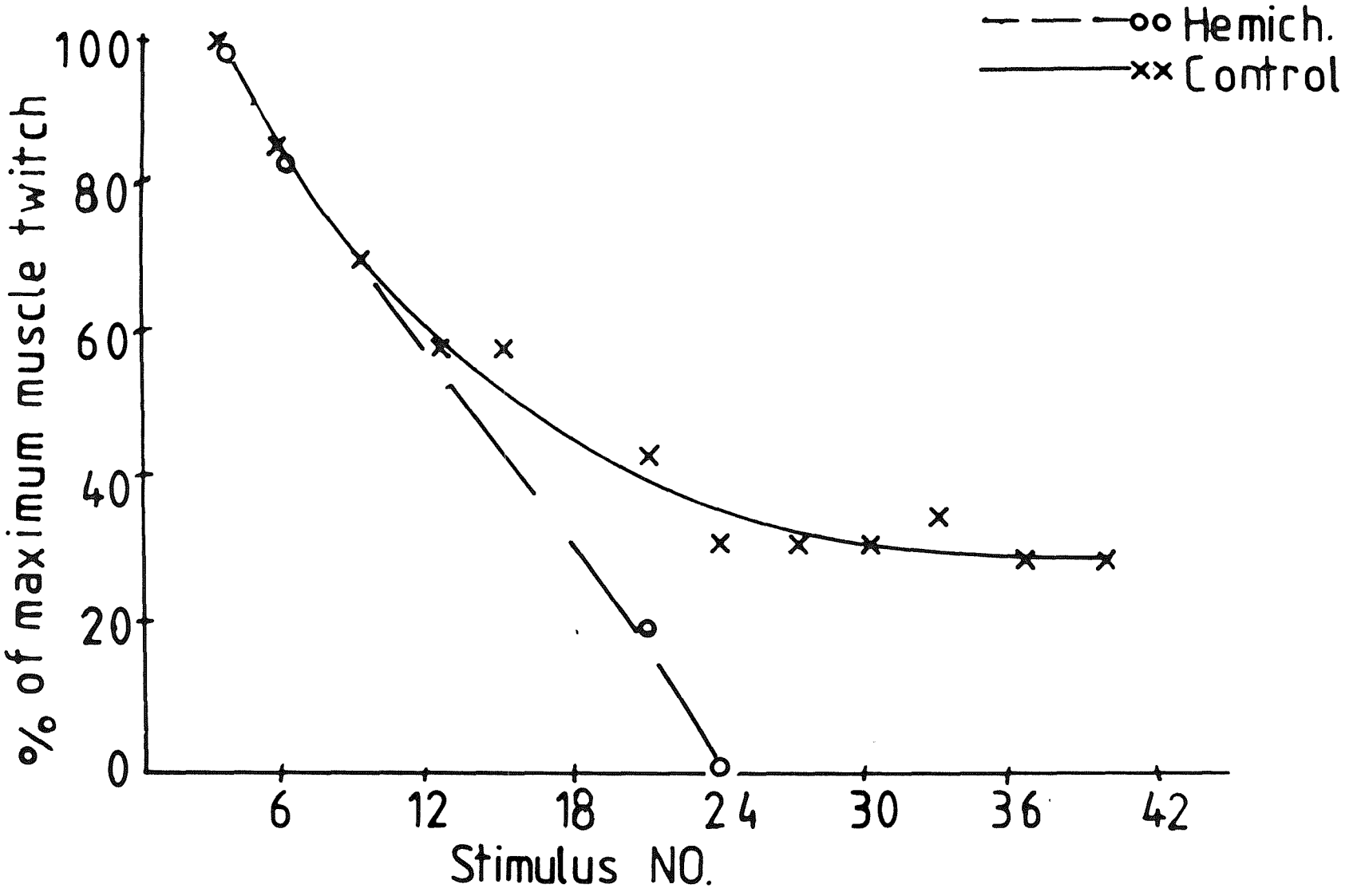

Hemich.
Control
% of maximum muscle twitch
100
80
60
40
20
0
6
12
18
24
30
36
42
Stimulus NO.

Figure 15 Traces to show the effect of repeated stimulation on the amplitude of the earthworm muscle twitch in the presence and the absence of hemicholinium.

A Electrical stimulation for 35 minutes as a control.

B 0.4μM hemicholinium.

C Increase the voltage from 20 to 25V.

D Increase the voltage to 30V.

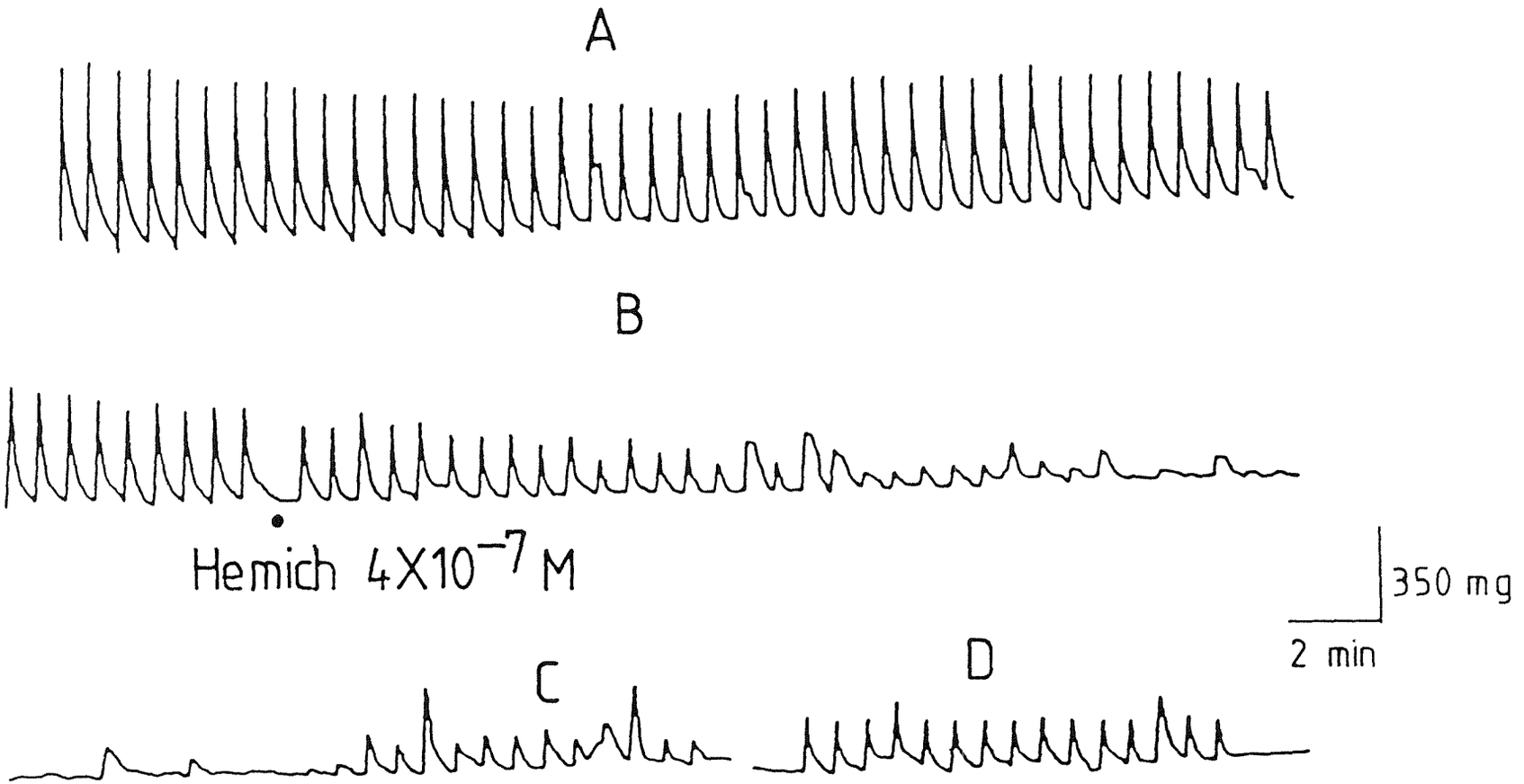

A
B
Hemich 4X10⁻⁷ M
350 mg
2 min
C
D

Figure 16 Shows the effect of hemicholinium on the muscle response to acetylcholine in the presence of 0.6μM physostigmine.

A & B 0.02mM acetylcholine as a control.

C_1 4μM hemicholinum-3 for 6 minutes.

C_2 4μM hemicholinium-3 for 11 minutes.

C_3 0.02mM acetylcholine.

D_1 0.04μM hemicholinium for 6 minutes.

D_2 0.04μM hemicholinium-3 for 11 minutes.

D3 0.04μM hemicholinium-3 for 13 minutes

D_4 0.02mM acetylcholine.

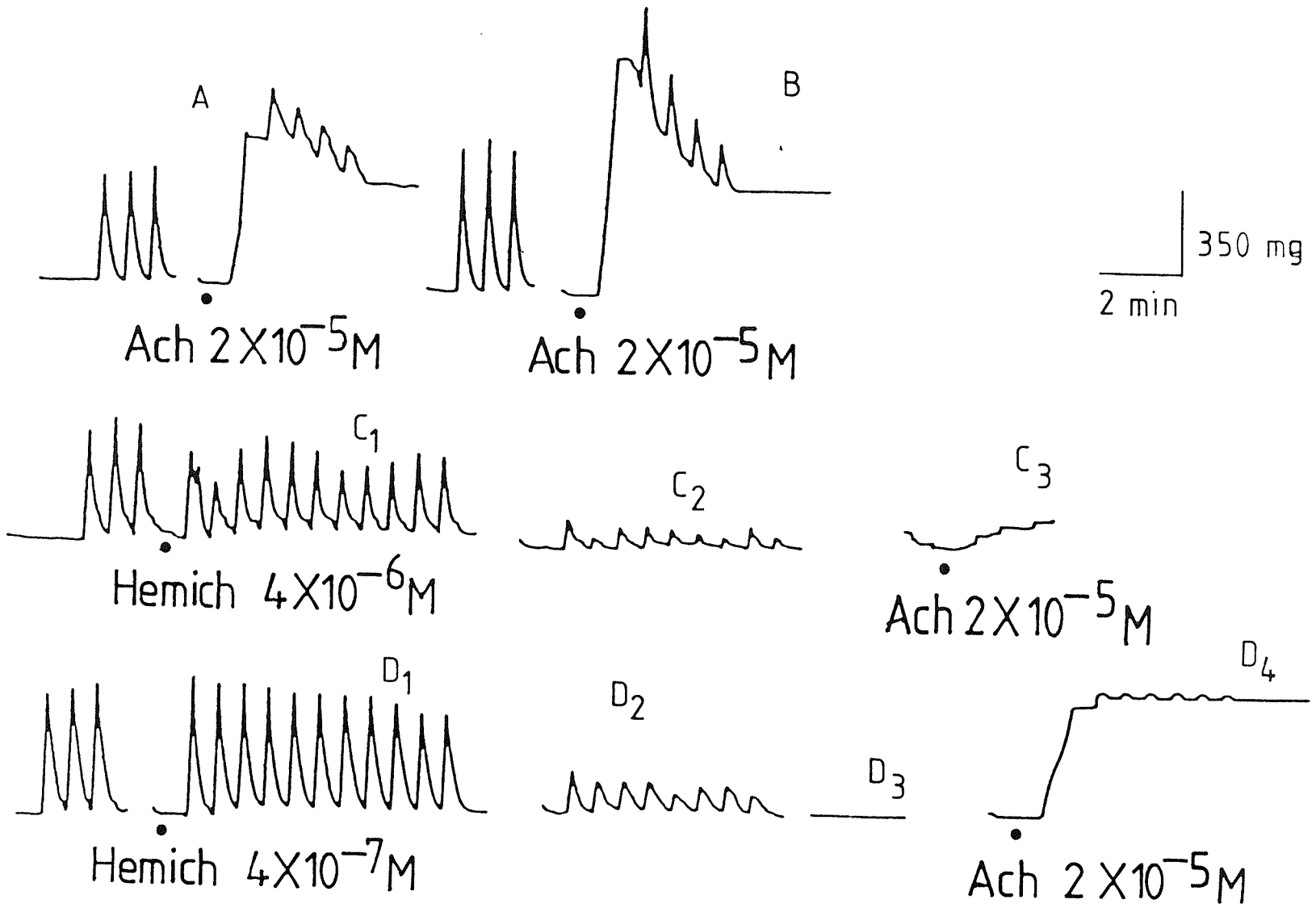

A
B
350 mg
2 min
Ach 2X10⁻⁵M
Ach 2X10⁻⁵M
C1
C2
C3
Hemich 4X10⁻⁶M
Ach 2X10⁻⁵M
D1
D2
D3
D4
Hemich 4X10⁻⁷M
Ach 2X10⁻⁵M

Figure 17 The action of 2μM α-bungarotoxin on the earthworm muscle twitch and on the response to acetylcholine in the presence of 0.6μM physostigmine.

A & B 0.1mM acetylcholine as controls.

C_1 2μM α-bungarotoxin applied to the muscle for 30 minutes the followed by electrical stimulation.

C_2 0.1mM acetylcholine response following 2μM α-bungarotoxin incubation.

D 30 0.1mM acetylcholine recovery after minutes.

E 0.1mM acetylcholine recovery after 60 minutes.

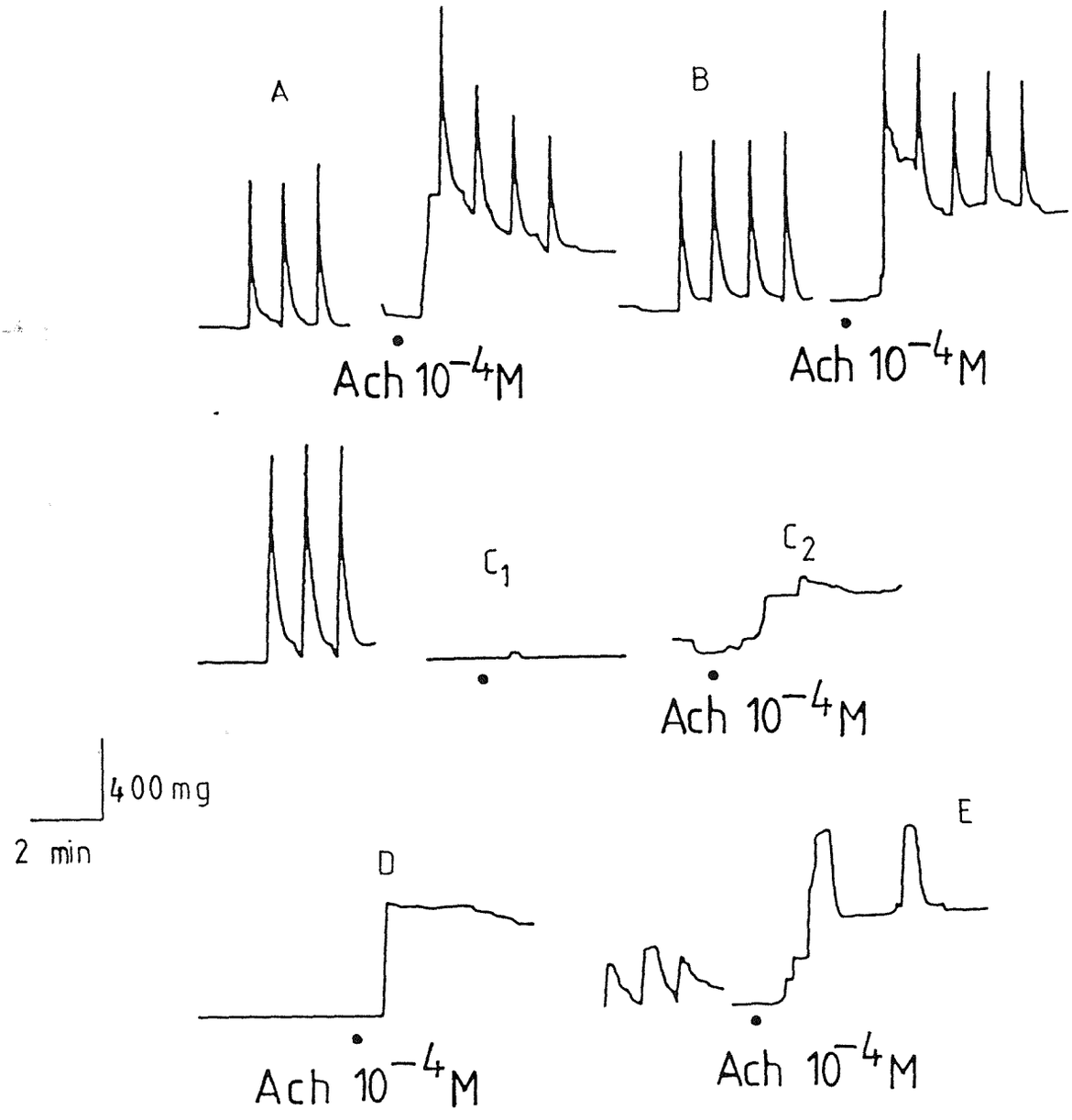

A
B
Ach 10^{-4}M
Ach 10^{-4}M
C_1
C_2
Ach 10^{-4}M
400 mg
2 min
D
E
Ach 10^{-4}M
Ach 10^{-4}M

Figure 18 The effect of α-bungarotoxin on the contractile response of earthworm to acetylcholine in the presence of 0.6μM physostigmine.

A & B 0.1mM Acetylcholine as controls.

C_1 2μM α-Bungarotoxin applied for 15 minutes and then followed by electrical stimulation.

C_2 0.1 mM Acetylcholine following exposure to 2μM α-bungarotoxin for 45 minutes.

D 0.1mM Acetylcholine recovery after 30 minutes.

E 0.1mM Acetylcholine recovery after 60 minutes.

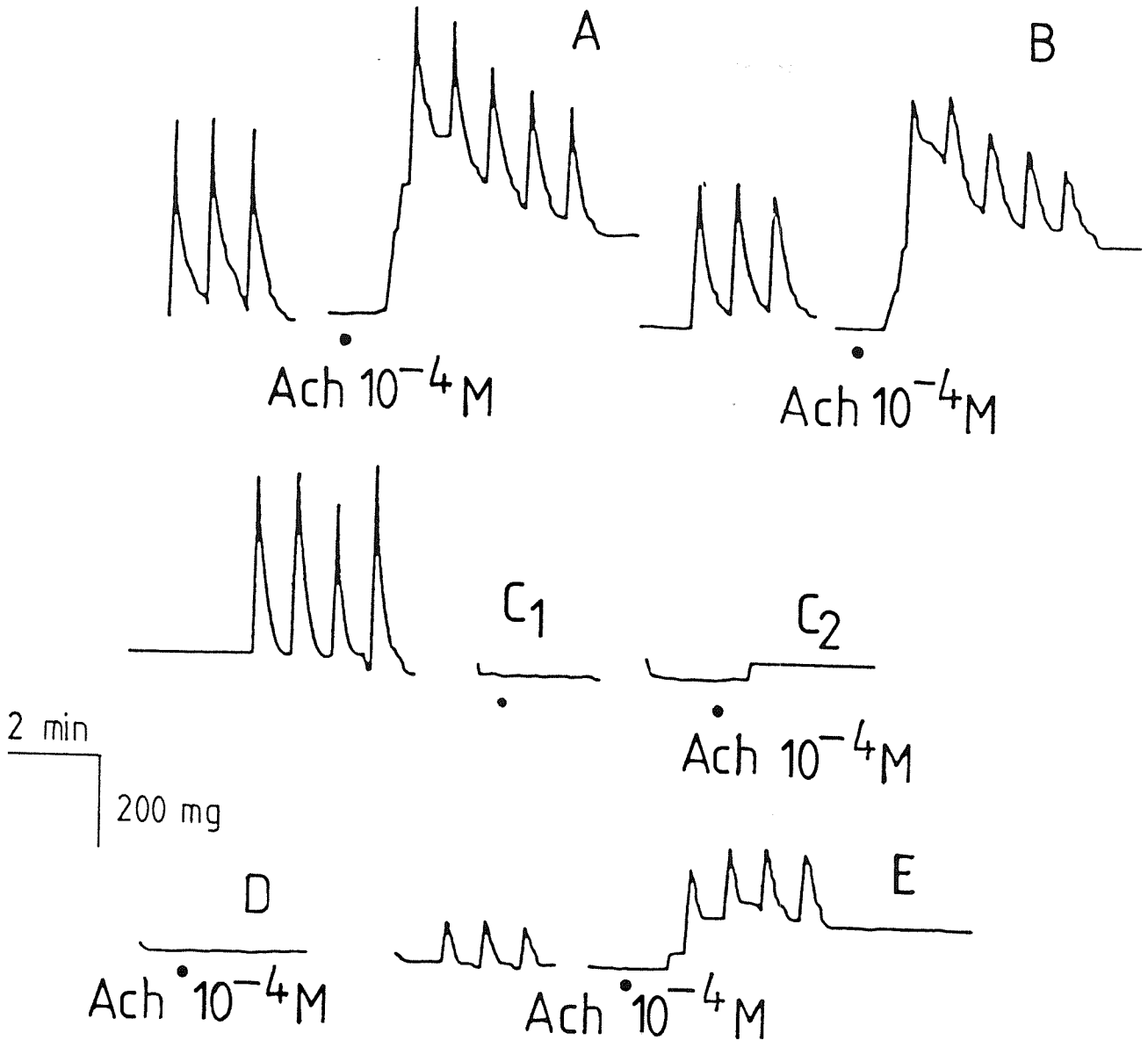
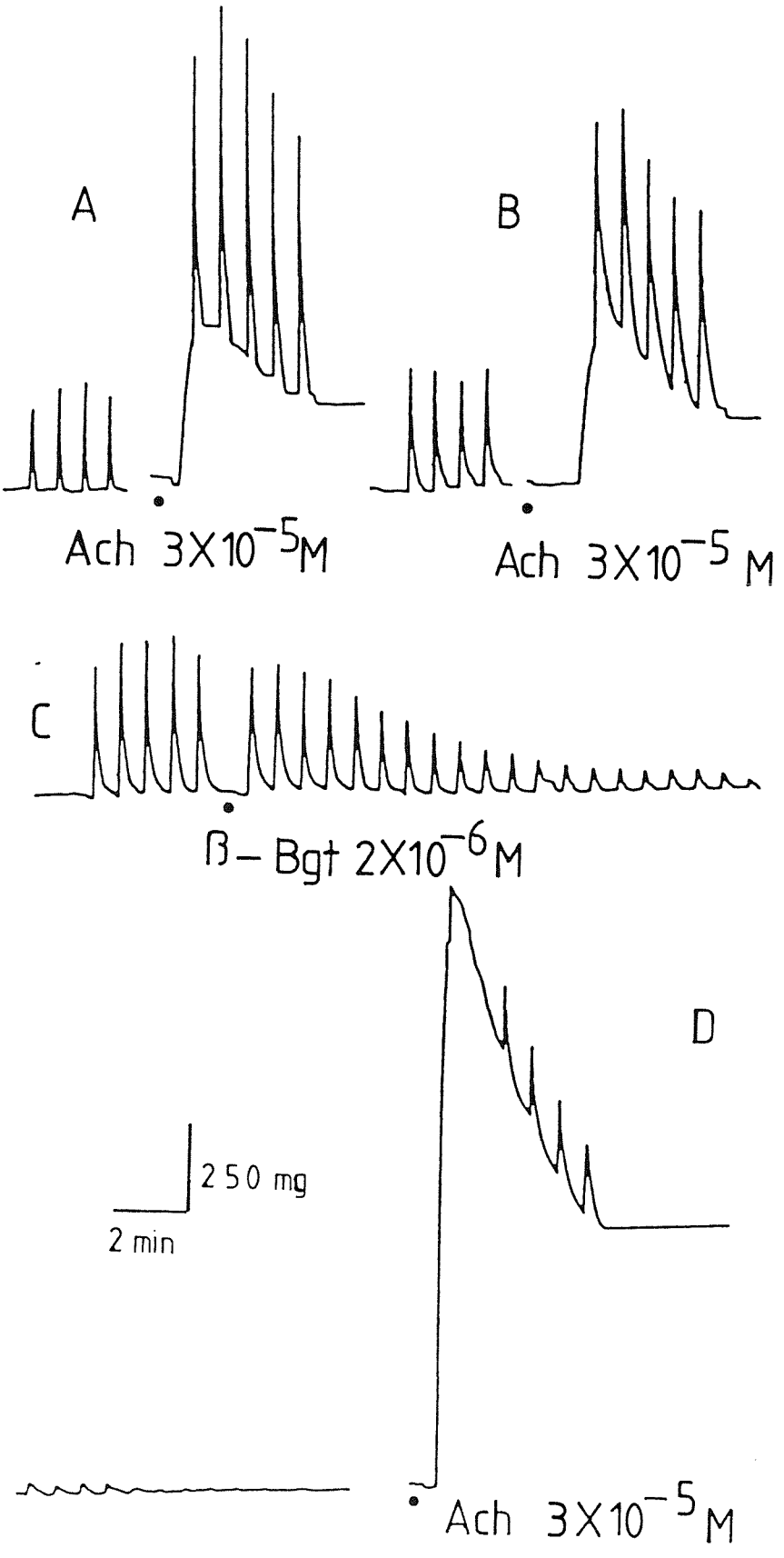

A
B
Ach 10^{-4}M
Ach 10^{-4}M
C_1
C_2
Ach 10^{-4}M
2 min
200 mg
D
E
Ach 10^{-4}M
Ach 10^{-4}M

Figure 19 The action of 2μM β-bungarotoxin on the earthworm muscle twitch with continuous stimulation and on the response to acetylcholine in the presence 0.6μM physostigmine.

A & B 0.03mM acetylcholine control.

C 2μM β-bungarotoxin applied for 15 minutes.

D 0.03mM acetylcholine in the presence of the toxin.


A
B
Ach 3X10⁻⁵M
Ach 3X10⁻⁵ M
C
β– Bgt 2X10⁻⁶M
D
250 mg
2 min
Ach 3X10⁻⁵M

DISCUSSION

The results from the present study show that the acetylcholine receptor on the body wall muscle of *Lumbricus* cannot be classified as either nicotinic or muscarinic. This conclusion is true in terms of both cholinomimetics and cholinolytics. For example, nicotine and muscarine are both very much less potent than acetylcholine but are of the same order of potency, that is, 100 and 230 times respectively less potent than acetylcholine. In terms of the characteristics of the response nicotine resembles acetylcholine to a greater extent than muscarine since its maximum effect approaches that of acetylcholine and the time to peak of the contraction elicited by nicotine is similar to that of acetylcholine. Muscarine has a very slow time to peak of the response and the maximum response is far less than that of acetylcholine, table 3. The other muscarinic agonist tested, bethanechol, also induced a small contraction with a fairly slow time to peak of the response. The responses to acetylcholine and electrical stimulation would appear to be due primarily to sodium since removal of external sodium from the saline virtually abolished the response. The response in calcium free saline was also almost completely abolished but this is possibly associated with the requirement for calcium in muscle contraction.

In terms of the main antagonists tested, tubocurarine, atropine and gallamine, all three had similar PA values against acetylcholine and so were similar in their ability to antagonise acetylcholine. Flacke and Yeoh (1968b) found a difference between the PA values for tubocurarine (5.68) and gallamine (4.44) against acetylcholine on leech body wall muscle. This value for gallamine agrees well with the value of PA obtained for *Lumbricus* body wall muscle. In

their study Flacke and Yeoh suggest there are separate receptors for acetylcholine and for succinylcholine and decamethonium. Further studies are required before one can comment as to whether a similar position occurs in *Lumbricus*. Gallamine antagonism against acetylcholine on *Lumbricus* body wall muscle was readily reversed on washing. Gallamine also completely blocked the response to nicotine while atropine at a similar concentration only reduced the nicotine response by about half. This suggests that atropine was less effective against nicotine compared to gallamine. Mecamylamine and hexamethonium also reduced the response to acetylcholine. All five antagonists reduced the electrically induced twitch contractions, providing evidence that they may be due to the release of acetylcholine. Interestingly in their study Gardner and Cashin (1975) failed to obtaine a block of acetylcholine using tubocurarine, gallamine or atropine.

Of the agonists tested, carbachol was clearly the most potent in this study and this finding agrees with data quoted in the review of Gardner and Walker (1982) where carbachol was the most potent agonist, being about 50 times more potent than acetylcholine. However in this case this was compared to acetylcholine in the absence of physostigmine and so allowing for this, the potencies in the present study are similar to those quoted above. Physostigmine enhances the response to acetylcholine at least ten times in *Lumbricus*. In the leech physostigmine enhances the response to acetylcholine around 1000 times (Flacke & Yeoh, 1968a) indicating that in the leech the cholinesterase activity is greater than in the earthworm. In the present study nicotine was less potent than the values quoted by Gardner and Walker (1982). However in the leech nicotine is about 20 times less potent than acetylcholine in the presence of physostigmine (Flacke and Yeoh 1968a), a value more in agreement with the current study. Carbachol

was 10 times more potent than acetylcholine in the leech, again a value which agrees well with the relative potency of around 8 from the present study. Succinylcholine and decamethonium were both around 200 times less potent than acetylcholine on leech muscle which suggests they are slightly less potent on leech than on earthworm body wall muscle. Although tetramethylammonium was considerably less potent than acetylcholine on *Lumbricus* muscle it did resemble acetylcholine in possessing a rapid rise time for the response and had a maximum contraction which resembled that of acetylcholine, suggesting that it was a full agonist.

Hemicholinium has been used in many studies to inhibit choline uptake into cholinergic nerve terminals and induce depletion of stores of acetylcholine (MacIntosh, et al, 1956; Elmqvist and Quastel 1965; Evans and Wilson,1964). In the present study incubation with hemicholinium resulted in a loss of the twitch contraction due to electrical stimulation of the body wall muscle. This finding would be consistent with the hypothesis that the electrically induced twitch resulted from the release of acetylcholine from cholinergic nerve terminals in the muscle. This effect of hemicholinium occurred at concentrations which had little postsynaptic action against acetylcholine. However the concentration of hemicholinium is critical since raising the concentration results in a postsynaptic action which antagonises acetylcholine.

The snake venom, α-bungarotoxin has been used in many preparations to irreversibly antagonised the action of acetylcholine(Berg, et al 1972; Mebs, et al 1972; Miledi & Potter 1971; Magazanik 1976). This toxin antagonises the action of acetylcholine at certain acetylcholine receptors on gastropod neurones and *Limulus* central neurones (Kehoe, et al 1976; Walker & Roberts 1984). There have in contrast

been relatively few studies on β-bungarotoxins though this toxin has been suggested to enhance transmitter efflux from nerve terminals, for example, glutamate, (Smith, et al 1980). However other workers have found that β-bungarotoxin blocked neuromuscular transmission by inhibiting acetylcholine release from motor nerve terminals (Chang, Chen & Lee 1973) possibly by altering the membrane conformation associated with calcium transport (Lin Shiau, Yang & Lee 1978) and/or hydrolysing phospholipids in nerve terminal membranes(Strong, Heuser, Oberg & Kelly 1978). In the present study β-bungarotoxin abolished the muscle twitch which could be expected if it were cholinergic and were inhibiting release. In addition β-bungarotoxin potentiated the response to acetylcholine and this potentiation was long lasting, remaining following washing. On *Lumbricus* body wall muscle, α-bungarotoxin abolished the twitch contraction following electrical stimulation and also reduced the response to acetylcholine. With shorter exposure time to α-bungarotoxin there was partial recovery of the acetylcholine response following washing but with longer exposure times the block was complete and there was relatively little recovery. In general the block with this toxin is fairly irreversible (Miledi & Potter 1971) but does depend on exposure time. The action of α-bungarotoxin has been investigated against carbachol and succinylcholine on leech body wall muscle (Ross & Triggle 1972). They found that while α-bungarotoxin had no effect against carbachol it did block the action of succinylcholine providing further evidence for two types of cholinoreceptor on this muscle.

The present study provides further evidence of a role for acetylcholine as a excitatory transmitter onto *Lumbricus* body wall muscle. For example, the action of hemicholinium and β-bungarotoxin on the twitch response would both support this possibility. α-Bungarotoxin also blocked the twitch and the response to acetylcholine which provides additional

evidence. Evidence from agonist and antagonist studies would suggest an acetylcholine receptor which cannot easily be classified as either muscarinic or nicotinic. This latter observation contrasts with the situation in the leech where the receptor on the body wall muscle appears to be nicotinic. From a comparative view point since it is very likely that acetylcholine is an excitatory transmitter onto body wall muscle in leeches then it is equally probable that it is also a peripheral transmitter in the earthworm.

CHAPTER 4

NEUROPHYSIOLOGICAL STUDY ON SOME ANTHELMINTIC COMPOUNDS ON SNAIL NEURONES.

INTRODUCTION

The intracellular fluid of *Helix* neurone has a different composition from the hemolymph therefore there are ionic gradients across the nerve membrane. The ionic distribution depends on permeability, electrochemical gradient and the activity of ions across the membrane. The equilibrium potential for an individual ion can be calculated from the Nernst equation if the intracellular and extracellular ions are known. Responses to neurotransmitters and applied drugs can be mediated via a direct change in the membrane conductance to an ion or ions by activating receptors and so causing ion channels to open or close and therefore the distribution of ions across the membrane can change. When membrane conductance to a specific ion is increased the ions will tend to move down their electrochemical gradient, therefore changing the cell membrane potential towards the equilibrium potential of the ion.

Acetylcholine can excite and depolarise the cell membrane or inhibit and hyperpolarise the cell membrane. Chad et al (1979) carried out ramped voltage clamp investigations of the ions involved in these responses on *Helix* neurones. The inhibitory hyperpolarising response was found to be mediated via an increase in chloride conductance with a reversal potential of -64mV. They found two types of acetylcholine excitatory response, one which is mediated purely by an increase in sodium permeability and a second type which involves an increase in both sodium and chloride conductance. In other molluscan species acetylcholine can also give an inhibitory response that is produced by increase in potassium permeability (Katchman et al, 1980;

Swann and Carpenter, 1975).

Where the inhibitory responses are associated with an increased membrane conductance, the ions involved would be chloride or potassium. These ions have equilibrium potentials more negative than the membrane potential in molluscan neurones (Marmore, 1975). For D-responses the likely candidates are sodium and calcium ions.

In terms of ion substitution the replacement ion must be impermeable to the cell membrane. The external ion should be exchanged with another ion of similar charge or by removing a similar number of ions with positive charges. The first case is most used. This type of replacement does not affect the ionic strength of the saline. The ions available for substitution are, for example, sulphate, isothionate, citrate, acetate etc. Christoffersen and Skisted (1975) reported that part of the effect of removing chloride ions is due to the calcium binding properties of some of the replacement ions used. Wilson et al (1977) reported that Tris antagonised the action of ionophortically applied acetylcholine on neurones of *Aplysia californica* at concentration 5 to 10mM. They found that the time required for Tris to act varied between 1 to 10 minutes. The site of action of Tris, whether acting intracellular or extracellular is not clear. Wilson et al (1977) suggested that Tris enters the cell membrane and interfers with the transmitter-receptor interaction and could antagonise the binding of acetylcholine to its receptor. According to Colquhoun (1981) Tris is a non-selective blocker which binds rapidly and equally to open and shut channel states without changing the rate of channel opening or closing.

A number of compounds have been developed as anthelmintic agents but their precise mode of action has not

been fully elucidated in all cases. However the possibility that at least certain of these compounds might act at the level of the acetylcholine receptor has been suggested from a number of studies. For example, levamisole is a potent anthelmintic whose pharmacological mode of action is yet to be rigorously determined. It causes rapid contraction of nematode muscles followed by spastic paralysis and at high doses, death (Thienpont et al, 1966). Levamisole's primary biological effects, especially as an anthelmintic, seems to be neurological. Levamisole depolarises nematode muscles (Aceves et al, 170). Its action on nematode muscle can be blocked by mecamylamine and pempidine, vertebrate cholinergic ganglion blocking agents (Coles et al, 1974), but its action is not well blocked by d-tubocurarine or atropine (Aceves et al 1970). Lewis et al (1980) showed that the effect of levamisole on the body muscle of the nematode *Caenorhabditis elegans* is most certainly attributable to cholinergic stimulation.

Pyrantel tartrate was the most promising of a new class of anthelmintics announced by Austin et al (1966). On nematodes, pyrantel acts like a cholinergic agonist (Aubry et al, 1970), and is similar in potency to levamisole. Pyrantel had a strong nicotinic depolarising effect on nematode muscle and much weaker nicotinic depolarising blocking effect on vertebrate skeletal muscle. However, Aubry et al (1970) pointed out that piperazine caused a relaxation of *Ascaris* strip preparations and in common with tubocurarine blocked the response to acetylcholine and pyrantel analogues on this preparation.

Voltage and current clamp studies have demonstrated that morantel, pyrantel and levamisole depolarise *Ascaris* muscle cells and increase their permeability (Harrow and Gration,1985). These authors presented evidence that the

three compounds act at acetylcholine receptors on this muscle.

Amidantel (Bay d 8815) has been described as the most promising representative of a new class of P-aminophenylamidine anthelmintic drug (Wollweber et al, 1979). This compound was shown to be effective against nematodes, filariae and cestodes in rodents (Wollweber et al, 1979), and was particularly effective against hookworm and ascarids in dogs (Thomas, 1979). Amidantel is rapidly deacylated in vivo to the corresponding free amine (Bay d 9216) which also exhibits anthelmintic activity (Thomlinson, 1985). It has been reported by these authors that effects of amidantel and deacylated amidantel may be antagonised by nicotinic antagonists e.g. d-tubocurarine and gallamine.

Hycanthone is one of the few drugs known which can be used therapeutically for the treatment of Schistomiasis (Rosi et al 1967; Berberian et al, 1967). Hycanthone stimulates the uptake of serotonin by Schistosomes. Hillmam and Senft (1975) pointed out that hycanthone may act by blocking acetylcholine receptors. Their work was based largely upon measurements of the motor activity of *Schistosomes*, in which they found that the paralytic effect of carbachol was blocked by hycanthone. It has been reported by these authors that hycanthone is an inhibitor of acetylcholinesterase in *S. mansoni*.

The synapse is a vulnerable point at which drugs can act and modify the passage of information. A compound may act on the receptor for the transmitter ligand, at the level of the ionophore or at a third adjacent extraceptor site (Walker 1982). This author reported that a drug may act pre-synaptically to modify the release of the endogenous transmitter or affect the uptake system which may be present

either pre- or postsynaptically. Compounds can also modify the metabolism of the transmitter and so alter the duration of its response.

However, the site of action of antagonists either at the receptor or ionophore level, has been the aim of many investigations. This is linked with the possibility that the same ionophore population may be activated by a range of transmitters (Swann and Carpenter 1975).

There is evidence in the literature that the antagonist effect of d-tubocurarine on acetylcholine responses is to some extent contradictory as far as depolarisation (sodium dependent) and hyperpolarisation (chloride dependent) are concerned. Carpenter *et al.*(1977) suggested that the non-specific block made by d-tubocurarine of fast D and H responses of *Aplysia* neurones to different neurotransmitter substances was due to the interaction of d-tubocurarine with ionic channels for sodium and chloride ions respectively. These authors also suggested that d-tubocurarine blocks the responses to several transmitters when these responses are associated with an increase in conductance to the same ion. Pharmacological experiments on *Helix* neurones suggested that d-tubocurarine reacted specifically with D and H receptors of acetylcholine in this preparation (Yavari *et al*, 1979). Furthermore, using noise analysis, Asher *et al*,(1978) suggested that same antagonists, for example, d-tubocurarine and hexamethonium, bind preferentially to the activated acetylcholine receptor-complex. Therefore, they are interacting with both the receptor and the ionophore.

In the present study, it was decided to investigate the effects of a range of anthelmintic compounds on acetylcholine receptors located on central neurones of snail, *Helix aspersa*. The potencies of these compounds were

determined, using acetylcholine as the standard compound. The ionic mechanism was also investigated, to find out which type of mechanism is involved in the drug responses of the identified neurones used. The action of d-tubocurarine was also tested against the response of levamisole, morantel pyrantel and deacylated amidantel.

RESULTS

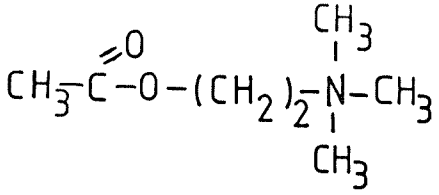
All recordings were made from identified neurones in the suboesophageal ganglionic mass of *Helix aspersa*. The structures of levamisole, morantel, pyrantel, amidantel and its deacylated derivate and hycanthone together with acetylcholine are shown in figure (20). These compounds were applied to "D" and "H" neurones in two ways. Either when the neurones where at resting membrane potential or the membrane was hyperpolarised to a certain value such as (-80 mV). The latter case gives more stable resting membrane potential which leads to more precise readings.

Levamisole and acetylcholine

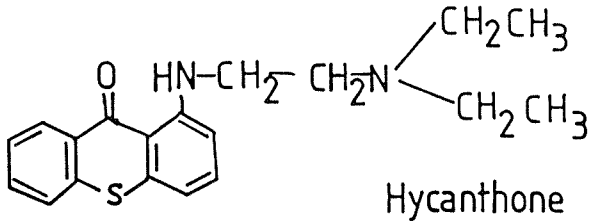
Levamisole has a direct effect on "H" neurones. This effect is long lasting and persisted even following washing for 15-20 minutes in some cases. The hyperpolarising action of levamisole was accompanied by an increase in membrane conductance. On "D" cells levamisole was relatively inactive with a potency ratio of greater than 100 compared to acetylcholine, table 4. Whereas levamisole on "H" neurones was 10.85 +\-0.56 (mean+\-S.E.) (n=7) times less potent than acetylcholine,table 4 and figure 21.

The ionic mechanism of the levamisole inhibitory response was investigated and compared to acetylcholine. When the bathing medium was changed to chloride free saline, the membrane potential was moved towards a more positive value and increased the firing rate and the inhibitory response to levamisole was reversed. On returning to normal

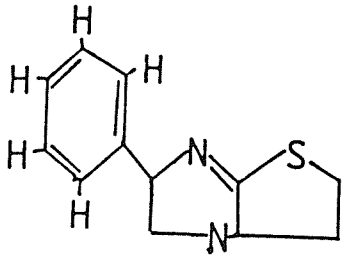
Figure 20 Shows the structures of acetylcholine, levamisole, morantel, pyrantel, amidantel, deacylated amidantel and hycanthone.



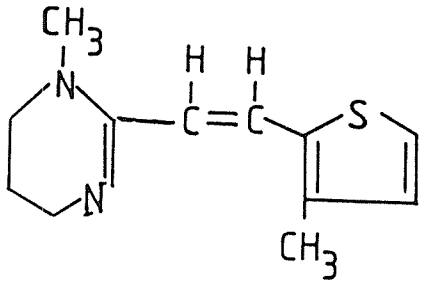
ACETYLCHOLINE



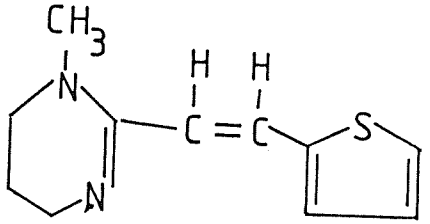
Hycanthone



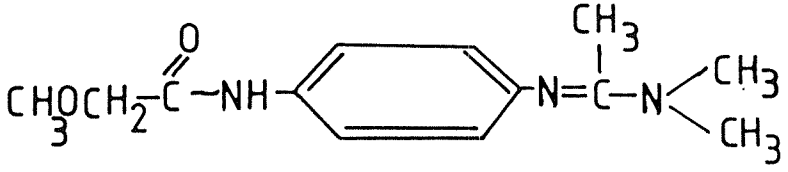
LEVAMISOLE



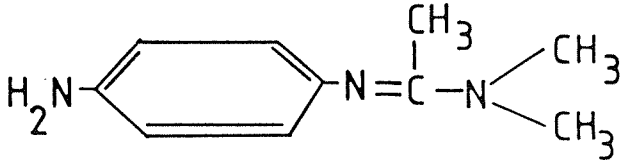
MORANTEL



PYRANTEL



Amidantel



Deacylated amidantel

saline, the recovery of the inhibitory response to levamisole was larger compared with the initial control. Similar results were obtained with acetylcholine.

To test whether levamisole might be acting through an acetylcholine receptor, the action of levamisole was tested while the preparation was immersed in a solution containing 20µM d-tubocurarine. A typical experiment is illustrated in figure 22. The figure shows that the dose of d-tubocurarine greatly reduced the response to 100 µM acetylcholine and blocked the response of 1 mM levamisole. Interestingly, the dose of levamisole 1 mM did not paralyse the cell after the application of d-tubocurarine.

Generally, levamisole gradually depolarised "H" cells and reduced their activity. A typical observation is illustrated in figure (23). This figure shows typical acetylcholine dose-response traces from the cell E7 which acts as a control. When levamisole was applied in increasing concentration, the activity of the cell gradually declined. The resting potential was 46 mV and during the action of levamisole figure (23 D,E,F), the cell initially hyperpolarised to -57 mV and then gradually depolarised. 5.5 minutes after the addition of levamisole 1 mM, the cell was still silent and had to be depolarised via the bridge to an apparent membrane potential of -12 mV to induce activity. After addition of 1mM levamisole it can be seen that the cell resistance remains low. This is probably associated with the observation that cell activity fails to recover.

On "D" cells levamisole had a very weak excitatory effect which was long lasting but in contrast to "H" cells even concentrations as high as 10 mM did not induce a loss of cell activity.

D cells			H cells		
EC-50 (ACh) μM	EC-50 (compound) μM	Relative Potency	EC-50 (ACh) μM	EC-50 (compound) μM	Relative Potenc
27.0 ± 8.0	---- (Levamsole)	>100 (n=4)	33.8 ± 7.96	380.0 ± 98 (Levamsole)	10.85 ± 0.56 (r
27.6 ± 12.34	29.2 ± 11.2 (Morantel)	1.12 ± 0.14 (n=5)	57.6 ± 33.0	320.0 ± 96.2 (Morantel)	5.16 ± 0.6 (n=6
20.75 ± 1.78	48.0 ± 7.60 (Pyrantel)	2.56 ± 0.26 (n=5)	29.8 ± 5.6	88.2 ± 8.54 (Pyrantel)	3.53 ± 0.63 (n=3
6.87 ± 1.07	177.0 ± 27.0 (Deacylated Amidantel)	26.0 ± 1.0 (n=4)	10.3 ± 4.3	755.0 ± 281.5 (Deacylated Amidantel)	76.0 ± 28.0 (n=4
20.2 ± 4.12	---- (Amidantel)	>100 (n=4)	32.87 ± 8.40	---- (Amidantel)	>100 (n=4)

Table 4. A comparison of the EC_{50} values and relative potencies on anthelmintic compounds compared with acetylcholine. The potency of acetylcholine is taken as one and values greater than one indicate that the compound is less potent than acetylcholine. The EC_{50} values and resulting potency ratios were determined for each experiment. The mean values in the table were then determined from the individual experimental values.

Figure 21 Dose response curves to compare the potencies of acetylcholine(x-x) and levamisole(o-o) on cell F-18 which is inhibited by both compounds. In this experiment the EC_{50} values for acetylcholine and morantel were 28μM and 370μM respectively.

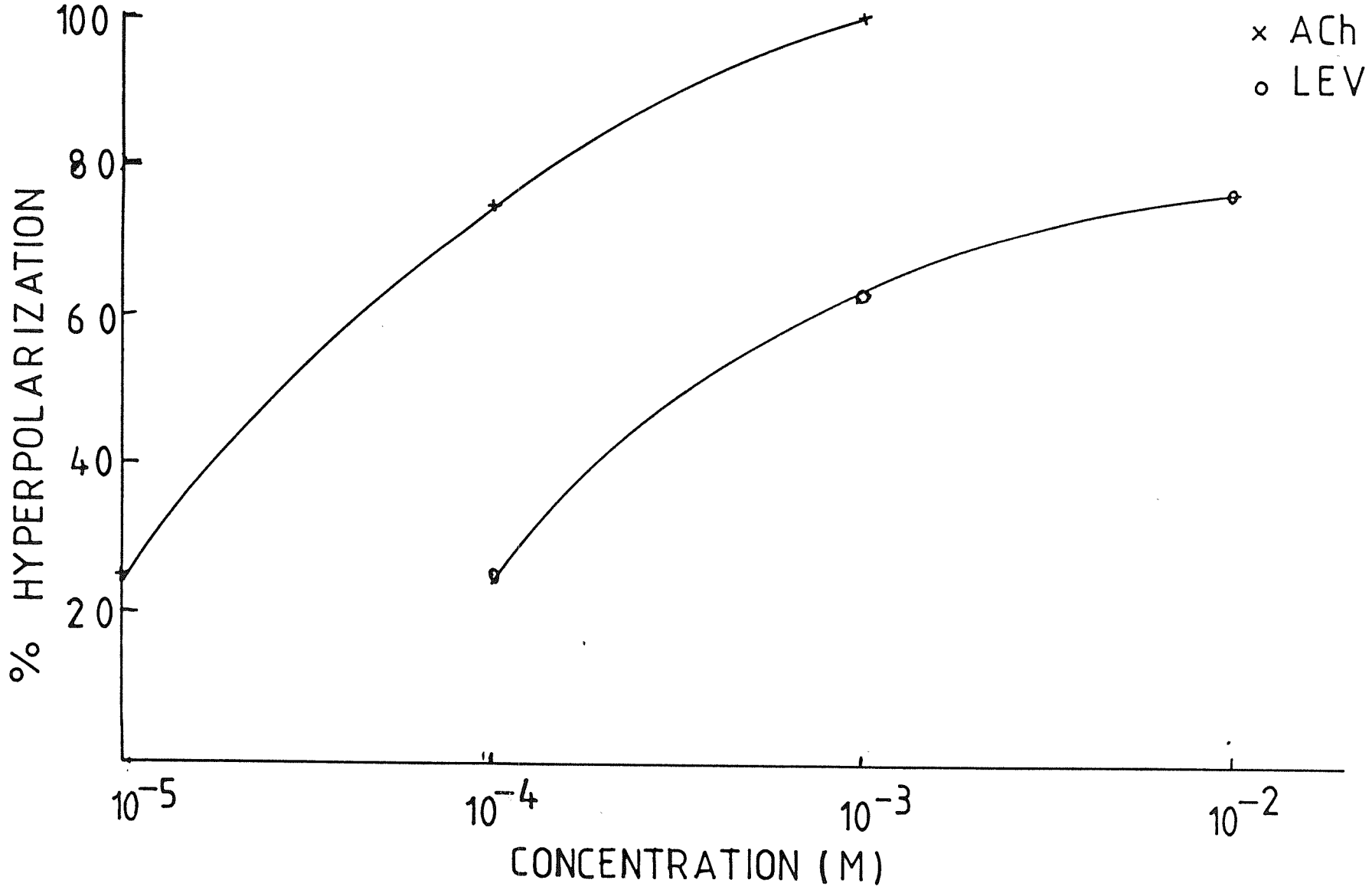

100
80
60
40
20
% HYPERPOLARIZATION
× ACh
o LEV
10⁻⁵
10⁻⁴
10⁻³
10⁻²
CONCENTRATION (M)

Figure 22 Traces to show the effect of 20μM d-tubocurarine on the inhibitory responses to acetylcholine and levamisole of cell F-40.

A control response to acetylcholine.

B & C Responses to acetylcholine and levamisole in the presence of d-tubocurarine .

D & E Recovery of the acetylcholine response and the response to levamisole following the removal of the d-tubocurarine.

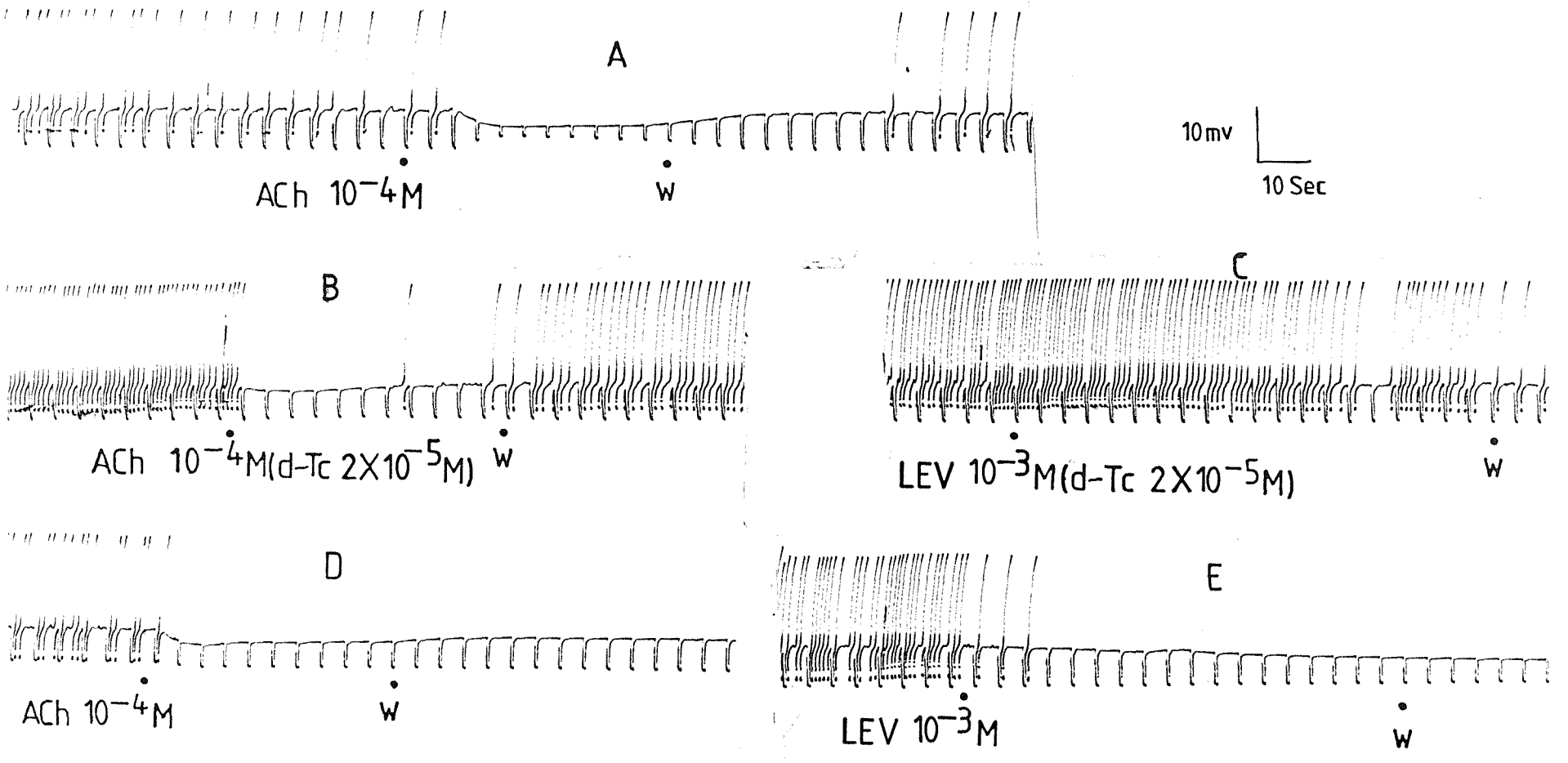

A
10 mv
10 Sec
ACh 10^{-4}M
W
B
ACh 10^{-4}M(d-Tc $2X10^{-5}$M)
W
C
LEV 10^{-3}M(d-Tc $2X10^{-5}$M)
W
D
ACh 10^{-4}M
W
E
LEV 10^{-3}M
W

Figure 23 Traces to show the effect of increasing concentrations of acetylcholine and levamisole on cell E-7, where levamisole affected the activity of this neurone.

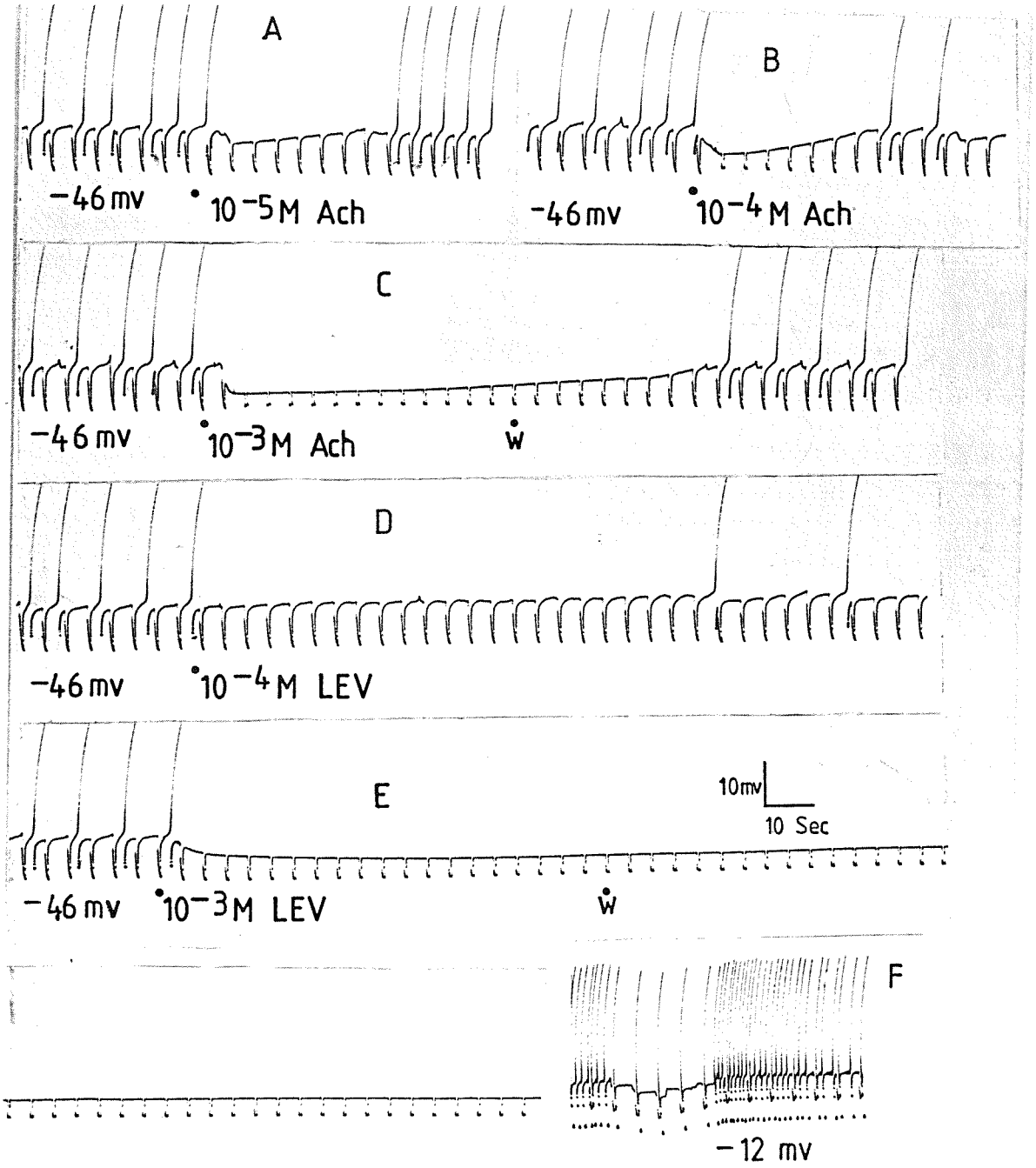
A,B & C Application of acetylcholine in increasing concentrations.

D The inhibitory response to 0.1mM levamisole.

E Shows the graded depolarisation of the cell membrane following the hyperpolarisation response to 1mM levamisole. The cell become silent. There was a decrease in input resistance of the cell compared with the control trace D.

F Shows the effect of levamisole after about 6 minutes. A positive current was injected via bridge to produce action potentials.

In traces A-E the membrane potentials was -46 mV while in trace F the apparent membrane potential was -12mV.


A
−46 mv
10⁻⁵ M Ach
B
−46 mv
10⁻⁴ M Ach
C
−46 mv
10⁻³ M Ach
W
D
−46 mv
10⁻⁴ M LEV
E
10mv
10 Sec
−46 mv
10⁻³ M LEV
W
F
−12 mv

Morantel and acetylcholine

Morantel was reasonably potent on both "D" and "H" cells, with potency ratios of 1.12+\-0.23 (mean+\-S.E.) (n=5) and 5.16+\-0.6 (mean+\- S.E) (n=6) respectively compared with acetylcholine, table (4). An example of the actions of morantel on cell F1 is shown in figure (24). From this it can be shown that morantel mimics the action of acetylcholine on "D" cells though the duration of the increase in activity is greater with morantel while the speed of onset of the response is fast in both cases.

Morantel induces an increase in conductance. Figure (25) shows two examples of dose-response curves for acetylcholine and morantel on cells F18 and F20 from which it can be deduced that morantel behaves as a full agonist and is around 5 times less potent than acetylcholine. Similarly morantel acts as a full agonist on cells excited by acetylcholine, figure (26). This figure represents the mean data from five experiments and gives slightly different values compared to those shown in table (4) since in the latter case EC_{50} values were obtained for each experiment and the values then averaged. In figure (26) the EC_{50} value for acetylcholine is 9 µM and that for morantel is 13 µM, giving a potency ratio of 1.44.

The ionic mechanisms associated with morantel excitation and inhibition were investigated. In sodium free saline the response to both acetylcholine and morantel on cell F1 were abolished, figure (27 C&D). On returning to normal saline both responses recovered, traces E and F. In chloride free saline both acetylcholine and morantel inhibitory actions were either greatly reduced or reversed. On returning to normal saline the responses to both compounds were enhanced figure 28.

The effect of 20 µM d-tubocurarine was investigated against both morantel excitation and inhibition. An example is shown in figure 29 where this concentration of d-tubocurarine greatly reduced the acetylcholine inhibition, trace C and completely blocked the morantel inhibition trace D as compare to the control traces A and B respectively. The d-tubocurarine effect was reversed following washing, traces E and F though it should be noted that the conductance increase was reduced, compare traces A and B with E and F. There is also an increase in cell conductance.

In experiments where the action of d-tubocurarine was tested, d-tubocurarine did not block either the action potentials or change input conductance (traces C and D)

Pyrantel and acetylcholine

Pyrantel was applied by bath addition to determine its effect on "H" and "D" neurones and also to determine whether pyrantel has any direct effect on the membrane potential and its conductance. The effects of pyrantel were observed on "H" and "D" cells, situated in the right parietal (F-cells) and the visceral ganglia (E-cells).

In terms of "H" cells, the cell membrane potential hyperpolarises and there is an increase in conductance in response to bath addition of acetylcholine. A hyperpolarisation in the cell membrane and increase in conductance was also obtained with pyrantel. However when the EC_{50} of the inhibitory response were compared to that of acetylcholine inhibitory response, the potency was 3.53+\- 0.63 (mean +\- S.E.) (n=5) times less potent than acetylcholine figure 30. This graph shows that pyrantel is a full agonist.

On "D" cells, acetylcholine applied to the bath in increasing concentrations, caused a depolarisation of the cell membrane and increase in conductance. Bath addition of morantel in increasing concentrations produced a similar depolarisation and increase in conductance to acetylcholine. The excitatory responses to pyrantel were compared in terms of potency to acetylcholine excitatory responses, the potency of pyrantel was 2.56+\-0.26 (mean +\- S.E.) (n=4) times less potent than acetylcholine. The potency ranking of the excitatory and the inhibitory responses are similar.

The ionic mechanisms for the pyrantel responses were the same as for morantel. Pyrantel was also blocked by d-tubocurarine. The effects of hexamethonium and mecamylamine were studied on the responses to acetylcholine and pyrantel on "D" cells and mecamylamine on the responses of both compounds on "H" cells. 4µM Hexamethonium, reversibly reduced the responses of both compounds. At this concentration, the reduced responses were 18-57% of control values on, for example cell F1. Mecamylamine, 1.5–3µM, likewise reduced both acetylcholine and pyrantel responses of both "H" and "D" cells, the reduced responses were 0-50% of control values.

Figure 24 Traces to compare the effect of increasing concentrations of acetylcholine and morantel on cell F-1 which is excited by both compounds. In this experiment the EC_{50} values for acetylcholine and morantel were both 1.9µM.

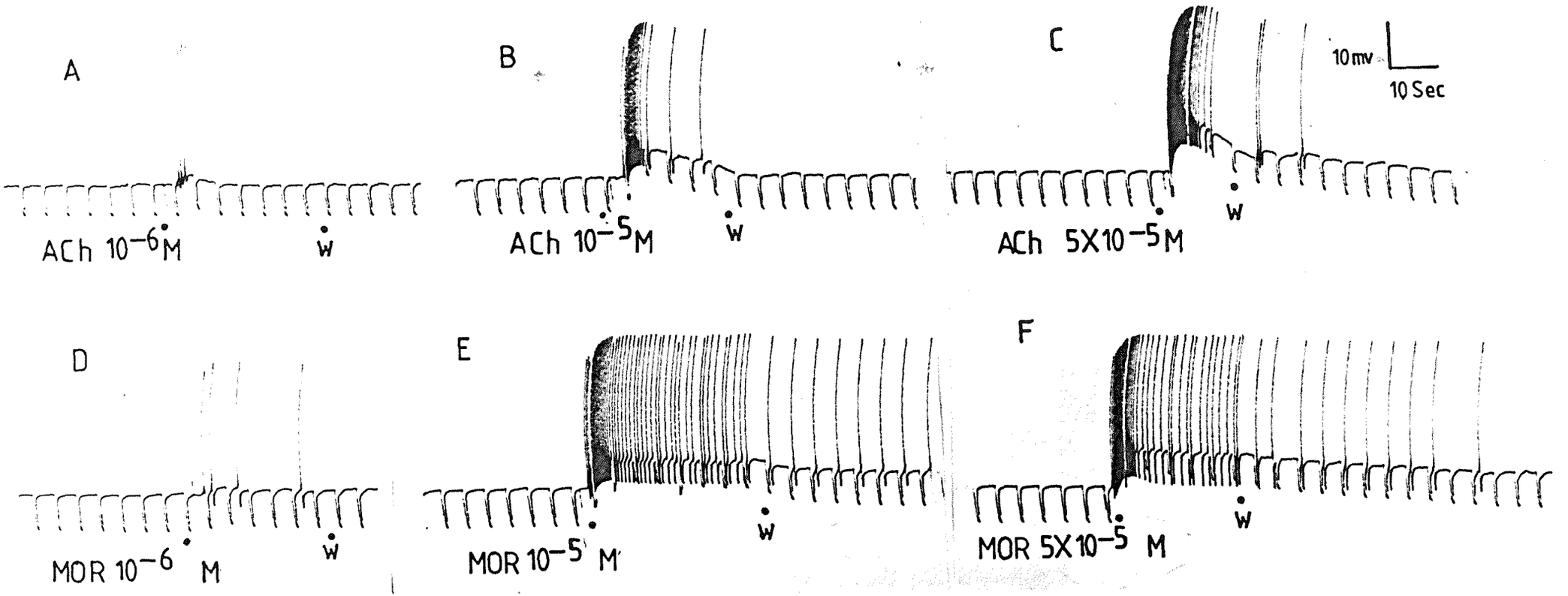

A
B
C
10 mv
10 Sec
ACh 10^{-6} M
W
ACh 10^{-5} M
W
W
ACh $5X10^{-5}$ M
D
E
F
MOR 10^{-6} M
W
MOR 10^{-5} M
W
W
MOR $5X10^{-5}$ M

Figure 25 Dose response curves to compare the potencies of acetylcholine and morantel on cell F-18 (left curves) and cell F-20 (right curves) where both cells were inhibited by both compounds. The EC_{50} values for morantel on F-18 was 56µM and for F-20 was 640 µM.

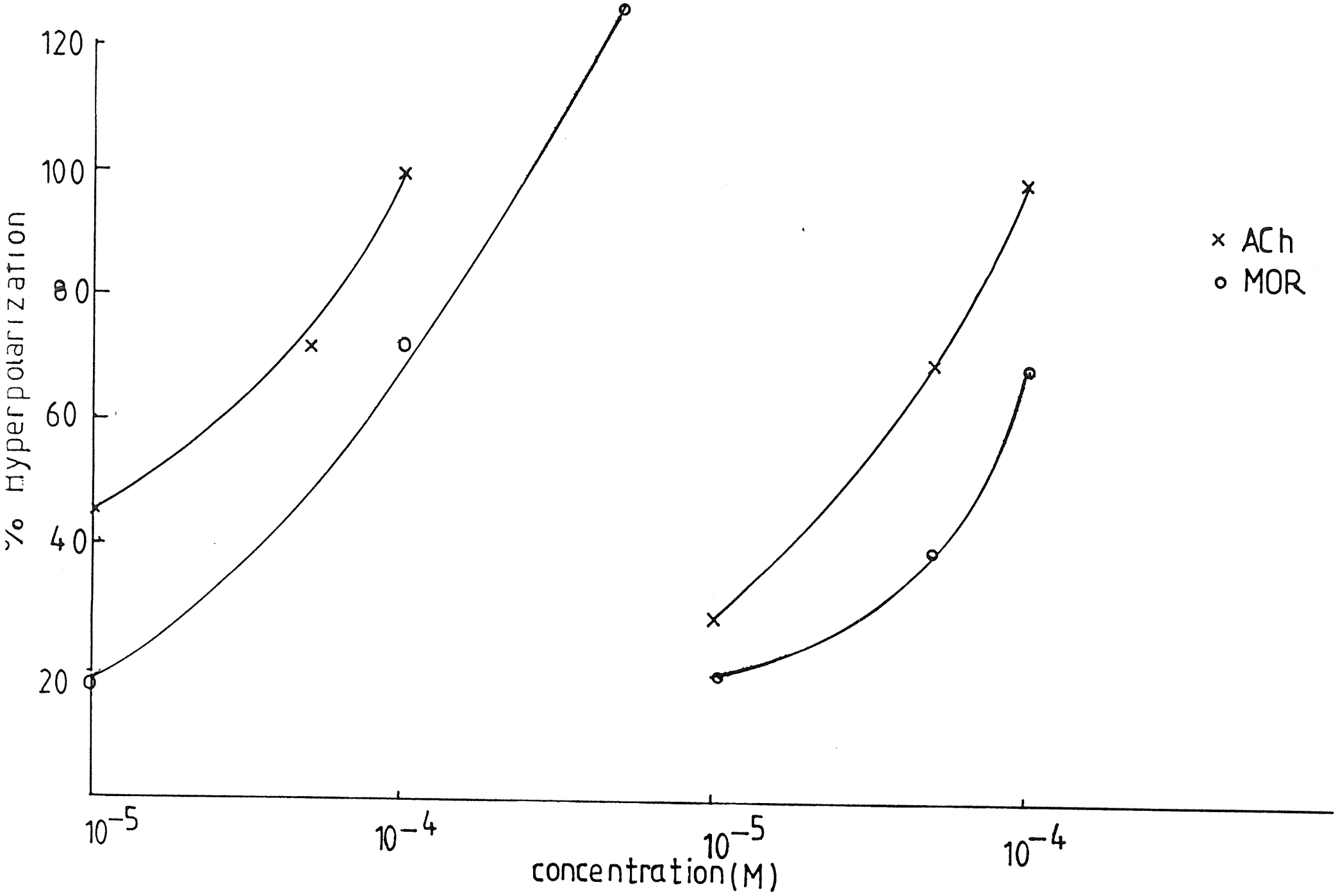

120
100
80
60
40
20
% Hyperpolarization
× ACh
o MOR
10^{-5}
10^{-4}
10^{-5}
10^{-4}
concentration (M)

Figure 26 Graph to compare the relative potencies of acetylcholine and morantel on cells excited by both compounds. The EC_{50} values and the relative potency were determined from the mean data shown in the graph. This method of determining the values gives a slightly different value from determining individual experiment values and then averaging them.(x) is curve for acetylcholine and (o) that for morantel

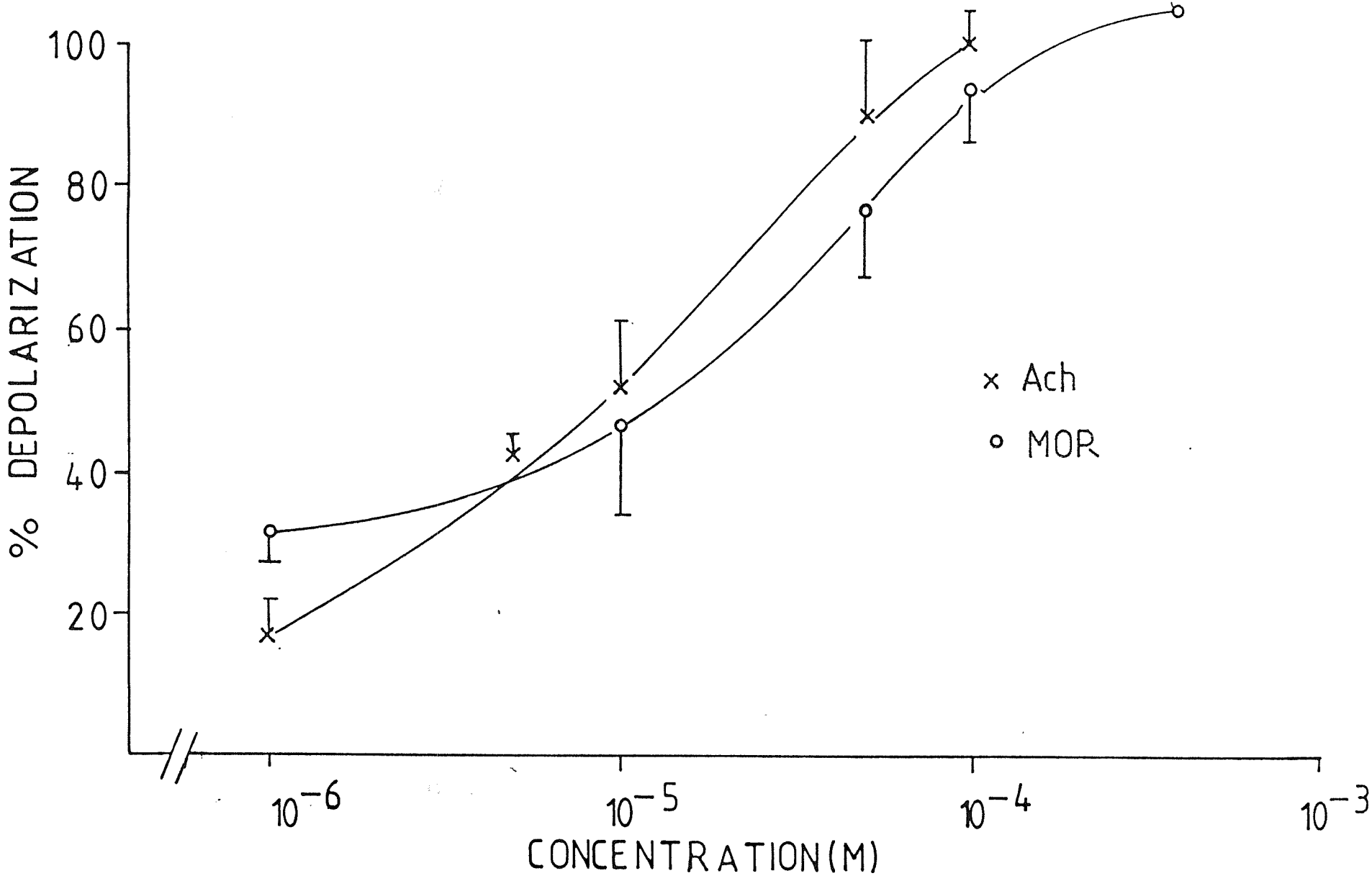

100
80
60
40
20
% DEPOLARIZATION
× Ach
o MOR
10⁻⁶
10⁻⁵
10⁻⁴
10⁻³
CONCENTRATION (M)

Figure 27 Recording from F-1 to show the effect of sodium free saline on the excitatory responses to acetylcholine and morantel both applied at 0.1mM.

A & B control responses to acetylcholine and morantel respectively in normal saline.

C & D Responses to acetylcholine and morantel respectively in sodium free saline.

E & F Recovery of the excitatory responses to acetylcholine and morantel.

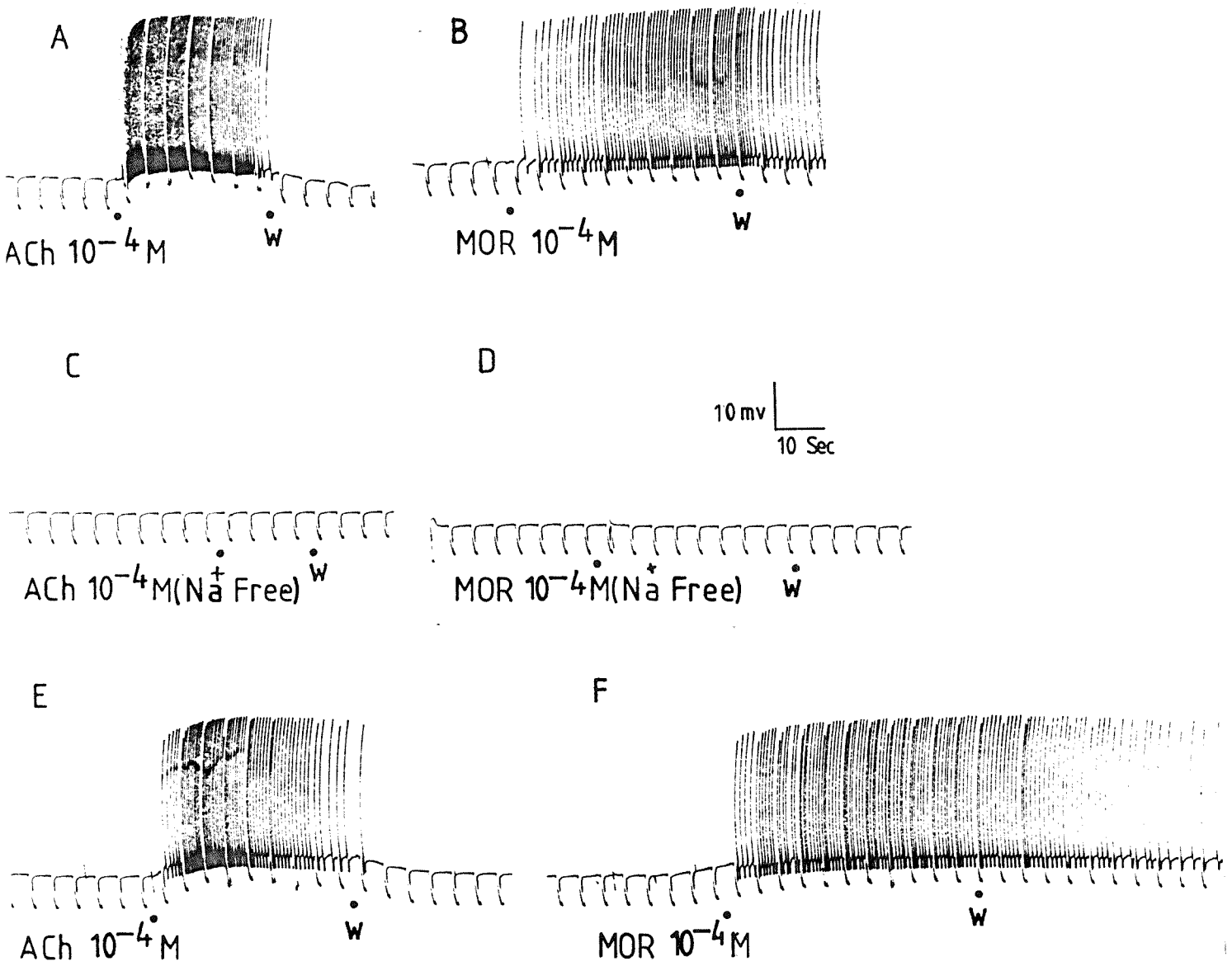

A
B
ACh 10^{-4}M
W
MOR 10^{-4}M
W
C
D
10 mv
10 Sec
ACh 10^{-4}M(Na^+ Free)
W
MOR 10^{-4}M(Na^+ Free)
W
E
F
ACh 10^{-4}M
W
MOR 10^{-4}M
W

Figure 28 Traces to show the effect of removing external chloride on the inhibitory responses to acetylcholine and morantel on cell E-6 both applied at 0.1mM.

A & B Control responses to acetylcholine and morantel respectively in normal saline

C & D Responses to acetylcholine and morantel respectively in chloride free saline.

E & F Recovery of inhibitory responses to acetylcholine and morantel following the return to normal saline. In both cases it can be seen that the responses are larger compared with the initial controls, traces A and B.

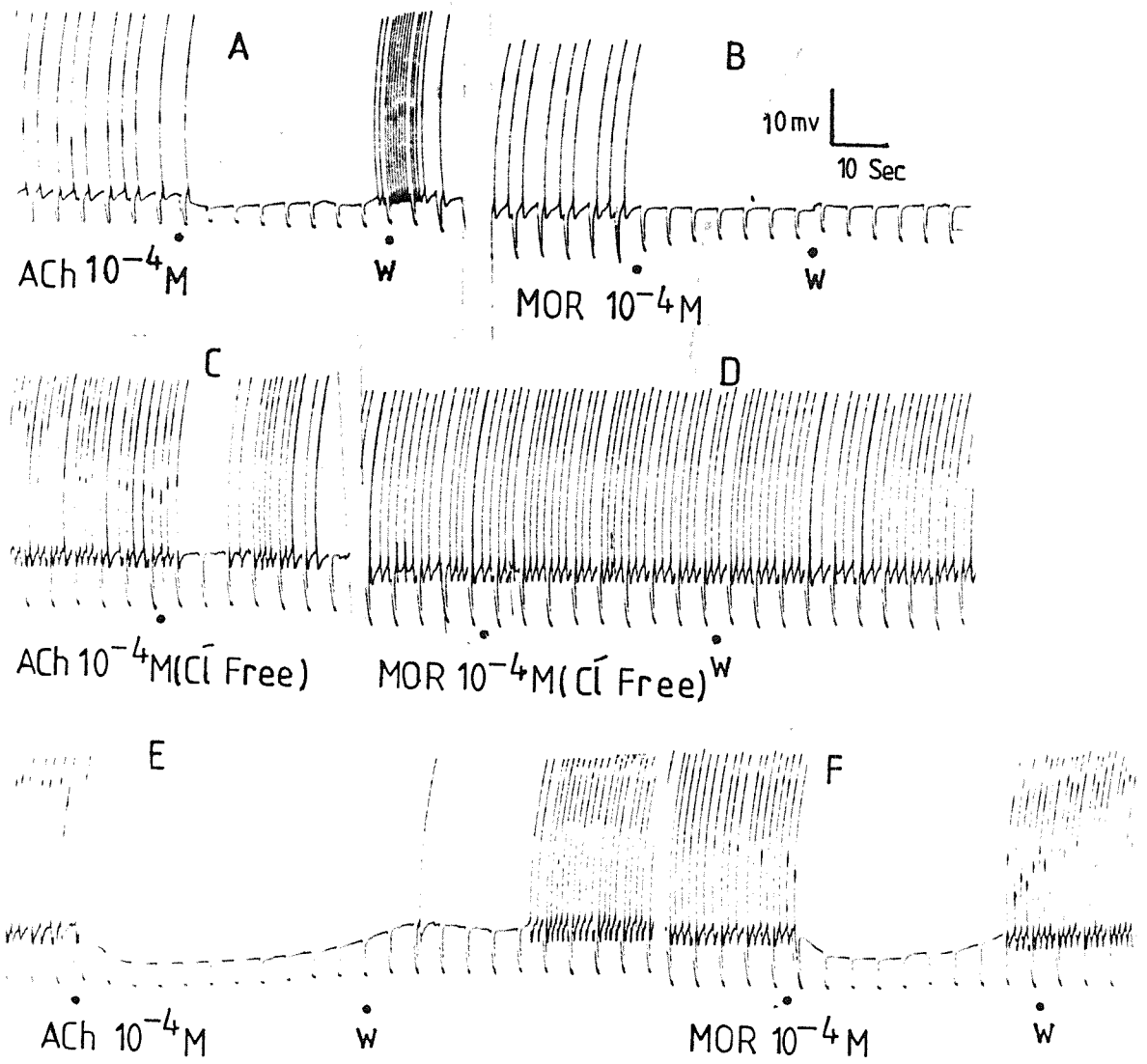

A
B
10 mv
10 Sec
ACh 10^{-4}M
W
MOR 10^{-4}M
W
C
D
ACh 10^{-4}M(Cl Free)
MOR 10^{-4}M(Cl Free)
W
E
F
ACh 10^{-4}M
W
MOR 10^{-4}M
W

Figure 29 Traces to show the effect of 20μM d-tubocurarine on the inhibitory resposes to acetylcholine and morantel of cell F-15

A and B Control responses to acetylcholine and morantel respectively.

C and D Responses to acetylcholine and morantel in the presence of d-tubocurarine .

E and F Recovery of the responses following removel of the d-tubocurarine.

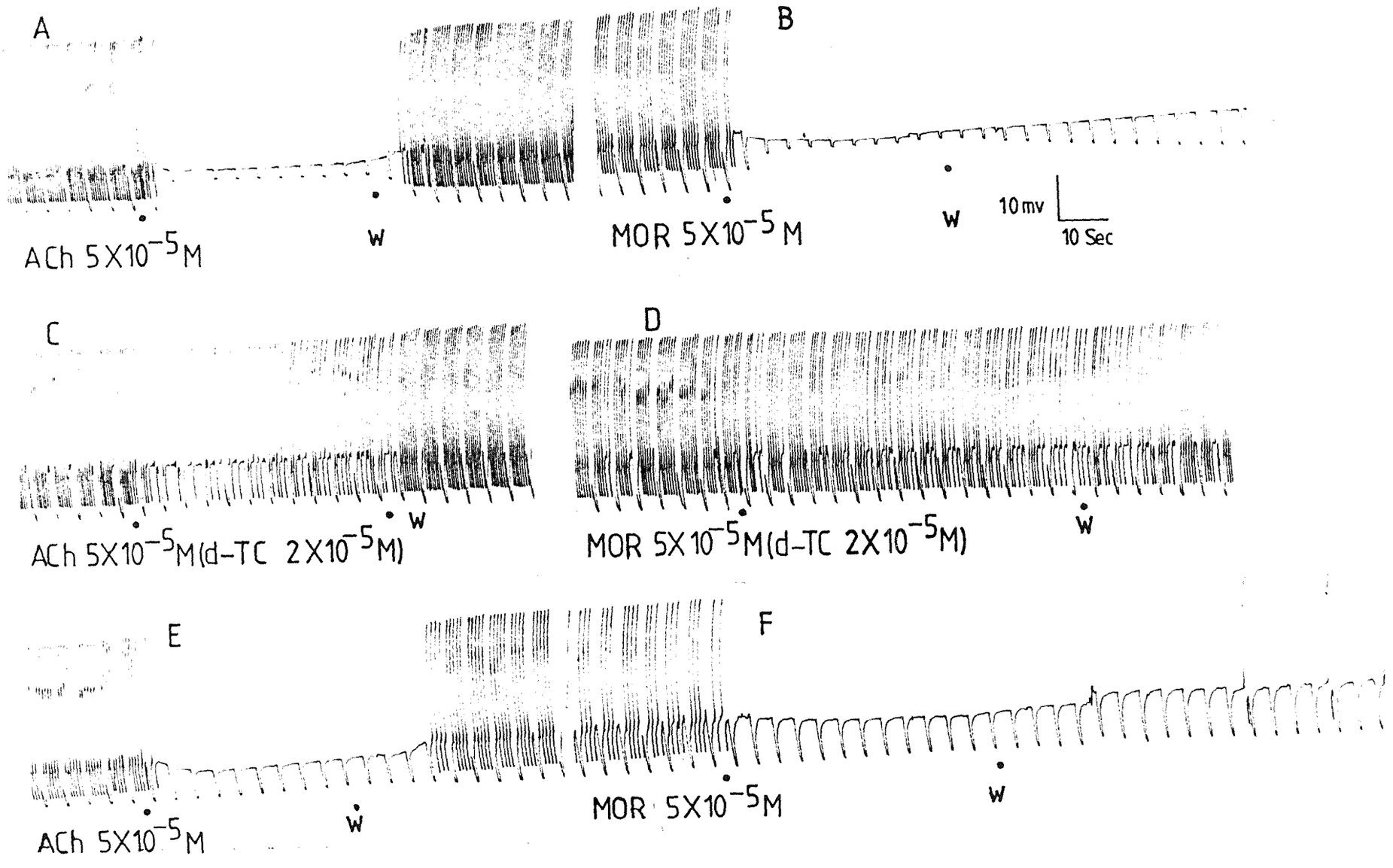
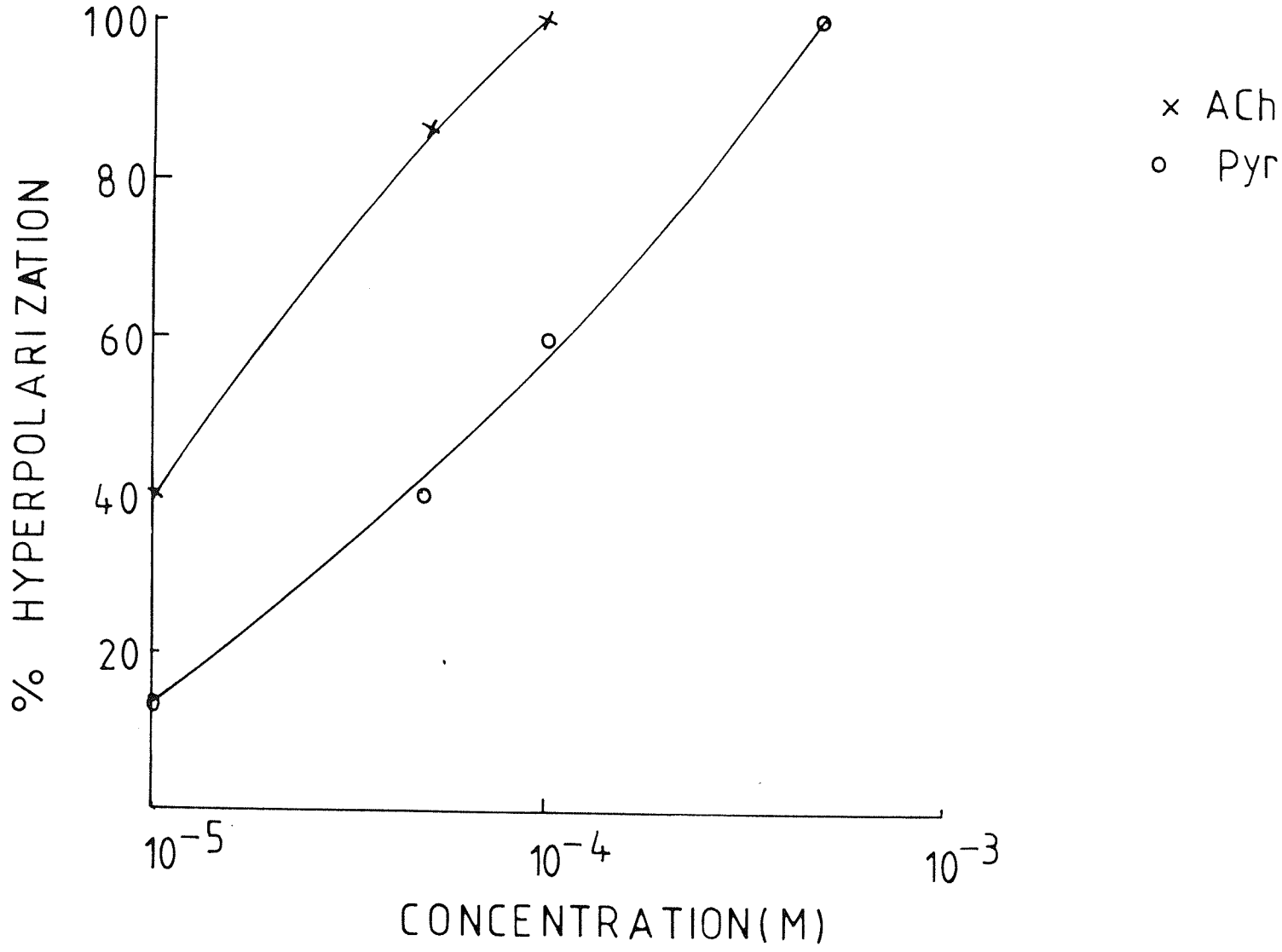

A
B
ACh 5X10⁻⁵M
W
MOR 5X10⁻⁵M
W
10 mv
10 Sec
C
D
ACh 5X10⁻⁵M(d-TC 2X10⁻⁵M)
W
MOR 5X10⁻⁵M(d-TC 2X10⁻⁵M)
W
E
F
ACh 5X10⁻⁵M
W
MOR 5X10⁻⁵M
W

Figure 30 Dose response curves to compare the potencies of acetylcholine and pyrantel on the cell F-18, the EC_{50} values being 0.02mM, and 0.1mM respectively.


100
80
60
40
20
% HYPERPOLARIZATION
10^{-5}
10^{-4}
10^{-3}
CONCENTRATION(M)
× ACh
o Pyr

Amidantel and acetylcholine

The effect of amidantel was tested on both "H" and "D" cells and its potency compared to that of acetylcholine. Amidantel possessed weak acetylcholine-like activity, for example 10mM amidantel induced only a slight effect in each case. Amidantel is more than 100 times less potent than acetylcholine on both "H" and "D" neurones table (4).

Deacylated amidantel and acetylcholine

Deacylated amidantel possessed clear acetylcholine-like activity on both the excitatory and the inhibitory receptors of acetylcholine table (4). Application of acetylcholine in increasing concentrations to the exposed cell (F1) gave a depolarising response and increase in conductance figure (31, A,B,C). The application of deacylated amidantel in increasing concentrations to the same cell was seen to mimic the excitatory action of acetylcholine figure (31, D ,E , F and g)

Dose-response curves constructed from changes in membrane depolarisation induced by increasing quantities of acetylcholine and deacylated amidantel, indicated that deacylated amidantel is 26.98+\-1.0 (mean +\- S.E.) (n=4) times less potent than acetylcholine.

On the inhibitory receptors of acetylcholine, acetylcholine and deacylated amidantel were applied to the bath. Acetylcholine hyperpolarised the cell membrane and increased the membrane conductance. Deacylated amidantel produced a similar hyperpolarisation and increase in conductance to that of acetylcholine.

Dose-response relationships to these two compounds from

changes in membrane hyperpolarisation shows that deacylated amidantel is 76.28+\- 3.25 (mean +\- S.E.) (n=4) times less potent than acetylcholine figure (32).

The effect of changing ions was also examined on the "D" and "H" neurone responses of deacylated amidantel. The excitation was sodium dependent while the inhibition was chloride dependent. In figure 33, traces A and B show control responses to both compounds which are reversed in chloride free saline. Both responses are enhanced on returning to normal saline.

The action of d-tubocurarine was tested against the excitatory and the inhibitory responses to acetylcholine of an identified neurone. 50µM d-tubocurarine antagonised the excitatory and the inhibitory responses to acetylcholine 5µM, 50µM respectively. The same concentration of d-tubocurarine also antagonised the responses to 0.5 mM deacylated amidantel.

Figure 31 Traces to show the effect of increasing concentrations of acetylcholine and deacylated amidantel on cell F1, which is excited by both compounds.

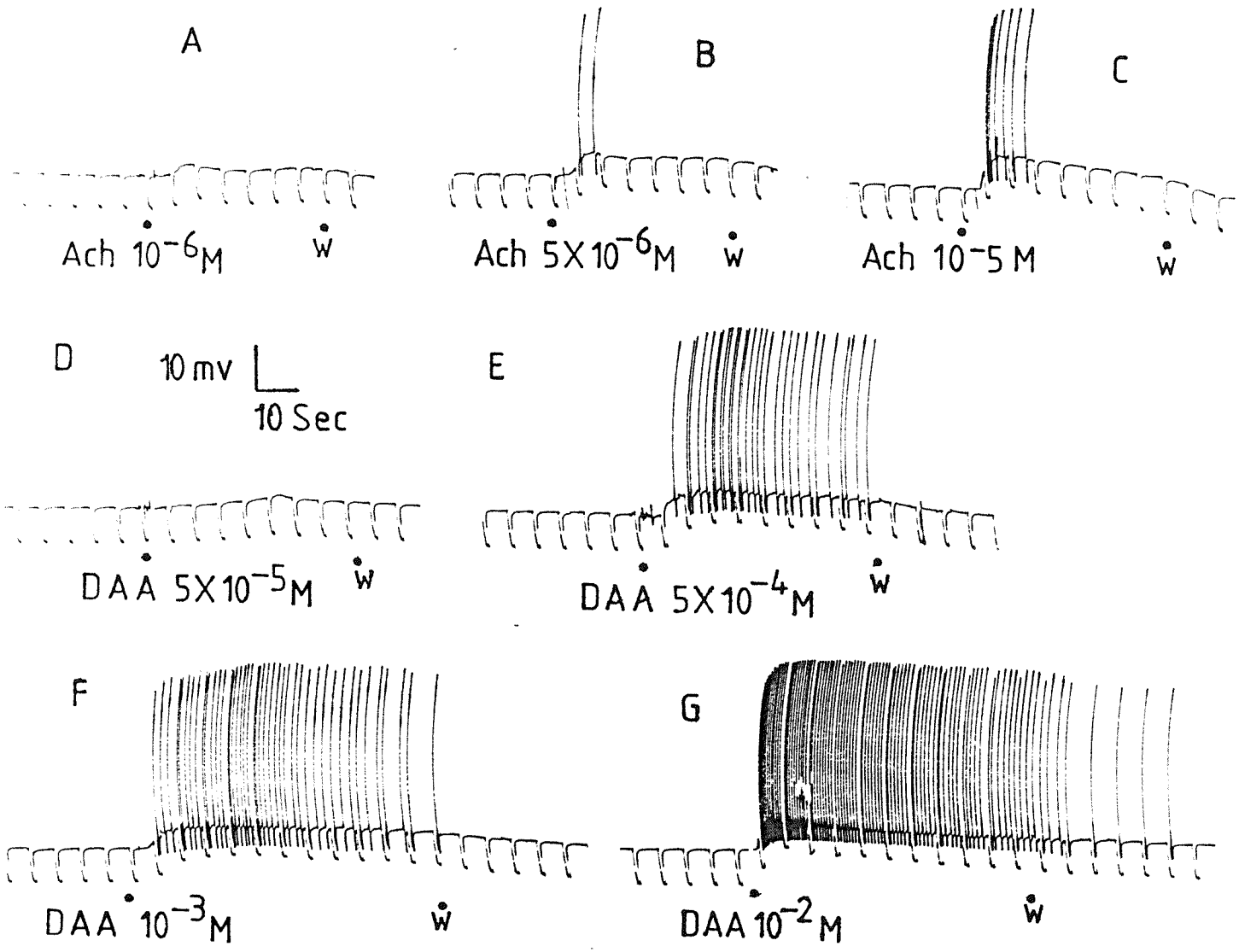

A
B
C
Ach 10^{-6}M
W
Ach $5X10^{-6}$M
W
Ach 10^{-5}M
W
D
10 mv
10 Sec
E
DAA $5X10^{-5}$M
W
DAA $5X10^{-4}$M
W
F
G
DAA 10^{-3}M
W
DAA 10^{-2}M
W

Figure 32 Dose response curves to compare the potency of acetylcholine and morantel on cell E4, which is inhibited by both compounds. In this experiment the EC_{50} values for acetylcholine and deacylated amidantel were 6.4µM and 500µM respectively.

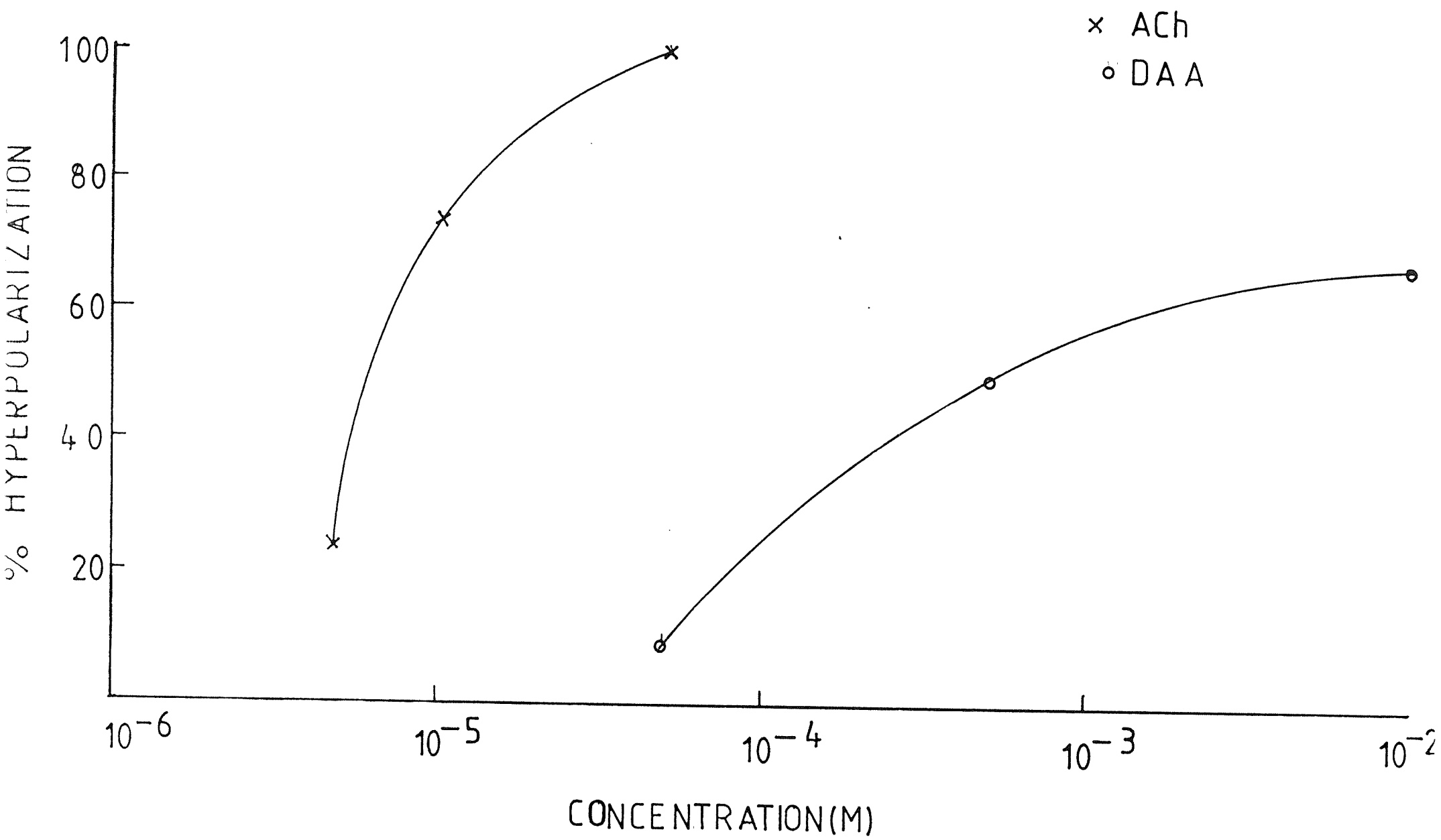
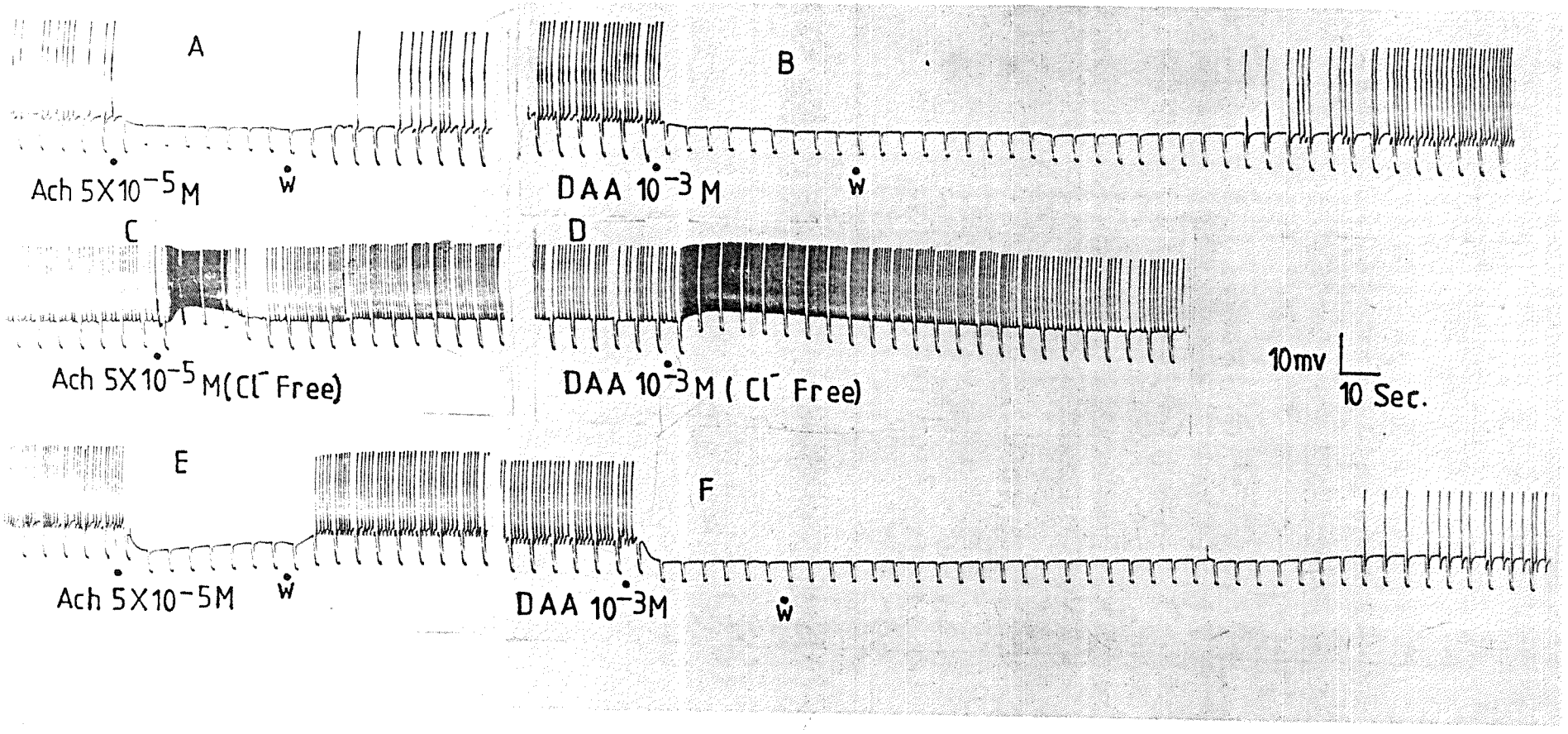

× ACh
○ DAA
100
80
60
40
20
% HYPERPOLARIZATION
10^{-6}
10^{-5}
10^{-4}
10^{-3}
10^{-2}
CONCENTRATION (M)

Figure 33 Recording from F-9 to show the effect of removing external chloride on the inhibitory responses to acetylcholine and deacylated amidantel.

A & B Control responses to acetylcholine and deacylated amidantel respectively in normal saline.

C & D Responses to acetylcholine and deacylated amidantel respectively in chloride free saline.

E & F Recovery of the inhibitory responses to acetylcholine and deacylated amidantel following the return to normal saline.


A
Ach 5X10⁻⁵M
W
B
DAA 10⁻³M
W
C
Ach 5X10⁻⁵M(Cl⁻Free)
D
DAA 10⁻³M (Cl⁻Free)
10mv
10 Sec.
E
Ach 5X10⁻⁵M
W
F
DAA 10⁻³M
W

Hycanthone and acetylcholine

The action of hycanthone was tested on both "D" and "H" cells. It was found to have no effect on "D" cells but it did alter the activity of "H" cells though the response appeared to depend possibly on the concentration applied or the cell type, figure (34).

Relatively low concentrations, 1 mM, mimicked the action of acetylcholine, trace D, while 10mM tended to excite the cell, trace B. which is probably a non-specific action unrelated to acetylcholine receptor interaction. In figure (34), trace A and B are from one cell while traces C--E are from a second cell. As can be seen from trace E, 10mM hycanthone, stopped cell activity with little hyperpolarising effect on the membrane potential and in fact tended to depolarised the cell. As can be seen hycanthone failed to alter membrane conductance in trace E. Hycanthone was further examined for antagonist activity and appeared to terminate the inhibitory effect of acetylcholine when applied soon after the peak of the response. This suggests that it may be interacting with activated channels which in the case of inhibitory responses would be chloride channels. However further experiments are required on this action.

Figure (35) shows an experiment where hycanthone was shown to affect the open channel directly. 0.1 mM acetylcholine hyperpolarised the cell membrane and increased the input conductance. The addition of 10 µM hycanthone reduced the input conductance and terminated the action of acetylcholine (figure 35B). Traces A and C show that 10 µM acetylcholine induced a long hyperpolarisation. The input conductances recovered after about 70 seconds, and the action potentials restored after washing. Trace B shows the action potentials were restored after 15 seconds following

the application of hycanthone. It can also be seen that after the addition of hycanthone the input conductance quickly recovered.

Figure 34 Shows the effect of hycanthone and acetylcholine on two "H"neurones. Traces A and B from one cell F-14 while traces C-E are from second cell, F-30.

A Control of 0.1mM acetylcholine

B 10mM hycanthone gave D-response instead of H-response

C A control response to 0.01mM acetylcholine.

D 1mM hycanthone gave acetylcholine-like response.

E 10mM hycanthone stops cell activity and tends to slightly depolarised the cell and block the response to acetylcholine.

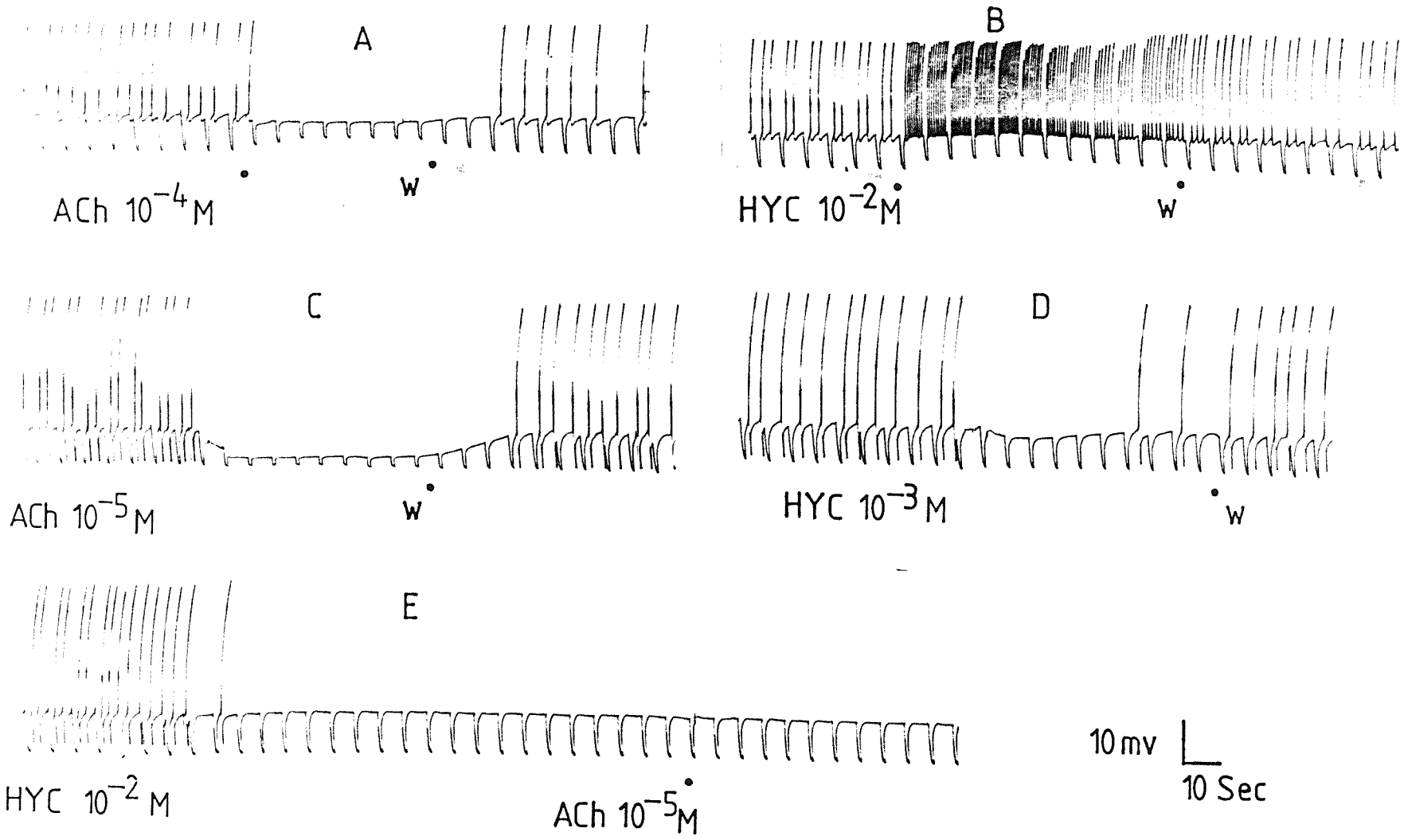
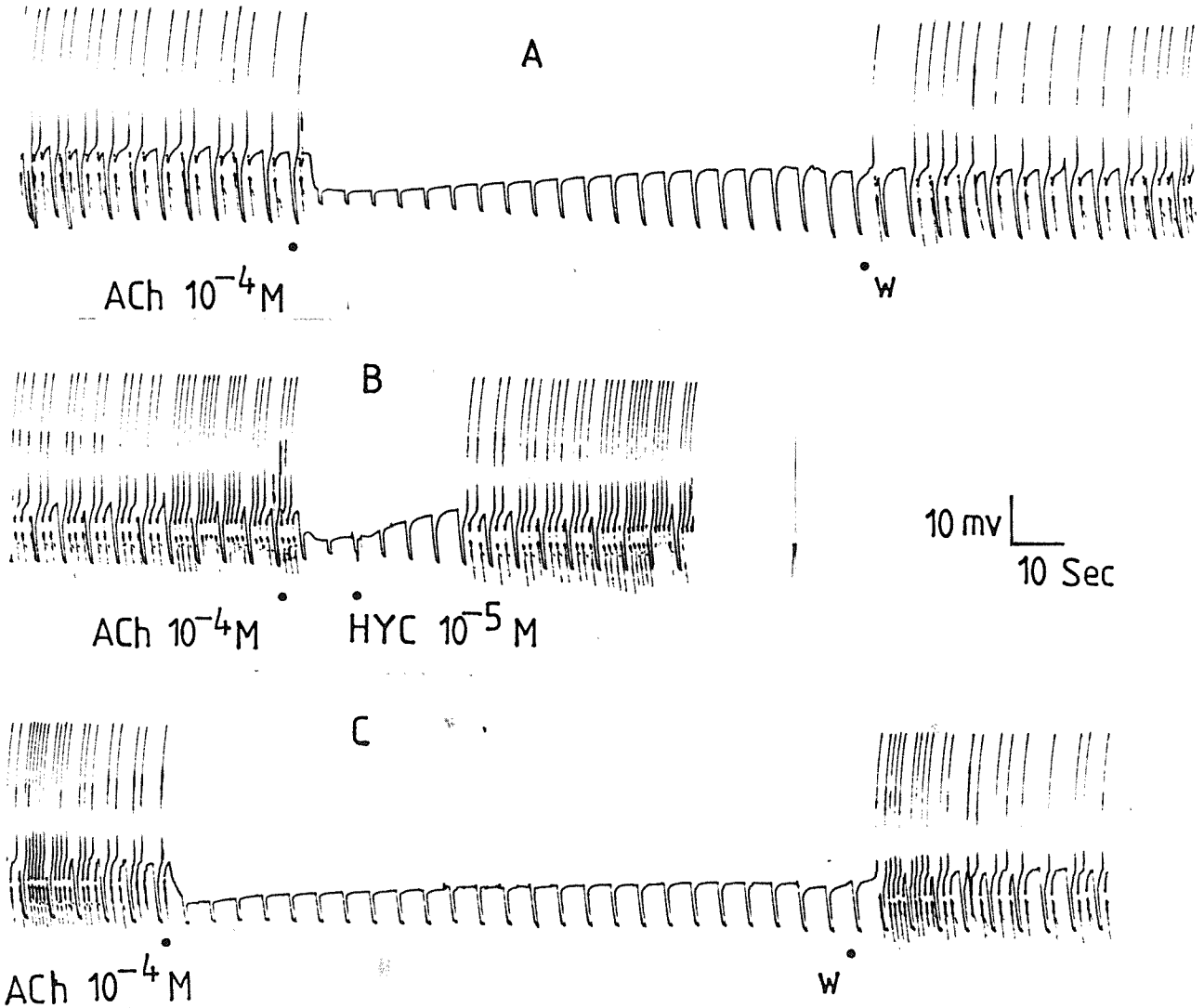

A
ACh 10^{-4} M
W
B
HYC 10^{-2} M
W
C
ACh 10^{-5} M
W
D
HYC 10^{-3} M
W
E
HYC 10^{-2} M
ACh 10^{-5} M
10 mv
10 Sec

Figure 35 Recording from F-9 shows the effect of hycanthone on the open channels which are activated by acetylcholine.

A 0.1mM acetylcholine as control

B 0.1mM followed by the addition of 0.01mM hycanthone which terminated the depolarising response to acetylcholine

C Recovery response to 0.1mM acetylcholine.


A
ACh 10^{-4}M
W
B
ACh 10^{-4}M
HYC 10^{-5} M
10 mv
10 Sec
C
ACh 10^{-4}M
W

DISCUSSION

The present study shows that several of the compounds tested possessed clear acetylcholine-like activity on *Helix* central neurones. None of the compounds proved to be more potent than acetylcholine though morantel was approximately equipotent with acetylcholine on "D" cells. There appeared to be differences in potency with this compound between "D" and "H" cells since in the latter case morantel was about 5 times less potent than acetylcholine. In contrast pyrantel, which lacks a methyl group but otherwise is the same as morantel does not appear to distinguish between the two cell types since its potencies on "D" and "H" cells are very similar, (table 4). This indicates that morantel and pyrantel have a higher efficacy for acetylcholine receptors compared with other anthelmintics tested, for example, deacylated amidantel. From the dose response curves it would appear that both compounds are full agonists on *Helix* neurones and from the ionic studies both compounds appear to activate receptors linked to the same ion channels as acetylcholine. Since d-tubocurarine antagonises both compounds on both cell types it is likely that pyrantel and morantel interact at the acetylcholine receptor complex. For example, on several mammalian tissues Eyre (1969) found evidence for pyrantel interacting with acetylcholine receptors. In these studies pyrantel was blocked by hexamethonium and in some cases atropine. He also observed that pyrantel inhibited cholinesterase which would fit in with observation that the responses to pyrantel are prolonged compared with acetylcholine. Recently Harrow and Gration (1985) found that the acetylcholine and pyrantel/morantel induced contractions of *Ascaris* muscle were blocked by d-tubocurarine and they found evidence for

competition between acetylcholine and morantel were at least in terms of threshold responses about 100 times more potent than acetylcholine. The *Ascaris* muscle acetylcholine receptor would appear to have a greater efficacy for pyrantel/morantel than the receptor on *Helix* neurones. Since it has been suggested that acetylcholine the receptor on *Ascaris* muscle resembles the vertebrate nicotinic ganglion type (Lewis *et al*, 1980), it was decided to test the action of hexamethonium and mecamylamine on an anthelmintic response. Pyrantel was selected since its potency was similar to acetylcholine on *Helix* neurones, table 4. Both hexamethonium and mecamylamine reversibly antagonised the action of pyrantel, again suggesting anthelmintic compounds can interact with an acetylcholine receptor which resembles the vertebrate nicotinic ganglion type.

In the present study levamisole was about 100 times less potent than acetylcholine on *Helix* "H"cells while possessing very little excitatory activity on "D" cells. This is in contrast to the finding on *Ascaris* muscle where at threshold response, levamisole is twice as potent as acetylcholine (Harrow and Gration 1985), again indicating a difference between *Ascaris* and *Helix* acetylcholine receptors. As with pyrantel, tetramisole (dl isomer where levamisole is only the l isomer) has acetylcholine-like activity on a range of mammalian tissues. For example, it induces atropine sensitive contractions of the guinea pig ileum, it augments twitches of the phrenic nerve-diaphragm preparation, it contracts the chick semispinalis muscle where its action is blocked by tubocurarine and it induces a fall in the blood pressure which is partly antagonised by atropine. Overall levamisole can have a wide range of action (Symoens et al., 1979). Interestingly it has been claimed that mutants of *Caenorhabditis* which are resistant to levamisole lack pharmacological acetylcholine receptors

(Lewis *et al.*, 1980).

There is relatively little information on the mode of action of the anthelmintic amidantel . However recently Tomlinson et al., (1985) have investigated the action of this compound and its deacylated derivative on whole and cut *Caenorhabditis*. They found that the deacylated analogue was 2-4 times more potent than amidantel while in the present study it is likely that the potency ratio is even greater. Deacylated amidantel was more potent on "D" than on "H" cells, table 4. Tomlinson *et al.*, (1985) concluded that these compounds resembled levamisole in the mode of action and since they consider that levamisole acts at the acetylcholine receptor then amidantel and its deacylated derivative also act at the same site. They found that their agonist effect was blocked by both d-tubocurarine and gallamine. They also observed that both compounds were inhibitors of cholinesterase and that this cholinesterase did not resemble either vertebrate "true" or "pseudo" cholinesterase which confirmed the earlier finding of Johnson and Russell (1983).

The results of this study also show that in sodium free saline acetylcholine, morantel, pyrantel and deacylated amidantel excitatory responses were abolished indicating that the initial response is due to the passage of sodium ions into the cell. When the external source of ions was removed, inward movement of ions ceased and the net driving force was insufficient to produce a net outside flux. An alternative explanation could be that external sodium ions are required in some direct way for the response to occur or that the Tris ion acts to block any other component of the response normally present. A similar finding was obtained by James *et al* (1979) on leech Retzius cells where there was no conductance change in the absence of sodium. Witte *et*

al (1985) suggested that complete replacement of sodium ions by Tris did not change the hyperpolarisation response to acetylcholine when the chloride concentration of the bath was kept constant. They also reported evidence based on the work of Wilson *et al* (1977) that both the depolarising and the hyperpolarising acetylcholine responses were reduced by Tris at lower concentrations. They explained their results as being due to competition between acetylcholine and Tris at the receptor site. They supported their conclusion with the observation that acetylcholine and Tris have similar structure (Rudman *et al* 1978).

The final compound tested is an antischistosomal compound and several mechanisms of action have been proposed (Enomoto and Edwards,1985). One of these is that hycanthone blocks the acetylcholine system in *Schistosomes* (Hillman and Gibler, 1975). Acetylcholine relaxes *Schistosome* muscle and so may function as an inhibitory transmitter (Baker *et al.*, 1966; Tomosky et al., 1974). Hycanthone, 1-10 µM, stimulates schistosome motility (Senft and Hillman 1973). Enomoto and Edwards in a very elegant study concluded that hycanthone acted primarily on the "transient state" i.e. the acetylcholine bound but closed conformation, of the acetylcholine receptor ion channel of frog neuromuscular junction. However they found that it also acted on the presynaptic nerve terminal and on the "open" conformation of the acetylcholine receptor ion channel complex. In the present study hycanthone at high concentrations did mimic the action of acetylcholine on "H" cells but in addition it did appear to terminate the inhibitory action of acetylcholine though not preventing its action. That is, hycanthone did not act primarily as a blocker though further work is required on this point. The finding that it does have some acetylcholine-like activity does not appear to have been previously reported. Further experiments are

required to determine whether hycanthone blocks the open channels. A possible method is to use ionophoretically applied acetylcholine. Having established a standard response acetylcholine, to pules of hycanthone can be added to see whether the amplitude of the acetylcholine responses decline in presence of hycanthone. It will be interesting to see whether or not hycanthone will terminate the action of acetylcholine.

As pointed out in the introduction there is evidence to suggest that the antagonist action of d-tubocurarine can be either at the acetylcholine-receptor site, acetylcholine transmitter gated channels or at both. In this study d-tubocurarine was found to reversibly antagonised the excitatory and the inhibitory responses to acetylcholine and the anthelmintic compounds. d-Tubocurarine did not block the action potentials of the voltage gated channels.

From the results obtained in this study the *Helix* brain would appear to offer a potentially useful model for analysing the mode of action of anthelmintic agents which may act to modify transmission in the nervous system.

CHAPTER 5

CHEMICAL CHARATERISTIC OF L-GLUTAMATE RECEPTOR ON HELIX NEURONES AND A STUDY ON THE ELECTOGENIC PUMP

INTRODUCTION

Glutamate exists in the arthropod pre-synaptic terminals and stimulation of the excitatory axons causes a release of glutamate in adequate quantities from pre-synaptic terminals of, for example, crayfish to activate somatic muscle (Kawagoe et al 1981, 1982). However, pharmacological identification of the excitatory transmitter has not yet been satisfactorily made at this site because of the lack of selective glutamate antagonists. Some spider venoms have been found to abolish both the glutamate response and excitatory junction potentials at the lobster neuromuscular junction (Abe et al. 1983; Jackson and Usherwood 1988) and are proving useful tools for the study of the glutamate receptor. A number of studies suggest that L-glutamic acid may act as an excitatory neurotransmitter in different groups of invertebrates (Kerkut, 1967; Walker 1986).

The best evidence for this view comes from studies on the arthropod nerve-muscle synapses. For example, Takeuchi & Takeuchi (1964) and Usherwood (1969) have shown that there are glutamate receptors at excitatory synaptic junctions of crustacean and insect leg muscle. The action of L-glutamate has been tested on gastropod neurones and has been found to excite some cells and inhibit others (Kerkut and Walker,1961, 1962b).

A structure-activity study on the inhibitory glutamate receptors of an identified neurone in *Helix aspersa* (Parmentier and Case, 1972) showed that the configurational requirements for the receptor were similar to those proposed

by Curtis and Watkins (1960) in cat spinal neurones, that is a three points electrically charged model. The structure activity relationship of the L-glutamate response was investigated on *Onchidium* G-H cells (Kato, et al, 1983). They reported that α-NH2,α-COOH, and γ-COOH groups of L-glutamate are all essential, and the relative positions of these groups are important for the interaction of L-glutamate with its receptor sites.

Electrogenic Pump

Molluscan neurones like all other cells have a semi-permeable cell membrane which allows selected ions to diffuse down electrochemical gradients. Therefore,active transport mechanisms must exist within the membrane to restore and maintain the resting ionic concentrations. If the active transport of ions in and out of the cell is balanced electrically (for example, if the movement of charges in and out are coupled 1-1),then the transport mechanism or the "pump" will have no net effect upon the membrane potential. and be termed electroneutral• However, evidence is accumulating that ionic pumps are rarely 1-1 coupled. For example in the squid axon it has been calculated that the ionic pump would be of the order of 2-3 mV (Hodgkin and Keynes, 1955). When the net flow of ions is greater in one direction across the cell membrane than in the other, a current is produced and the pump is "electrogenic".

Kerkut and Thomas (1965) first documented an electrogenic sodium transport system in *Helix* neurones, using low-resistance microeletrodes to inject sodium into the cells and stimulate the pump. A large hyperpolarisation

resulted from sodium (but not potassium) injection, and this was inhibited by oubain, parachloromercuribenzoate or the removal of external potassium. Subsequently, Thomas (1969) duplicated these findings under much more rigorous conditions using an ion-sensitive microelectode to monitor internal Na.

Gormar and Marmor (1970a) suggested the internal sodium level of *Anisodoris* G-cells may be lowered by prolonged exposure to Na+ free saline and such treatment eliminated all signs of electrogenic pump activity. Their data were supported by Baker, et al, (1969) who suggested that changes in the external concentration of sodium in squid axons may also directly affect the level of sodium pump activity. Whereas Thomas (1972) found no such effect in *Helix* neurones.

However, when a nerve is activated there is an entry of sodium ions and if this activation is strong enough this entry induces an electrogenic potential. For example, a burst of tetanic stimulation will produce a post-tetanic hyperpolarisation (Ritchie and Straub, 1956). A post-tetanic hyperpolarisation (PTH) has been demonstrated in crayfish stretch receptor (Nakajima and Takahashi, 1966) and this can result in hyperporlarisation of up to 11mV.

There is evidence that compounds applied to neurones can induce hyperpolarisation which can be linked to sodium pump activation. For example dopamine when applied to cell R-15 of *Aplysia* induces a hyperpolarisation and part of this response is blocked by ouabain, indicating that the dopamine response of this cell has an electrogenic component (Asher, 1968). Catecholamines, for example dopamine, adrenaline and noradnaline, can also activate an electrogenic sodium

pump in *Lymnaea* neurones and this effect is inhibited by strophanthidin (Kazacheno *et al* 1979).

The pump may be inhibited by reducing substrate by blocking metabolic activity, or by the application of cardiac glycosides. The removal of external potassium which acts as a substrate for the pump inhibits electrogenic transport in all molluscan neurones with a demonstrated electrogenic pump (Marmor, 1971a,b). When the sodium pump is active this inhibitory effect of removing external potassium can overshadow the ionic potential and cause a depolarisation. The presence of potassium in the external solution is very important since in the absence of potassium, sodium injection fails to stimulate the pump (Kerkut and Thomas, 1965 ; Thomas 1969) and ouabain no longer has any effect (Gorman and Marmor, 1970a).

Metabolic activity can be inhibited by cooling cells and in most molluscan neurones cooling to 0-5 °C is sufficient to inhibit electrogenic transport. For example, the resting potential of the *Anisodoris* G-cells at 0-5 °C can be predicted accurately by the constant field equation for 0-200 mM external potassium (Marmor 1971a). As would be expected cooling eliminates the effects of ouabain and external potassium upon electrogenic activity (Gorman and Marmor, 1970a). Lowering PO_2 also inhibits sodium pump activity (Kerkut and York, 1969) as does DNP (Snover and Carpenter, 1969), and other non-specific inhibitors. The most specific inhibitors of Na-K transport mechanism are the cardiac glycosides (Glynn, 1964) of which ouabain is the most widely used in electrophysiology. Ouabain is rapidly effective in the blocking of the Na pump of molluscan neurones and causes a depolarisation when the sodium pump is active (Gorman and Marmor 1974).

Some of the effects of ouabain and its reversibility appears to depend upon the duration of the exposure to the drug. Ouabain is clearly reversible in its effects on the *Anisodoris* G-cell following exposure for up two hours (Gorman and Marmor 1970b). It has been reported to be poorly reversible in *Aplysia* (Carpenter and Alving, 1968; Carpenter,1970) and in *Helix* (Moreton, 1969) although Kerkut and York (1969) noted in *Helix* that the effects were reversible if ouabain was only left acting for 3-5 minutes. The degree of reversibility also depends on the concentration used, lower concentrations being more easily reversed.

In this chapter the ionic mechanism of an inhibitory response to L-glutamate was investigated. The nature of an electrogenic pump was also investigated. The potency ratio for L-glutamate and its analogous for both excitatory and inhibitory responses was calculated.

RESULTS

L-glutamate inhibition

The ionic mechanism of the inhibitory response of L-glutamate was investigated seven times on cell F1. At the normal resting potential of the cell L-glutamate induced hyperpolarisation of the cell membrane and this was associated with an increase in membrane conductance.

Figure (36) shows that when the cell was placed in chloride free acetate saline the cell membrane potential depolarised and the firing rate of the cell increased. The inhibitory response to 1mM L-glutamate was reversed to a depolarisation followed by an afterhyperpolarisation of 8-10mV (figure 36C). On returning to normal saline the inhibitory response to 1mM L-glutamate was enhanced (7mV) compared with the control (4mV). In terms of input conductance, for example trace (B), glutamate induced change in input conductance by about 60% (0.06µS) compared with the resting conductance (0.1µS). In trace (F) the changed in input conductance was about 80% (0.08µM) of control value (0.1µS).

Structure activity study on L-glutamate receptor

L and D-glutamate analogues were tested on both the inhibitory and excitatory receptors of L-glutamate. These analogues were tested on identified neurones and their structure given in (figure 37). The potency ratio was measured by producing comparable responses of these analogues with L-glutamate. The results of this study are

Figure 36 Traces of intracellular recordings from cell F-1 to show the ionic mechanism of the inhibitory response to 1mM L-glutamate.

A The excitatory response to 0.01mM acetylcholine (control).

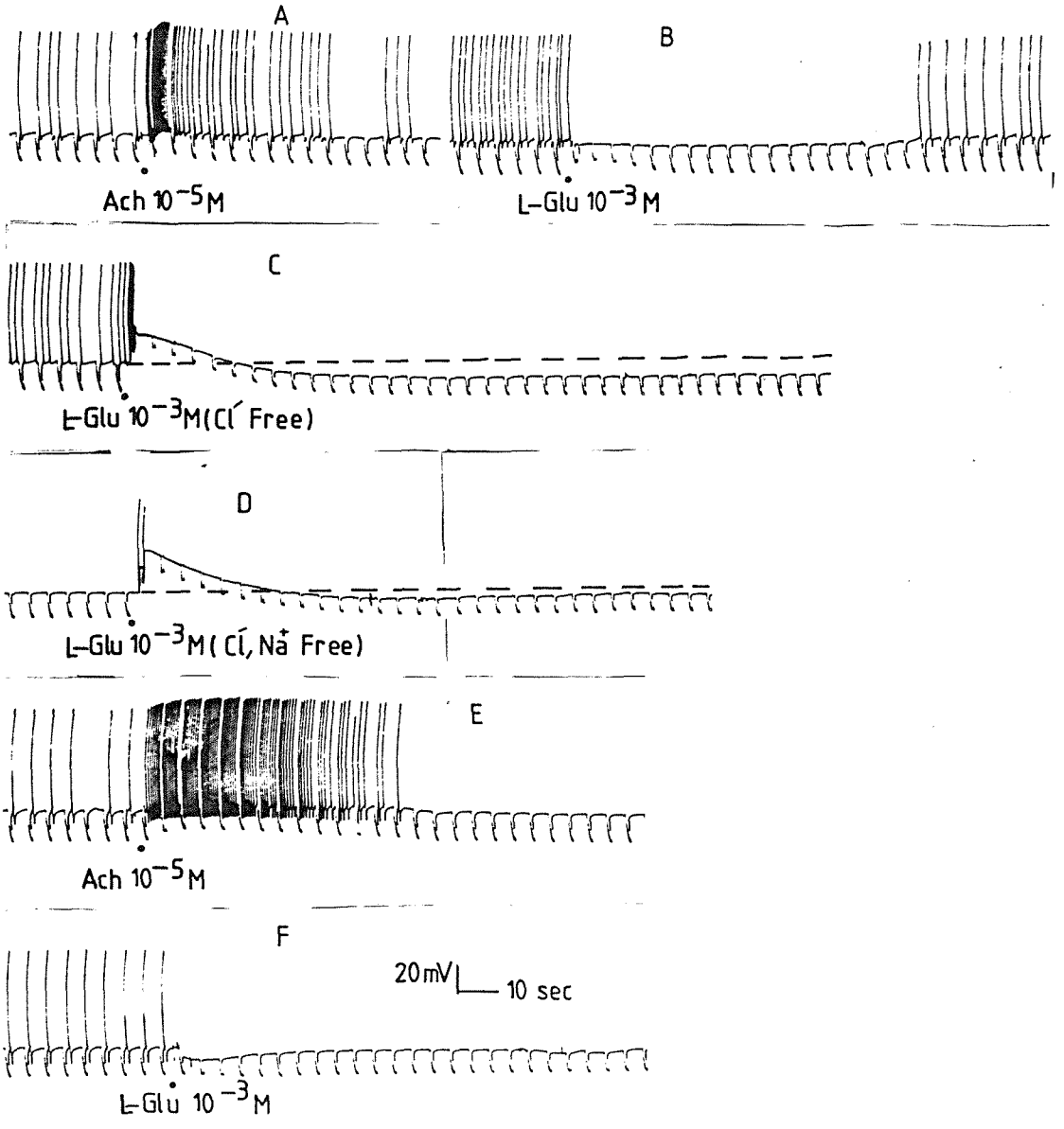
B The inhibitory response to 1mM L-glutamate (control).

C Chloride free saline reversed the inhibitory response to 1mM L-glutamate, with an afterhyperpolarisation of 10 mV.

D In the presence of chloride and sodium free saline. The action potentials were stopped and the afterhyperpolarsation was reduced to 5mV.

E The reapplication to 0.01mM acetylcholine.

F The reapplication to 1mM L-glutamate showed enhancement of the inhibitory response to 10 mV compared with the control (5mV).


A
B
Ach 10^{-5}M
L-Glu 10^{-3}M
C
L-Glu 10^{-3}M(Cl´ Free)
D
L-Glu 10^{-3}M(Cl, Na^{+} Free)
E
Ach 10^{-5}M
F
20mV
10 sec
L-Glu 10^{-3}M

summarised in table (5).

1. *N-methyl-D-aspartic acid (NMDA)*

NMDA was tested on both the excitatory and the inhibitory receptors of L-glutamate and induced a weak response. NMDA was more than 100 times less potent than L-glutamate (table 5). In Mg++ free saline the membrane potential was slightly hyperpolarised (2mV). The response to NMDA in the absence of Mg++ was unchanged compared with the normal saline.

2. γ-N-cyclopentylamide-L-glutamate.

This compound was tested on the inhibitory receptor on the identified neurones F9 and 3 cells F1. This compound was 100 times less active than L-glutamate on these neurones. The cyclopentyl group reduced the activity of L-glutamate.

3. γ- Thio-L-glutamic acid.

This compound with both the D and L configurations were tested on both inhibitory and excitatory receptor sites of L-glutamate. Four F1 cells were used for the inhibitory response. Figure (38) shows that γ-thio-L-glutamic acid mimics the biphasic response of L-glutamate by depolarising/hyperpolarising the cell membrane and increasing the input conductance. The potency of this compound on F1 was 1.5+\-0.11 (n=4) less potent than that of L-glutamate.

γ-Thio-D-glutamic acid mimics the action of L-glutamate on both excitatory and inhibitory receptor

Compound	Excitation	Inhibition	Potency ratio
1. L-glutamate			1
2. NMDA	weak	weak	>100
3. γ-N-Cyclopentylamide-L-glutamate	weak	weak	100
4. γ-Thiol-L-glutamic acid	excitation	inhibition	1. 5+/-0.25 (n=4)
5. γ-Thio-D-glutamic acid	excitation	inhibition	1.72+/-0.25 (n=3)
6. γ-N-Norbornylamide-L-glutamate	-	-	inactive
7. γ-Morpholinylamide-L-glutamate	-	-	inactive
8. γ-N-Cyclohexylamide-L-glutamate	-	-	inactive
9. γ-N-terttiary butylamide-L-glutamate	-	-	inactive

Table 5

A structure-activity study on a range of L-glutamate analogues to compare their actions and potencies to L-glutamate on Helix neurones. The D-glutamate analogue (γ-thio-D-glutamate acid) was also tested.

sites. The cells used were F1 4 cells for the inhibitory response and F16 for the excitatory response of L-glutamate. The potency of this compound on F1 was 1.72+\-0.25 (n=3) less potent than L-glutamate.

4. γ-N-tertiary butylamide-L-glutamate.

This compound was tested on the inhibitory receptor of L-glutamate. This compound appeared to be completely inactive on 3 F1 and F30 cells.

5. γ-N-Norbornylamide-L-glutamate.

This compound was tested on five neurones(3 times F1, and once on E5, and E4) which are inhibited by L-glutamate. The results indicated that this was inactive on these cells.

6. γ-N-morpholinylamide-L-glutamate

The potency of this compound was investigated on four cells (3 times on F1 and once on F30). The results obtained indicated that this compound was inactive on these neurones.

7. γ-N-cyclohexylamide-L-glutamate.

This compound was tested on E5, F16, E4, F1 and unidentified F cell. These cells did not respond to γ-N-cyclohexylamide-L-glutamate.

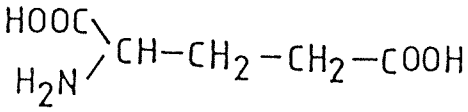
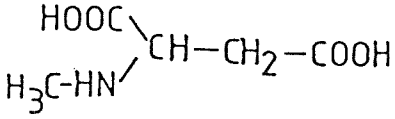
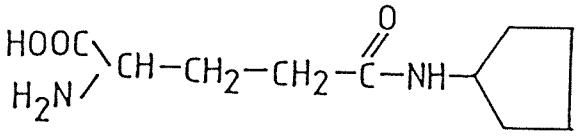
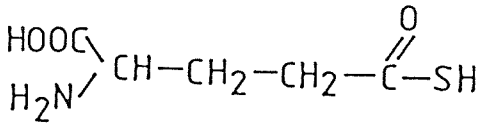
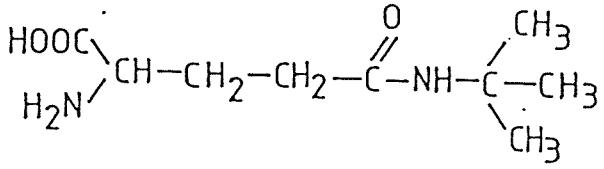
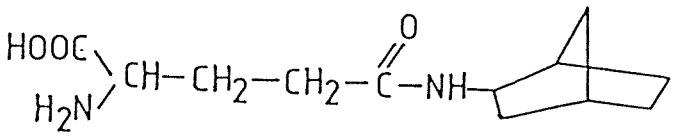
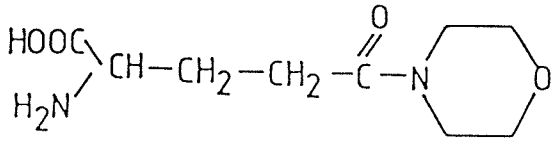
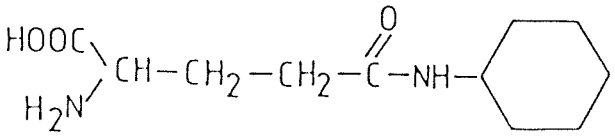
Compound	Structure
L-Glutamic acid	
NMDA	
γ-N-Cyclopentylamide-L-glutamate	
γ-Thioglutamic acid	
γ-N-Tertiary butylamide-L-glutamate	
γ-N-Norbornylamide-L-glutamate	
γ-Morpholinylamide-L-glutamate	
γ-N-Cyclohexylamide-L-glutamate	

Figure 37 Structure of L-glutamate analogues

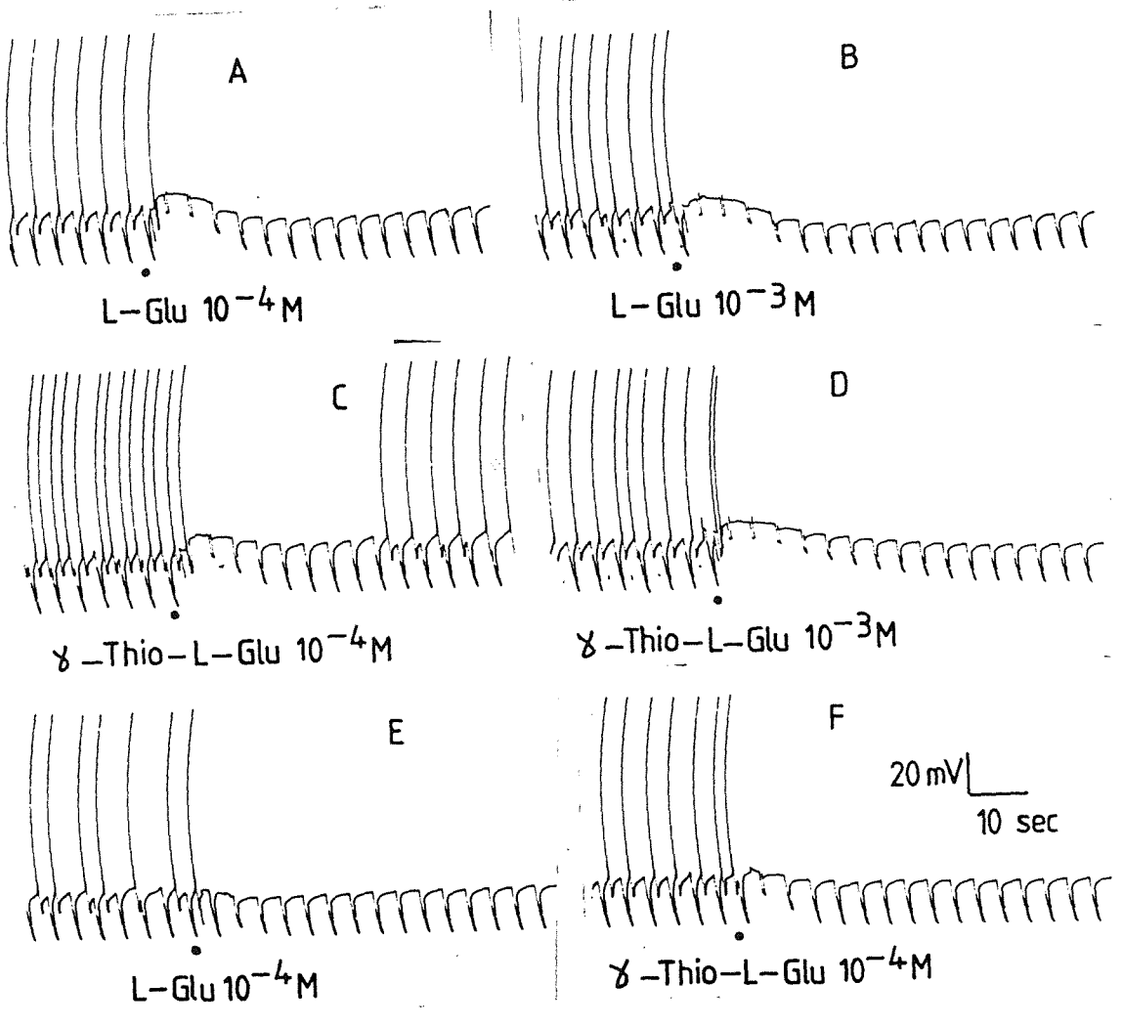
Figure 38 Traces of intracellular recordings from neuron F-1 to show a potency comparison between L- glutamate and its analogue γ-Thio-L-glutamic acid.

A & B show the effect of increasing concentrations of L-glutamate on cell F-1.

C & D Show the effect of an increasing concentrations of γ-Thio-L-glutamate. This compound mimics the action of L-glutamate.

E Reapplication of 0.1mM L-glutamate.

F Reapplication of 0.1mM γ- thio-L-glutamic acid.


A
L-Glu 10^{-4}M
B
L-Glu 10^{-3}M
C
γ-Thio-L-Glu 10^{-4}M
D
γ-Thio-L-Glu 10^{-3}M
E
L-Glu 10^{-4}M
F
20 mV
10 sec
γ-Thio-L-Glu 10^{-4}M

Electrogenic Sodium Pump

The presence of an electrogenic sodium pump was investigated on cell F1. This neurone is excited by acetylcholine and inhibited by L-glutamate. In chloride free saline the response to L-glutamate is reversed. In these experiments the effect of chloride, sodium, and potassium free saline on a possible electrogenic pump induced by L-glutamate was investigated.

Figure (36) shows that when the external chloride was removed the inhibitory response to 1mM L-glutamate was reversed to a depolarisation followed by an afterhyperpolarisation of (8-10mV) figure (36 C). In the absence of both chloride and sodium (figure 36 D) the action potentials disappeared and the depolarisation to 1mM L-glutamate was slightly enhanced (25 mV) compared with the response to the same concentration of L-glutamate in chloride free media (22 mV). The afterhyperpolarisation was also reduced from 8 mV to 4 mV. On returning to normal saline the inhibitory response to 1mM L-glutamate was increased (7 mV) compared with the control (4 mV) and the action potentials returned (figure 36 B & F).

The experiment illustrated in figure 39 shows the effect of potassium free saline on the afterhyperpolarisation. L-glutamate 1mM was applied to the bath in chloride free saline. The reversed response to L-glutamate was followed by afterhyperpolarisation figure 39B of 8mV. This afterhyperpolarisation was either reduced greatly or absent in the presence of potassium free saline. In the experiment illustrated in figure 39 the afterhyperpolarisation of 8 mV was reduced to 2mV in chloride, potassium free saline. It was also observed that

the depolarisation to 1mM L-glutamate was increased in chloride, potassium free saline compared to the same concentration of L-glutamate in chloride free media figure (39 B & C). In this experiment the depolarisation in trace"B" was 4 mV while that in trace"C" was 12mV. On returning to normal saline the response of L-glutamate was enhanced 7 mV compared with the control 3 mV figure (39 trace D & A).

Strophanthidin was applied to the bath in chloride free saline and was left in contact with the preparation for five minutes prior to the application of L-glutamate. This compound was tested on four identified neurones. Srophanthidin was applied in different concentrations. At high concentrations, for example 0.1mM the depolarisation was reduced to about 50% compared to control and the afterhyperpolarisation which followed the application of 1mM L-glutamate was decreased by about 80% compared with the control. At low concentrations, for example, 1µM strophanthidin shown in figure 40 both the afterhyperpolarisation and the depolarisation to 1mM L-glutamate were reduced figure (40 trace C & D). It was also observed that strophanthidin prolonged the depolarisation to 1mM figure (40 D). The effect of strophanthidin was partialy reversed after washing out with saline for 15 minutes figure (40 E & F).

Figure 39 Effect of potassium and chloride free saline on the electrogenic sodium pump from cell F-1.

A The inhibitory response to 1mM L-glutamate in normal saline.

B The reversed (depolarisation) inhibitory response to 1mM L-glutamate in chloride free saline. The dashed line refers to the afterhyperpolarisation.

C The afterhyperpolarisation is greatly reduced in chloride and potassium free media.

D The inhibitory response to 1mM L-glutamate returns in normal saline.

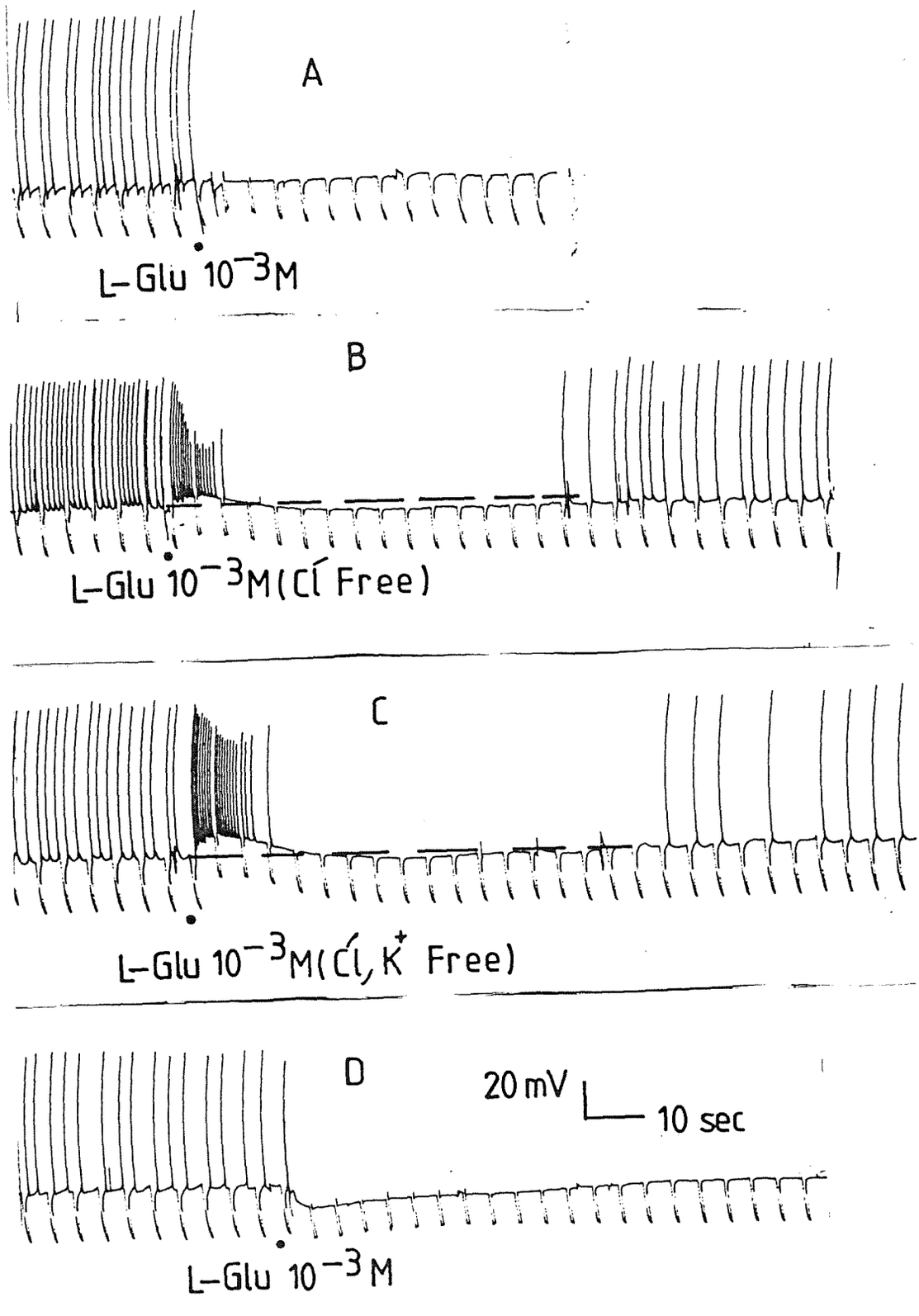

A
L-Glu 10^{-3}M
B
L-Glu 10^{-3}M(Cl Free)
C
L-Glu 10^{-3}M(Cl, K^+ Free)
D
20 mV
10 sec
L-Glu 10^{-3}M

Figure 40 The effect of strophanthidin on the afterhperpolarisation which followed the application of 1mM L-glutamate.

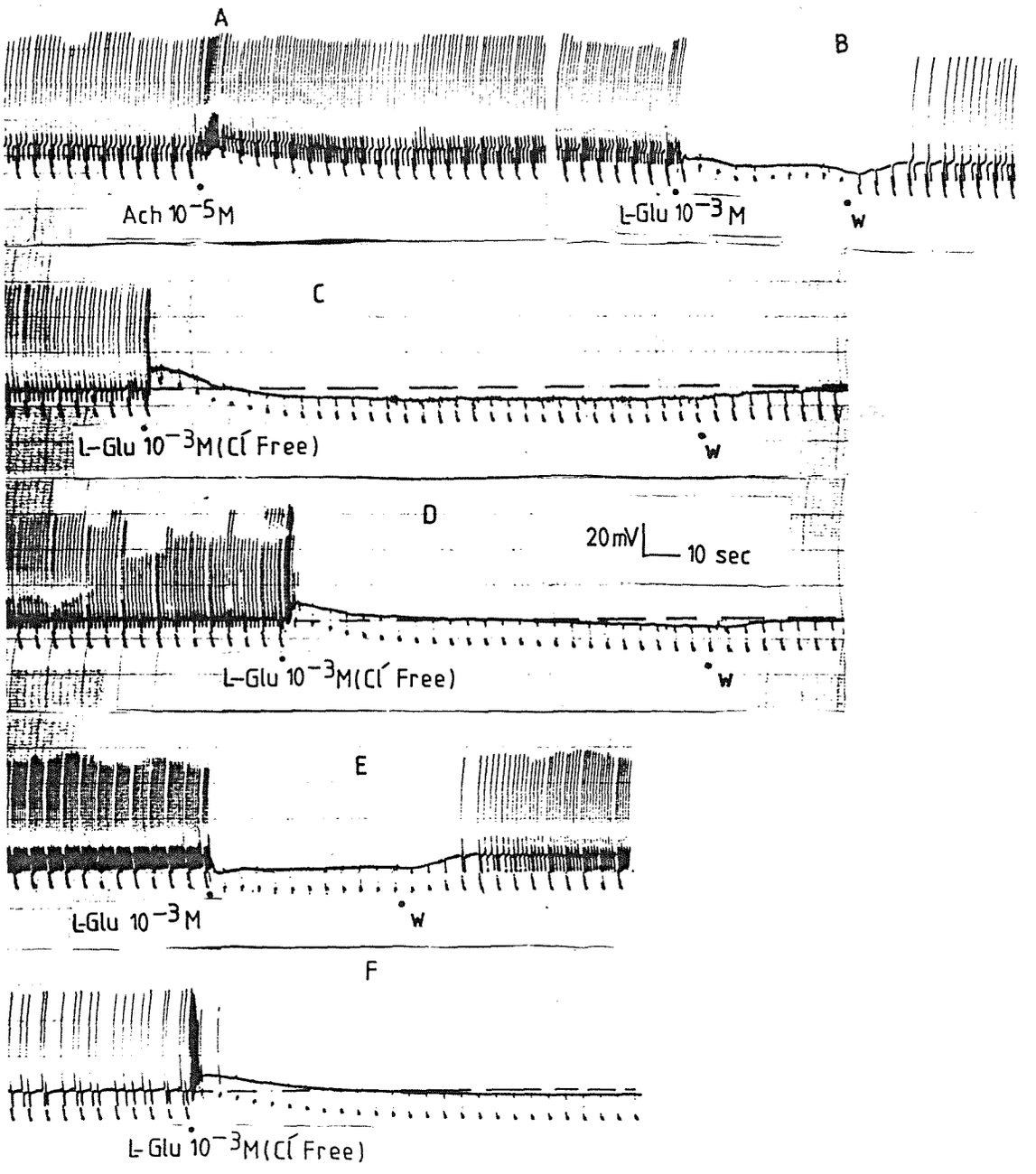
A & B Control excitatory response 10 mV to acetylcholine and inhibitory response 6mV to L-glutamate.

C 1mM L-glutamate in chloride free saline.The dotted line shows the level of the afterhyperpolarisation which followed the application of L-glutamate (6 mV).

D Following the addition of 1µM Strophanthidin the afterhperpolarisation was greatly reduced (2 mV) and the response to 1mM L-glutamate was slightly prolonged compared with the trace (C).

E Shows the inhibitory response to L-glutamate was enhanced by 5 mV compared with the control (B).

F L-glutamate in chloride free saline to show that the effect of strophanthidin is partially reversible.


A
B
Ach 10^{-5}M
L-Glu 10^{-3}M
W
C
L-Glu 10^{-3}M(Cl Free)
W
D
20mV
10 sec
L-Glu 10^{-3}M(Cl Free)
W
E
L-Glu 10^{-3}M
W
F
L-Glu 10^{-3}M(Cl Free)

Discussion

The results confirm that the inhibitory response of L-glutamate on the cell F1 is chloride mediated. When this cell was placed in chloride free acetate saline the membrane was shifted towards more positive values and the response to L-glutamate was reversed. The reason is that the internal chloride leakes out of the cell and the inhibitory response to L-glutamate reverses to excitation. This would suggest that the chloride equilibrium potential had become more positive than membrane potential. This indicates that the cell membrane is permeable to chloride ions. It is also observed that the depolarising response to L-glutamate was slightly enhanced either in chloride, sodium free saline or in chloride, potassium free saline compared with the same concentration of L-glutamate in chloride free saline. The explanation is that the ECl moved towards a more positive value after the application of L-glutamate in chloride free saline and the result of this was an efflux of intracellular chloride from the cell. In the presence of zero external chloride, the ECl was more positive than at the start the experiment. When the same concentration of L-glutamate was applied in the absence of either chloride and sodium or chloride and potassium the response to L-glutamate was enhanced because of the higher ECl. Therefore the higher the ECl value the more enhancement of the depolarising response to L-glutamate, after washing out each dose.

The depolarising response of L-glutamate was enhanced in chloride free saline i.e. 15mV depolarisation compared with the inhibitory response to L-glutamate 5mV for the same concentration in normal saline. These results are in

agreement with Kerkut and Thomas (1963) who observed a shift of 22 mV to the positive for acetylcholine when *Helix* neurones were exposed to saline containing no chloride saline. Similarly, Gardner and Kandle (1977) found the average shift in IPSP reversal potential was 42 mV in a depolarising direction when *Aplysia* neurones were bathed in chloride free sea water saline.

On returning to normal saline the addition of L-glutamate brought about a hyperpolarision of 10 mV (figure 36) a greater hyperpolarisation than in the original condition 5 mV (figure 36). This was due to chloride diffusing from the cell when it was in chloride free saline and thus affecting chloride gradient across the membrane on return to normal saline.

The potency of L-glutamate varied from one neurone to another. For example L-glutamate was less potent on cell F1 compared with neurone F9. Both these cells are inhibited by L-glutamate. It was also observed that the interaction of L-glutamate with the receptor site on F1 was slower than that of F9. Piggott et al (1975) investigated the effect of L-glutamate analogues on three identified *Helix* neurones (F1, E4, and F30) and found there were differences between the excitatory receptors of F30 and the inhibitory receptors of E4 and F1. The excitatory receptors required the glutamate molecule to be in a conformation resembling L-aspartic acid for optimum activity whereas the inhibitory receptor site on F1 and E4 appeared to prefer the more extended conformation of ibotenic acid.

The structure-activity relationships in this study indicated that on all cell types L-glutamic acid was the most potent compound compared with its analogues. All the α-

NH_2, α-COOH positions in L-glutamate analogues were unchanged. The substitutions were made in the γ-COOH group. The γ-Thio-L-glutamic acid was approximately equipotent with L-glutamate on both the inhibitory and the excitatory receptors. Substitution of SH instead of OH in this compound did not affect the potency of L-glutamate. Interestingly the L and D forms of thio-glutamic acid are similar in potency to L-glutamate which is unlike the case of D and L structural configurations. D-glutamate was found to be eight to ten times less potent than L-glutamate on *Helix* neurones (Piggott et al. 1975). NMDA was more than 100 times less potent than L-glutamate on both the inhibitory and the excitatory receptors of L-glutamate. Substitution of methyl group on the α-NH_2 group greatly reduced the activity of this compound compared with L-glutamate. The presence of a methyl group may stabilize the membrane in an unionized state.

It is interesting to note that γ-N-cyclopentylamide-L-glutamate was more than 100 times less potent than L-glutamate whilst γ-N-cyclohexylamide-L-glutamate was inactive. The reason is that the cyclohexyl moiety in γ-N-cycloamide-L-glutamate is expected to be in a more stable chair conformation. Therefore it is reseasonable to assume that this compound may not be accommodated into the receptor site and so is not reactive. While γ-N-cyclopentylamide-L-glutamate has a near planar structure and hence shows some reactivity.

In this study, a possible electrogenic pump was investigated in the cell F1. This neurone tends to hyperpolarise to a greater extent than other cells either following the application of an excitatory compound or when the neurone is at resting membrane potential values. It was

also observed that this neurone can exhibit regular bursting activity. These observations suggest that F1 might have an electrogenic pump and are in agreement with observations by Airapetyan (1969) and Romey and Arvanitaki-Chalazonits (1970). They reported that *Helix* and *Aplysia* neurones are rhythmically active,firing bursts of action potentials at regular intervals. This spontaneous activity could be regulated by an electrogenic pump.

The nature of the possible electrogenic pump induced by L-glutamate in chloride free saline was investigated in this study. Chloride free saline allows us to obtain an afterhyperpolarisation following the reversed inhibitory action of L-glutamate. The electrogenic pump may be stimulated by this depolarisation to L-glutamate.

Generally the pump rate of an electrogenic sodium pump is proportional to the intracellular sodium. Thus loading the cell with sodium stimulates the pump and many studies on the electrogenic sodium pump have been carried out using sodium-loaded cells, unlike the work in this study which involved chloride efflux to activate the pump.

There is considerable evidence that increasing the internal sodium concentration either by injecting into the cell or by depolarising the cell membrane by an excitatory compound stimulates the pump (Thomas 1972). Whereas our results indicate that although the reversed hyperpolarisation to L-glutamate is chloride mediated an electrogenic pump appeared to be activated and an obvious afterhyperpolarisation occurred. This afterhyperpolarisation was reduced in sodium free saline. This would indicate that the afterhyperpolarisation is at least partly dependent on the external sodium and when it was replaced with Tris, it

is no longer possible for sodium to enter the cell when it depolarised. It would be interesting to leave the neurone in the media (sodium free saline) for an hour and to see whether or not the pump is completely blocked. Mullins and Brinley (1969) found no effect on the sodium efflux from dialysed squid axon. According to Baker et al (1969) removal of external sodium increases the affinity of the pump mechanism for external potassium ions.

Decreasing external sodium should decrease the passive sodium influx into the cell and thus reduce the sodium pump. Reducing external sodium will also reduce the energy required to operate the pump which will be smaller. The present study indicated that in the presence of potassium free saline the afterhyperpolarisation was either greatly reduced or absent. Further experiments are required to investigate the effect of increasing or decreasing the external potassium concentration on the membrane potential.

In our experiments strophanthidin greatly reduced the afterhyperpolarisation associated with the reversed response to L-glutamate in chloride free saline. This reversed response was prolonged by strophanthidin. These results suggest that strophanthidin blocked the activated hyperpolarisation in this neurone. These results are in agreement with those obtained by Kazchenko et al (1979) and Mat Jais et al (1986).

Boyle and Conway (1941) provided evidence from frog muscle which suggested that changes in chloride concentrations at constant osmotic pressure and at external potassium concentration produced only a transient alteration of the membrane potential and the membrane at equilibrium depended on the potassium gradient across the membrane .

Their results were developed by Hodgkin and Horowicz (1959) who concluded that in frog muscle both chloride and potassium have an influence on the membrane. The results agreed quantitatively with the idea that these two ions control the membrane potential. Kerkut and Thomas (1963) observed a shift of 22mV to the positive for the acetylcholine inhibitory response when the *Helix* neurones were exposed to chloride free saline.

CHAPTER 6

LOCAL ANAESTHETICS AND TRANSMITTER GATED CHANNELS

Introduction

Most local anaesthetic agents consist of a lipophilic group, and often an aromatic ring connected by an intermediate chain commonly including an ester or amide to an ionizable group usually a tertiary amine. Optimal activity requires a delicate balance between the lipophilic and hydrophilic strengths of these groups. In addition to the general physical properties of the molecules, specific stereochemical configurations can also be important; i.e. differences in potency of stereoisomers have been documented for a few compounds. Since ester links as in procaine are more prone to hydrolysis than amide links, esters usually have a shorter duration of action.

Local anaesthetics are weak bases. For therapeutic application, they are usually synthesised as salts for reasons of solubility and stability. In the body, they exist either as the uncharged base or as cation. The cation form is thought to be the most active form at the receptor site since cationic drugs can not readily leave closed channels, but the uncharged fraction is very important for rapid penetration of biological membranes since the local anaesthetic receptor is not accessible from the external side of the cell membrane (Katzung, 1984).

The smaller and more lipophilic the molecule, the faster the rate of the interaction with the sodium channel lipoprotein. Potency is also positively correlated with lipid solubility, as long as the agent remains to some extent water-soluble, since water solubility is required for diffusion to the site of action. Lidocaine, procaine, and

mepivacaine are more water-soluble than tetracaine, etidocaine and bupivacaine. The latter agents are more potent and have longer durations of action. They also bind more extensively to proteins and will displace or be displaced from these binding sites by other drugs.

Sodium channels are probably composed of lipoproteins that span the thickness of the nerve membrane(Benzer and Raftery,1972). These authors reported that the outer opening of the channel appears to be a relatively static structure. It is the site where drugs like tetrodotoxin (TTX) bind and block the sodium ion influx. Tetrodotoxin binds to the channel when they are open or closed, i,e. when the nerve membrane is at rest or stimulated(Schwarz, Ulbrich, and Wagner,1973).

The inner membrane end of sodium channels is probably the location of structures that open and close channels, the so-called "gating functions". Gating properties can be selectively affected by exposure of the inner surface of the nerve membrane to specific agents(Armstrong, Bezanilla, and Rojas, 1973).

The full extent to which drugs exert their pharmacological and therapeutic effects by acting at the ion channel level is not known. However it should always be taken into account as a possible way in which drugs can influence synaptic transmission. Studies where it is possible to distinguish between ion channels and ligand receptor sites of action will enhance our understanding of this matter.

Local anaesthetics such as procaine and lidocaine block action potential propagation along axons by acting at the

sodium ion channel(Hille 1966). The local anaesthetics used clinically differ widely in their chemical structure but all are lipid soluble and so can cross nerve sheaths and membranes to reach their site of action. A major advance came with the introduction of quaternary derivatives of local anaesthetics. Compounds such as QX-314 have a permanent positive charge and so cannot easily cross cell membranes. These drugs block sodium channels when applied inside the cell(Frazier et al 1970). Interestingly Strichartz(1973) found a close analogy between QX compound block of sodium channels and quaternary ammonium block of potassium channels. The drug receptor interaction requires the channel gate to be open for it to act. Thus after QX-314 is applied inside a myelined nerve fibre, the first voltage clamp test pulses elicit a nearly full-sized inward sodium current, indicating that no block has developed at rest. Subsequent pulses given at one second intervals produce smaller and smaller currents showing that the drug binds progressively during the depolarising pulses and does not dissociate at rest. This gradual development of block with repetitive stimulation is termed "Use-Dependent Block"(Courtney 1975).

Use-dependent block with QX-314 requires the channels to be opened. Thus it develops only when depolarising pulses are large enough to open the channels. The rate of block per pulse can be increased by giving a hyperpolarising pre-pulse before each test pulse and decreased by a small depolarising pre-pulse. These pre-pulses change the fraction of channels opening during each pulse by changing the extent of sodium inactivation.

The sodium channel can exist in three functionally distinct states or group of states: resting, active or

inactive. Both resting and inactivated channel states are nonconducting but channels that have been activated by prolonged depolarisation are refractory unless the preparation is repolarised to allow them to return to the resting state. These basic properties are known to be characteristic of channels in mammalian myelinated nerve, neurones in culture, skeletal muscle fibres in the cardiac conducting system and dissociated cardiac myocytes.

Studies have been carried out on the interaction of local anaesthetic agents and calcium ions. Since it is believed that calcium ions may have a regulatory role in the movement of sodium ions across the nerve membrane, it is conceivable that the local anaesthetic agents exert primary action on calcium and indirectly suppress sodium conductance. Blaustein and Goldenman (1966) have conducted voltage clamp studies with the lobster axon revealing that an increase in calcium decrease the ability of procaine to reduce sodium conductance. Conversely, a decrease in calcium concentration resulted in an increased inhibition of sodium conductance by procaine. This suggests that local anaesthetic agents may compete with calcium for some sites on the nerve membrane that controls the movement of sodium ions across the membrane. Additional support for this hypothesis was forthcoming from the studies by Kuperman, Alture and Chezar(1968). These investigators immersed isolated frog sciatic nerves in saline containing labeled calcium (^{45}Ca) for four to eight hours. The nerves were then placed in calcium-free saline, and the rate of calcium efflux from nerve to the bathing solution determined. Addition of 20 mM procaine to the nerve bath markedly accelerated the rate of efflux of ^{45}Ca from the isolated nerve. Similar results of an increased calcium release by procaine were obtained in isolated sartorius muscle. In

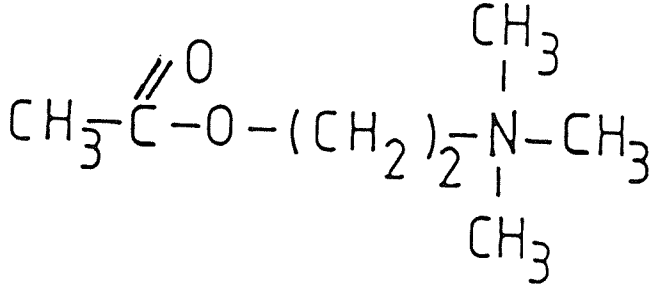
addition, a comparison of the calcium-release effect of procaine and tetracaine in sartorius muscle revealed that 5 mM tetracaine produced almost twice the increase in rate of calcium efflux as did 50 mM procaine. Thus, it is of interest that an approximate correlation appears to exist between the calcium displacement effect and local anaesthetic potency of procaine and tetracaine.

Some drugs with diverse structures decrease the agonist-induced ionic endplate conductance in a reversible manner. A clue to their mechanism of action was provided by the observation that such drugs,i.e., the local anaesthetics modified the decay time of end-plate currents (e.p.c.s.) and miniature end-plate currents (m.e.p.c.s.) (Ruff 1977). Since Anderson and Steven (1973) had demonstrated that the exponential time constant of m.e.p.c. decay is probably a measure of the randomly distributed life time of the acetylcholine-activated ionic channels, this observation indicated that these drugs in some way modified the life time of the acetylcholine ionic channels. Adams(1975) suggested that local anaesthetics did not interact with the acetylcholine binding site on the receptor but rather with the ionic channel associated with the receptor, a mechanism first proposed by Blackman(1970) to account for the action of hexamethonium on ganglion cells. Following Adams's model, a cationic local anaesthetic is envisaged as entering the ionic channel when the receptor-channel complex is activated to allow the flow of ions through the channel. Drug binding to the activated agonist-receptor complex is seen as not only blocking the channels, but simultaneously "freezing" the agonist-receptor channel complex in the open but blocked state, so that it can repeat its normal life cycle only when the antagonist dissociates from the ionic channel binding site.

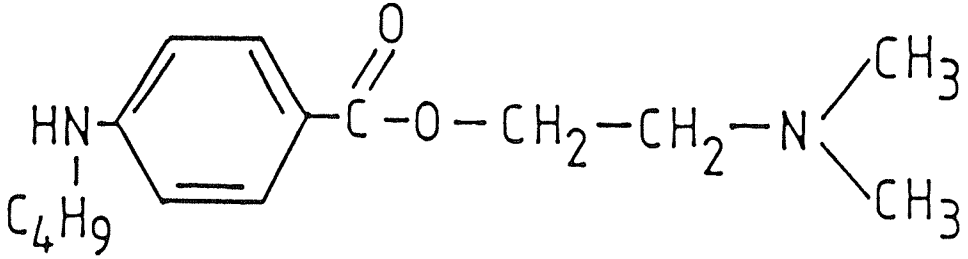
This chapter focuses on the action of the local anaesthetics; procaine and tetracaine both of which were suggested by many researchers to interfere with opening of ion channels in excitable membranes. The data in this chapter deals particularly with tetracaine. This compound possesses an agonist and antagonist action on snail neurones. These actions were investigated on the action potentials and on transmitter gated channels of acetylcholine and dopamine .

Representative structures of these compounds are presented in figure 41.

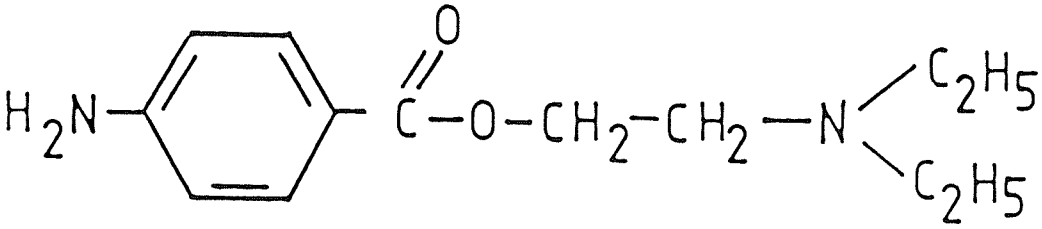
Figure 41 The chemical structures of acetylcholine, tetracaine, procaine and dopamine.



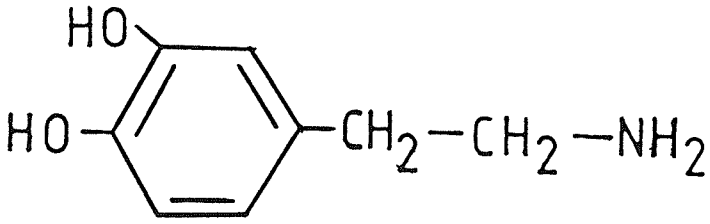
Acetylcholine



Tetracaine



Procaine



Dopamine

RESULTS

Figure 42 illustrates a series of control experiments in which acetylcholine was applied locally to neurone F-1. As can be seen the EC_{50} of the depolarising response to acetylcholine was stable for 150 minutes (Table 6). The acetylcholine was ionophoretically applied 4 times. Trace A shows a depolarisation of the membrane potential with associated action potentials. Neurone F-1 was left for about 70 minutes and acetylcholine was reapplied. Traces B, C, F, and G showed lack of action potentials at resting membrane potentials whereas the neurone induced spikes of action potentials when excited by acetylcholine. In trace (G) acetylcholine was applied after 180 minutes. The depolarising response declined slightly compared with the first control and its potency ratio was (1.31) compared with first control A (see Table 6). Therefore, the traces in this figure indicated that the ionophoretically applied acetylcholine did not desensitise dramatically. The data from this figure were plotted as a series of dose-response curves. The EC_{50} for each was constructed and the potency ratio is shown in table (6).

Table 6 shows the EC_{50} for repeated control applications of acetylcholine with times periods of up to 210 minutes. The EC_{50} ratios were similar. These data indicated that the ionophoretically applied acetylcholine did not produce desensitization.

Figure 43 illustrates the responses to repeated doses of ionophoretically applied acetylcholine. Doses were repeated every 25 minutes for 75 minutes. The EC_{50} for each

curve was constructed. The data indicated that the values of the EC_{50} were similar. The dose-response curve after 75 minutes has a slightly lower maximum peak compared with the others, but a similar EC_{50} value 380 compared with the initial value of 340. These data demonstrated the lack of desensitisation.

Figure 42 Intracellular recordings from F-1 illustrating a series of control depolarisations in response to ionophoretic application of a range of doses of acetylcholine.

A. shows the first trace of control depolarisation responses to ionophoretically applied acetylcholine.

B. After 70 minutes the depolarising responses were unchanged, and the action potentials disappeared at normal membrane potential.

C. shows graded responses to acetylcholine after 90 minutes.

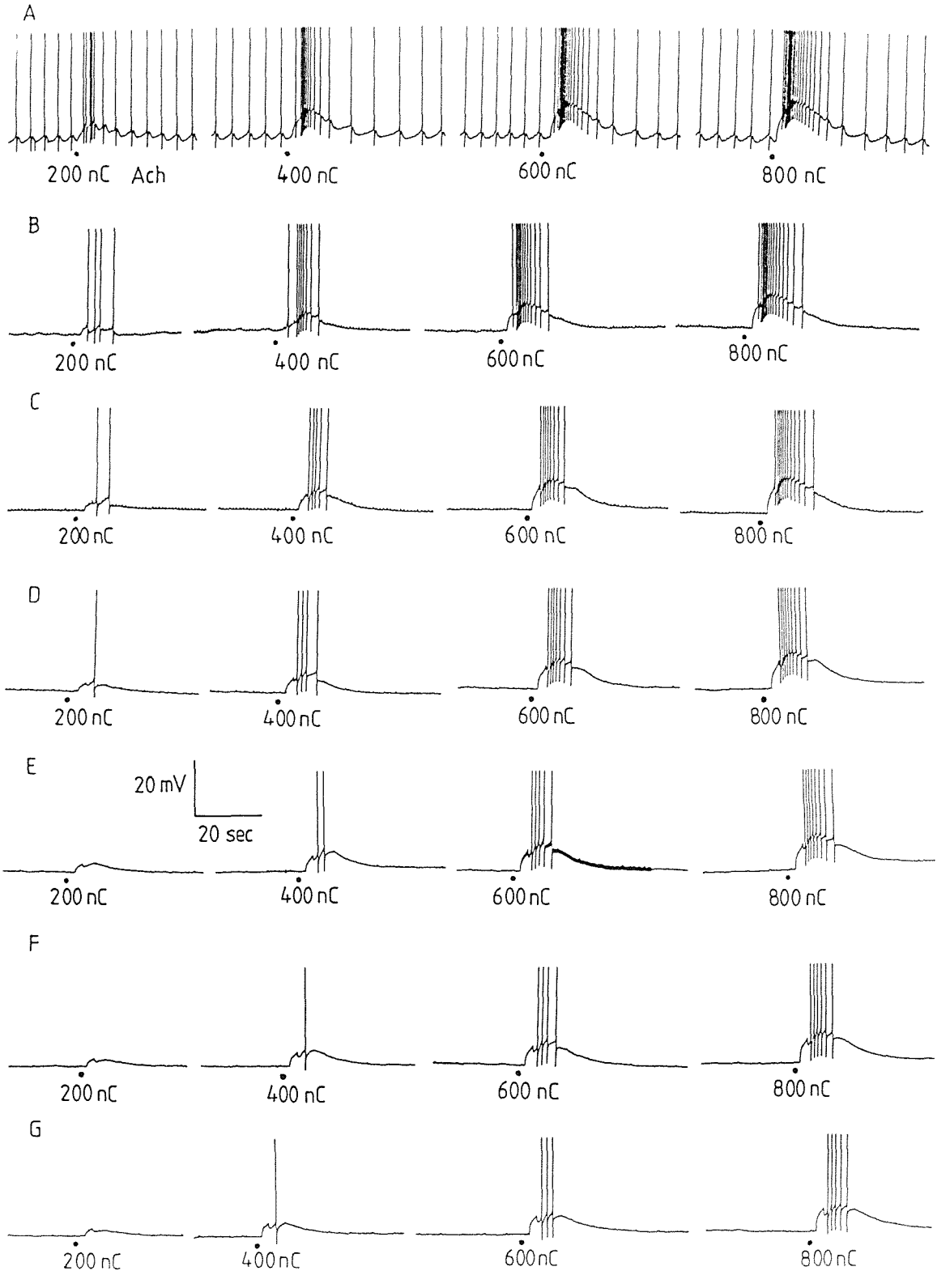
D. shows graded responses to acetylcholine after 110 minutes.

E. shows graded responses to acetylcholine after 130 minutes.

F. shows graded responses to acetylcholine after 150 minutes.

G. shows graded responses to acetylcholine after 180 minutes.

These control experiments were repeated up to H (table 6). Trace H is omitted.


A
200 nC Ach
400 nC
600 nC
800 nC
B
200 nC
400 nC
600 nC
800 nC
C
200 nC
400 nC
600 nC
800 nC
D
200 nC
400 nC
600 nC
800 nC
E
20 mV
20 sec
200 nC
400 nC
600 nC
800 nC
F
200 nC
400 nC
600 nC
800 nC
G
200 nC
400 nC
600 nC
800 nC

control	time/min.	EC_{50}/nC	P.R.
A	—	365	1
B	70	460	1.2
C	90	405	1.10
D	110	410	1.12
E	130	400	1.09
F	150	440	1.20
G	180	480	1.31
H	210	680	1.86

Table (6). Shows the EC_{50} values for a series of control experiments to ionophoretically applied acetylcholine. This data is taken from figure 42.

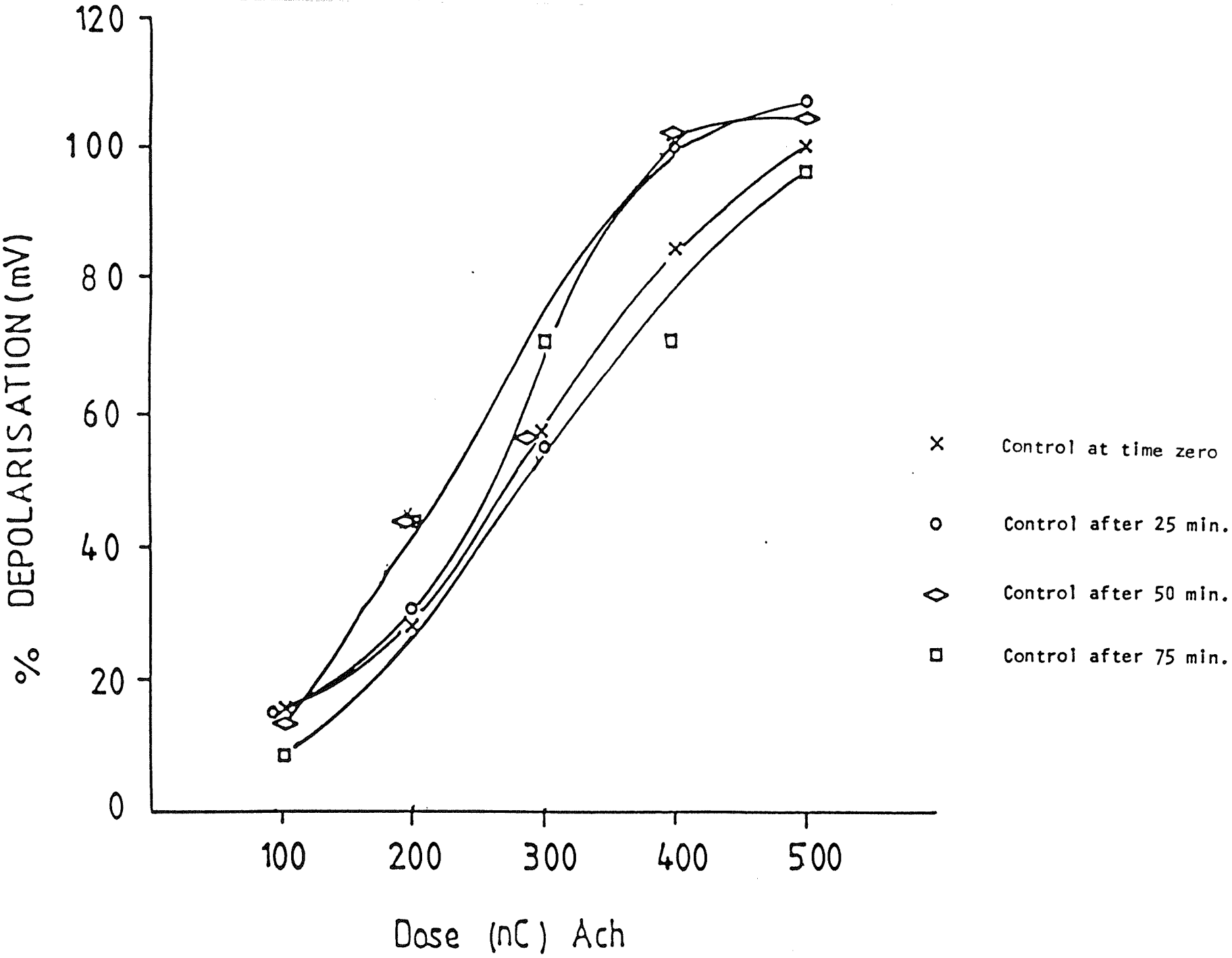
Figure 43 Dose response curves illustrating control experiments to ionophoretically applied acetylcholine up to 75 minutes, from F-1.

x. shows the first doses response curve of depolarisation to acetylcholine. The EC_{50} value for acetylcholine was 340nC.

o. Dose-response curve of depolarisation to acetylcholine after 25 minutes. The EC_5 value was 340nC.

◇ Dose-response curve of depolarisation to acetylcholine after 50 minutes. The EC_{50} value for acetylcholine was 260nC.

□ Dose-response curve of depolarisation to acetylcholine after 75 minutes. The EC_{50} value for acetylcholine was 380nC.


120
100
80
60
40
20
0
% DEPOLARISATION (mV)
100
200
300
400
500
Dose (nC) Ach
Control at time zero
Control after 25 min.
Control after 50 min.
Control after 75 min.

Action of procaine on H neurones

The sensitivity of H-neurones to procaine showed a degree of variation from neurone to neurone and also between the same neurone from different preparations. For example, in four experiments (E4, F9 and 2 cells F18), the responses to a test dose of procaine 1mM range from 2 to 22mV. In these experiments, the large hyperpolarisation produced by 0.1mM procaine is 22 mV (figure 44 H). However, procaine 0.01mM caused a depolarisation to the membrane of 2 mV and increased the frequency of the action potentials without obvious change in the membrane conductance (figure 44B). If procaine is applied at a higher concentration for example, 0.1mM, the second dose at a higher concentration has the same effect as the first one (figure 44 C,D), although the onset of the hyperpolarisation is delayed (trace, D). In the case of cell E-4 (traces E & F), 1mM procaine had a similar effect to 0.1mM acetylcholine.

In some experiments procaine appears to have an antagonist action on the response of acetylcholine. 1mM Procaine reduced the inhibitory response of 0.1mM acetylcholine by about 50%.

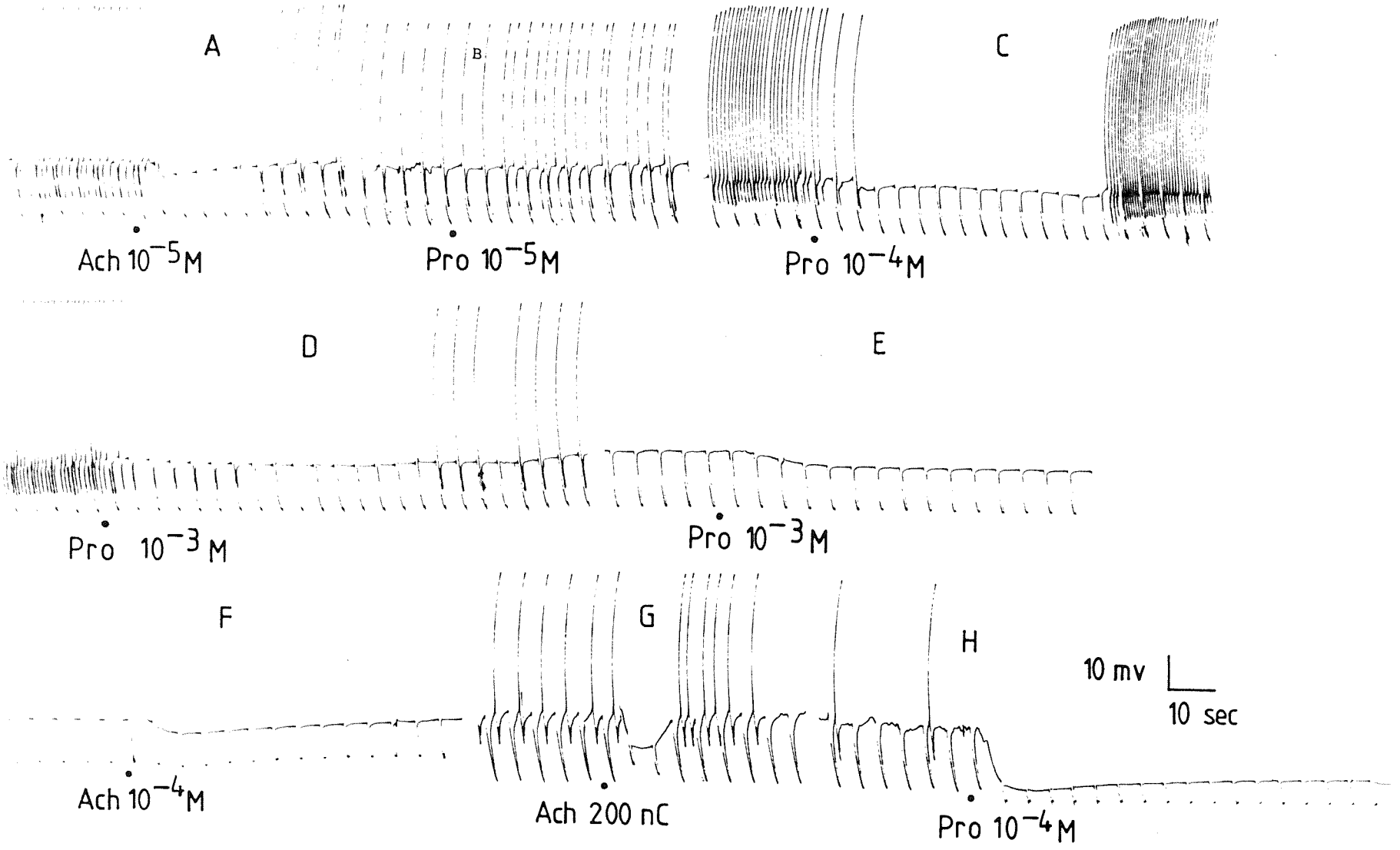
Figure (44, H) shows that high concentrations of procaine, (for example 1 mM) hyperpolarised the membrane and increased the conductance similar to that of acetylcholine,trace G. In this case procaine increased membrane conductance from 0.1 µS to 0.43 µS. This is shownin figure 44 traces A-D from cell F-18, traces E and F from cell E-4 and traces G and H from cell F-9.

Figure 44 Shows responses of H-neurone to procaine.

A,B,C and D Show the inhibitory responses of increasing concentrations to procaine, from cell F-18.
In traces A & B, acetylcholine and procaine were applied at the same concentration (0.01mM).

E & F. Shows the inhibitory responses to 1mM procaine and 0.1 mM acetylcholine respectively; procaine mimics the action of acetylcholine.Traces E & F from E-4.

G & H. Shows the inhibitory responses to ionophoretically applied acetylcholine and bath application to 0.1mM procaine respectively. Procaine increased the membrane conductance by about 200% of control. Traces G and H from F-9


A
B
C
Ach 10^{-5}M
Pro 10^{-5}M
Pro 10^{-4}M
D
E
Pro 10^{-3}M
Pro 10^{-3}M
F
G
H
10 mv
10 sec
Ach 10^{-4}M
Ach 200 nC
Pro 10^{-4}M

Tetracaine actions on "D" neurones

High concentrations 0.1mM of tetracaine, produced a depolarising effect on the cell membrane, whereas at low concentrations,tetracaine only induces a blocking effect. Figure 45 shows the effect of 0.1mM tetracaine on the neurone E2. Tetracaine has a long depolarising action on the cell compared with 0.01mM acetylcholine although it should be noted that the concentration of acetylcholine was lower. Application of 0.01mM acetylcholine produced an increase in input conductance of 0.02 µS compared with the resting conductance 0.10 µS, and a membrane depolarisation of 6 mV (trace A). Tetracaine 0.1mM increased the membrane conductance by 0.16 µS compared with the resting membrane conductance 0.1 µS, and a membrane depolarisation of 21 mV (trace B). On recovery both compounds were reapplied and the changed in conductance measured. Acetylcholine increased the membrane conductance by 0.05µS compared with the resting conductance 0.12 µS, and depolarised the membrane by 5mV. Tetracaine 0.1mM increased the input conductance by 0.13 µS compared with the resting conductance 0.10 µS and depolarised the membrane by 23 mV (trace D). These data indicate that tetracaine 0.1mM possibly slightly reduced the action of acetylcholine on the neurone, but further experiments are required.

In general, D-neurones which respond to acetylcholine did not depolarise when tetracaine was applied even with high concentrations, and in these cells the response to acetylcholine was blocked by tetracaine.

In parallel with the study of the depolarising effect of tetracaine, its inhibitory action on ionophoretic application of acetylcholine was also investigated. Partial

current dose-response curves to ionophoretically applied acetylcholine were obtained as a control then repeated in the presence of tetracaine 0.01 µM for different periods up to 50 minutes.

The data from these experiments are plotted in the form of ionophoretic current-voltage relations. The EC_{50} was obtained from the graphs. Figure 46 illustrates an experiment in which tetracaine 0.01 µM was applied, and the gradual decline of acetylcholine responses recorded. The initial effect of tetracaine 0.01 µM on the acetylcholine response was recorded after 7 minutes(trace B). After 50 minutes tetracaine 0.01 µM became fully effective blocking both action potentials and the response to acetylcholine (trace D).

The data from figure 46 are shown graphically in figure 47. It can be seen that the dose response curves are progressively shifted to the right with increasing time follows the application of 0.01µM tetracaine. The control EC_{50} values for acetylcholine from the dose-response curve was 270nC. The EC_{50} values for acetylcholine in the presence of 0.01 µM tetracaine after 7, 20 minutes, and 50 minutes were 510,750,and 1200nC respectively (figure 47 and table 7).

A further experiment was conducted where 0.01µM tetracaine was left for 30 minutes in the bath prior to the reapplication of the ionophoretically applied acetylcholine. The results indicated that the amplitude of the depolarising response to acetylcholine gradually decayed with time. The results from this experiment are shown in graphic form in figure 48. It can be seen that the slope of the acetylcholine dose-response curve shifted greatly to the

right after 80 minutes (curve □). The EC_{50} values for acetylcholine in the presence of tetracaine are higher than control value. For example, the EC_{50} values for acetylcholine in the presence of tetracaine after 30 and 50 minutes are 390nC and 1080nC respectively compared with EC_{50} control value 270nC.

Generally, in the presence of 0.01 µM tetracaine the response to acetylcholine became progressively smaller with time and the action potentials gradually disappeared. Further experiments were made on cell F-1 to determine whether tetracaine blocked the action potentials in the presence of acetylcholine. Tetracaine with increasing doses 0.01,0.1, and 1 µM was left for 50 minutes in the bath and during this time had no obvious effect on the action potentials whereas these concentrations of tetracaine reduced the action potentials gradually during the same period of acetylcholine application (Figure 49).

Table 7 summarise the action of tetracaine when tested against the excitatory response to acetylcholine on cells F6, F1, E2, and an unidentified F cell. The effect of 0.01 µM tetracaine was tested at 2, 7, 20, 30 and 50 minutes following the initial application. This concentration of tetracaine gradually reduced the EC_{50} value for acetylcholine from around 200 nC to over 1000 nC. The EC_{50} values are also expressed as a ratio compared to the control and this ratio gradually increases to a value around 4. The data would suggest that the ratio increases each time a dose-response curve is taken rather than being linked to the time following initial exposure to tetracaine. For example, the ratio following the first dose-response curve in the presence of tetracaine is similar whether it is taken 2

minutes, 7 minutes or 30 minutes after exposure.

Table 7A

Summary of the EC-50 values to ionophoretic applications of ACh on the excitatory receptor. The EC-50 values for ACh were calculated in the presence of 0.01 μM tetracaine with time and compared to the EC-50 value of ACh as standard control.

Cells	ACh (control)	ACh/tetracaine 0.01 µM					
		2 min	7 min	10 min	20 min	30 min	50 min
F6	270 nC	-	510	-	750	-	1200
E2	150 nC	-	300	-	570	-	-
F	180 nC	345	-	600	-	-	-
F1	270 nC	-	-	-	-	390	1080

Table 7A

Table 7B

Potency ratio of ACh in the presence of 0.01 μM tetracaine and its absence. The EC-50 values were calculated from table 7A. The potency ratios are expressed where ACh is taken as 1 and where a value greater than 1 means it is less potent than ACh.

Cells	ACh (cont)	ACh/tetracaine 0.01 μM					
		2 min	7 min	10 min	20 min	30 min	50 min
F6	I	-	1.88	-	2.77	-	4.44
E2	I	-	2.00	-	3.8	-	-
F	I	1.91	-	3.33	-	-	-
F1	I	-	-	-	-	1.4	4.00

Table 7B

Figure 45 Shows the excitatory action of acetylcholine and tetracaine on neurone E-2.

A. Shows the excitatory action to 0.01mM acetylcholine (7mV).

B. Shows the excitatory action to 0.1mM tetracaine (21mV).

C. Recovery to 0.01mM acetylcholine (5mV), after washing off the tetracaine. The depolarisation response to 0.01mM acetylcholine was slightly reduced compared with A.

D. Response to second application of 0.01mM tetracaine. The depolarisation response was slightly enhanced, compared with B.

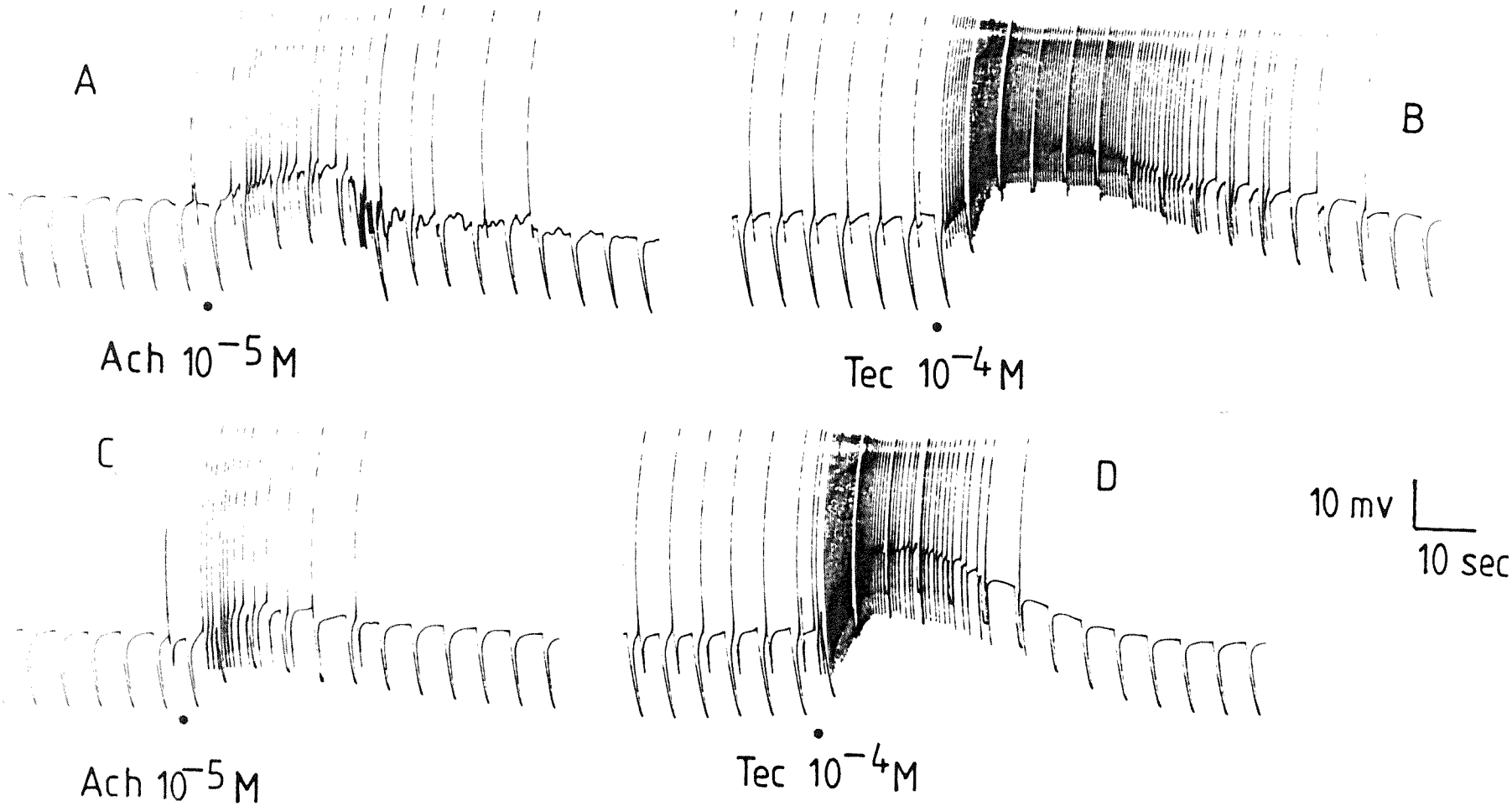

A
Ach 10^{-5} M
B
Tec 10^{-4} M
C
Ach 10^{-5} M
D
Tec 10^{-4} M
10 mv
10 sec

Figure 46 Effect of 0.1μM tetracaine on the excitatory responses to ionophoretically applied acetylcholine from neurone F-6.

A. Responses to a standard doses of acetylcholine.

B. Shows the effect of tetracaine on acetylcholine responses after 7 minutes.

C. Shows the effect of tetracaine on acetylcholine responses after 20 minutes.

D. Shows the effect of tetracaine on acetylcholine responses after 50 minutes.

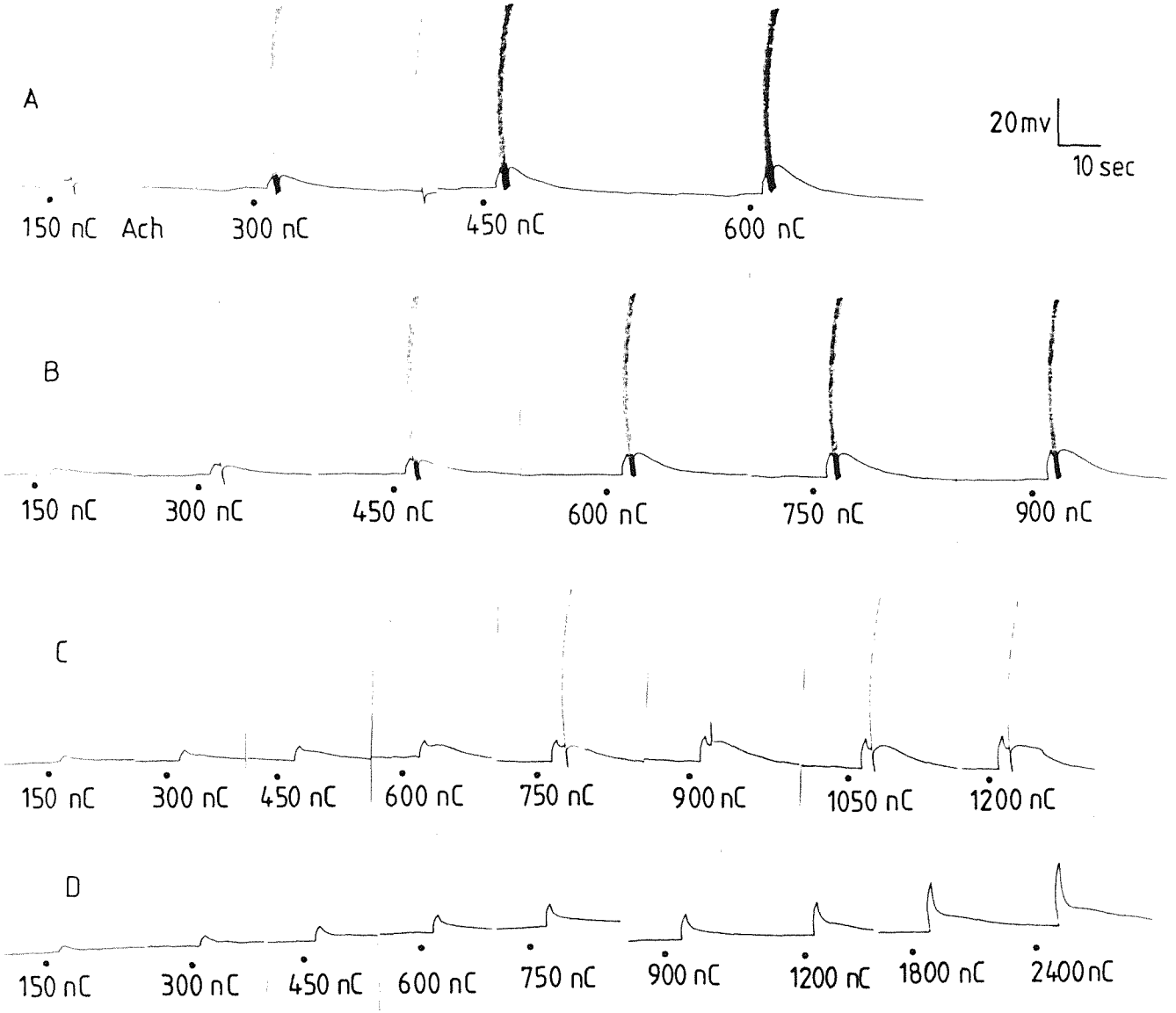

A
20mv
10 sec
150 nC
Ach
300 nC
450 nC
600 nC
B
150 nC
300 nC
450 nC
600 nC
750 nC
900 nC
C
150 nC
300 nC
450 nC
600 nC
750 nC
900 nC
1050 nC
1200 nC
D
150 nC
300 nC
450 nC
600 nC
750 nC
900 nC
1200 nC
1800 nC
2400 nC

Figure 47 Shift of the ionophoretically applied acetylcholine curves in the presence of 0.1μM tetracaine.

x. Partial acetylcholine dose-response curve.The value of EC_{50} for acetylcholine was 270nC

o. Acetylcholine dose-response curve 7 minutes after the addition of tetracaine. The value of EC_{50} for acetylcholine was 510nC.

▽ Acetylcholine dose-response curve 20 minutes after the addition of tetracaine. The value of EC_{50} for acetylcholine was 750nC.

◇ Acetylcholine dose-response curve 50 minutes after the addition of tetracaine of tetracaine. The EC_{50} value for acetylcholine was 1200nC.

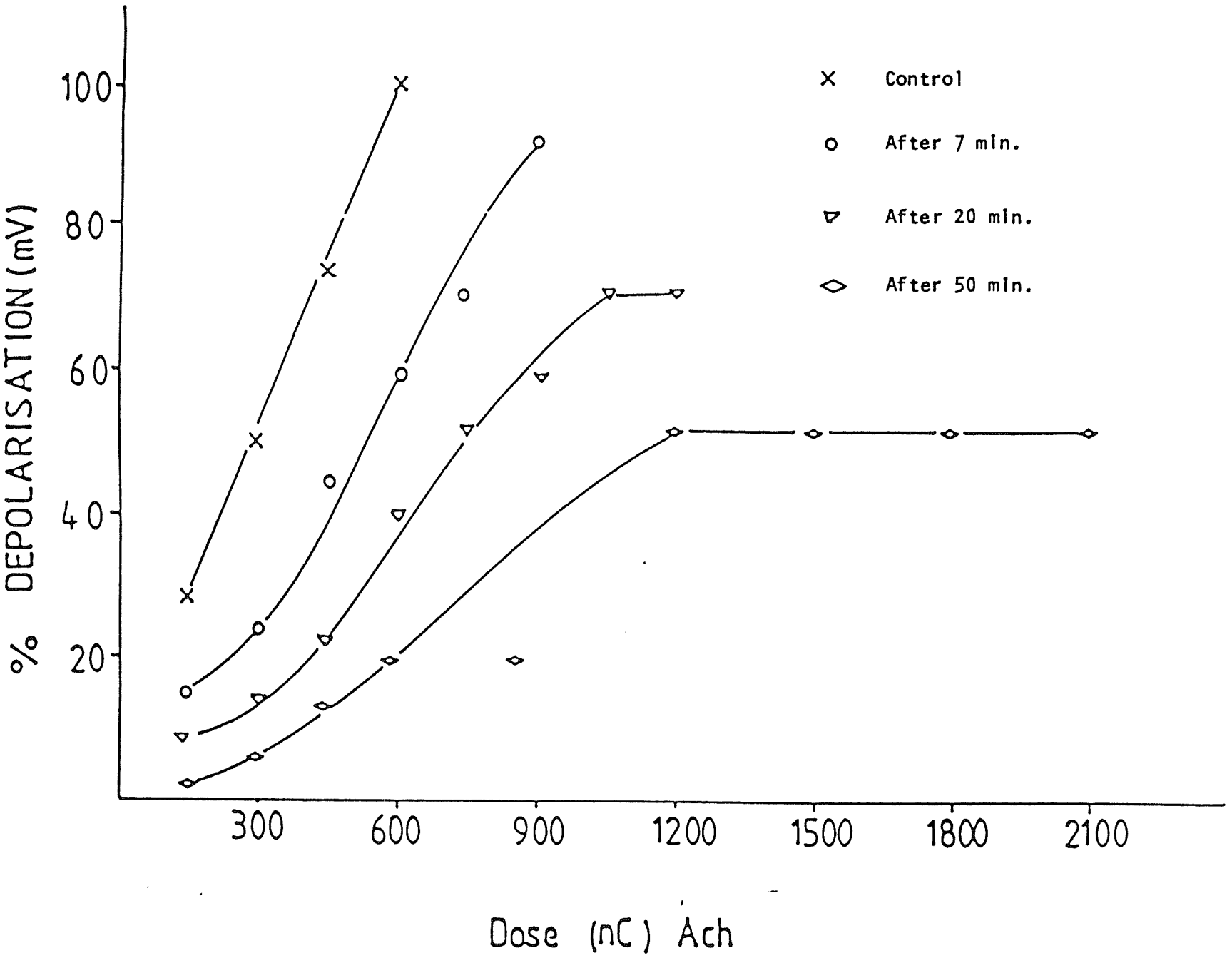

% DEPOLARISATION (mV)
100
80
60
40
20
300
600
900
1200
1500
1800
2100
Dose (nC) Ach
Control
After 7 min.
After 20 min.
After 50 min.

Figure 48 Dose-response curves showing the effect of 0.1μM tetracaine on the acetylcholine excitatory responses in neurone F-1.

x. Acetylcholine control curve. The EC_{50} vlaue for acetylcholine was 270nC.

o. Acetylcholine curve shifted to the right 30 minutes after the addition of tetracaine. The EC_{50} value for acetylcholine was 390nC.

◇ Acetylcholine curve shifted further to the right 50 minutes after the addition of tetracaine and the mixmum was depressed. The EC_{50} value for acetylcholine was 1080nC.

□ Acetylcholine curve shifted further to the right after 80 minutes and the miximum was depressed. The EC_{25} value for acetylcholine was 1200nC.

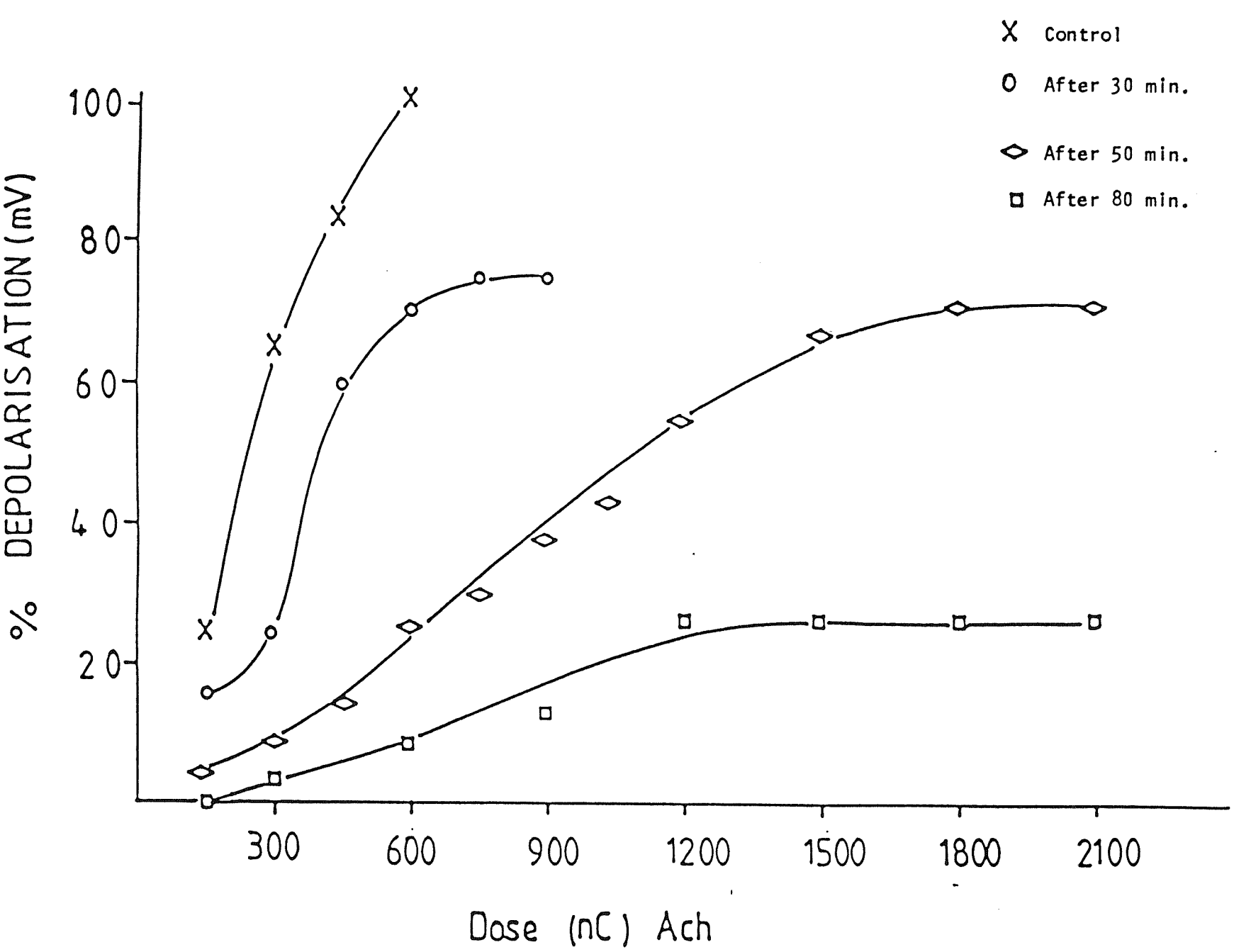
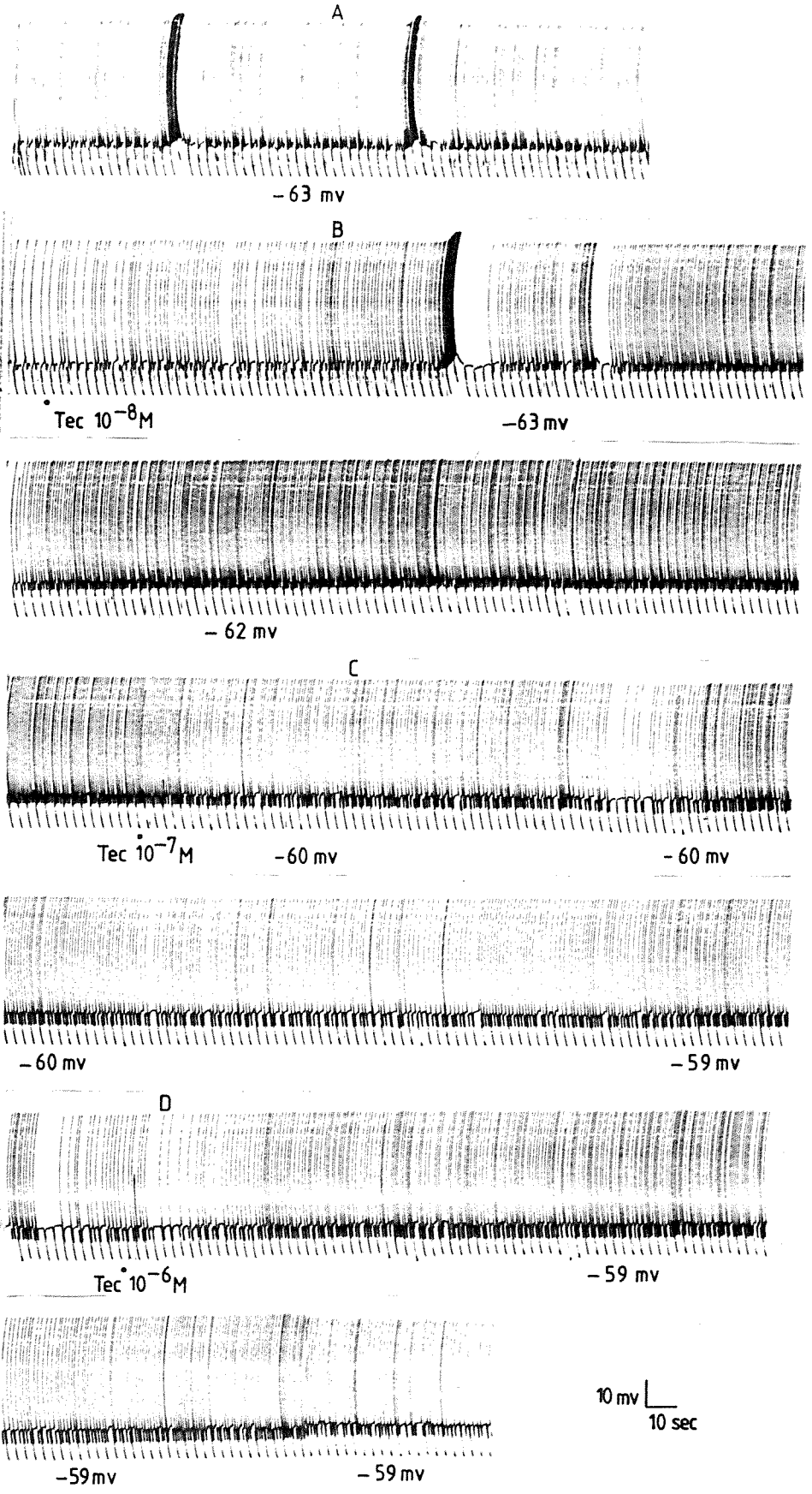

X Control
O After 30 min.
◇ After 50 min.
□ After 80 min.
% DEPOLARISATION (mV)
100
80
60
40
20
300
600
900
1200
1500
1800
2100
Dose (nC) Ach

Figure 49 Recording from F-1, illustrating the effect of 0.1μM tetracaine on the action potentials and bursting. The bursting disappeared 7 minutes after the application of tetracaine whereas the action potentials remained 50 minutes after the application of tetracaine.


A
−63 mv
B
Tec 10^{-8}M
−63 mv
−62 mv
C
Tec 10^{-7}M
−60 mv
−60 mv
−60 mv
−59 mv
D
Tec 10^{-6}M
−59 mv
−59 mv
−59 mv
10 mv
10 sec

Tetracaine action on "H" neurones

The antagonist action of tetracaine was also investigated on H-neurones. Figure 50 shows an example where tetracaine 0.01 µM did not affect the response to acetylcholine after 7 minutes, whereas it induced a gradual decrease of the action potential frequency during this time trace B. The initial effect of tetracaine 0.01 µM on the acetylcholine response occurred at the second dose-response application after 20 minutes.In this case tetracaine 0.01 µM reduced both the hyperpolarising response of acetylcholine and the action potentials. After 50 minutes the response to acetylcholine was greatly reduced (figure 50 trace E) and the maximum hyperpolarisation just reached the EC_{50} value of the control. This data can be expressed graphically figure 51. This figure shows that the first dose-response line (x-x) was not displaced to the right in the presence of tetracaine 0.01 µM. The EC_{50} value for acetylcholine as control was 135nC and the EC_{50} value for acetylcholine in the presence of tetracaine 0.01 µM was 120nC after 5 minutes line (o-o). The second, the third and the fourth curves for acetylcholine in the presence of tetracaine 0.01 µM were displaced to the right with time and their EC_{50} were 225, 360, and 600 nC respectively (Table 8).

Table 8 summarises the action of tetracaine when tested against the inhibitory response to acetylcholine on cell F-18. The effect of 0.01 µM tetracaine was tested at 5, 7, 20, 30, and 50 minutes following the initial application. This concentration of tetracaine increased the EC_{50} value for acetylcholine following the first dose-response curve in the presence of tetracaine where it can be seen the EC_{50} value slightly fell. This indicates that the tetracaine initially slightly potentiates the sensitivity of the cell to

acetylcholine. However subsequent dose-response curves show an increase in the EC_{50} value, up to a maximum ratio of around 5 after 50 minutes exposure to tetracaine.

Table 8a

The action of 0.01 µM tetracaine on the inhibitory responses to ionophoretically applied ACh. The EC-50 values for ACh in the presence of 0.01 µM tetracaine were constructed during incubation with tetracaine and compared with ACh as a control.

Cells	ACh (cont)	ACh/tetracaine 0.01 µM				
		5 min	7 min	20 min	30 min	50 min
F18	135 nC	120	-	225	360	600
F18	470 nC	420	-	900	-	-
F18	690	-	600	975	-	-

Table 8a

Table 8b

Potency ratio for ACh in the presence of 0.01 μM tetracaine. These ratios were constructed from the EC-50 values of ACh in table 8a.

Cells	ACh (control)	ACh/tetracaine 0.01 μM				
		5 min	7 min	20 min	30 min	50 min
F18	I	0.88	-	1.66	2.66	4.44
F18	I	0.89	-	1.91	-	-
F18	I	-	0.86	1.4	-	-

Table 8b

Figure 50 Shows the effect of 0.1μM tetracaine on the inhibitory responses to acetylcholine, from F-18.

A. Graded responses to ionophoretically applied acetylcholine as controls.

B. The inhibitory responses to acetylcholine after 5 minutes in the presence of 0.1μM tetracaine. These responses were slightly enhanced.

C. The inhibitory responses to acetylcholine after 20 minutes in the presence of 0.1μM tetracaine.

D. The inhibitory response to acetylcholine after 30 minutes in the presence of 0.1μM tetracaine.

E. The inhibitory responses to acetylcholine after 50 minutes in the presence of 0.1μM tetracaine.

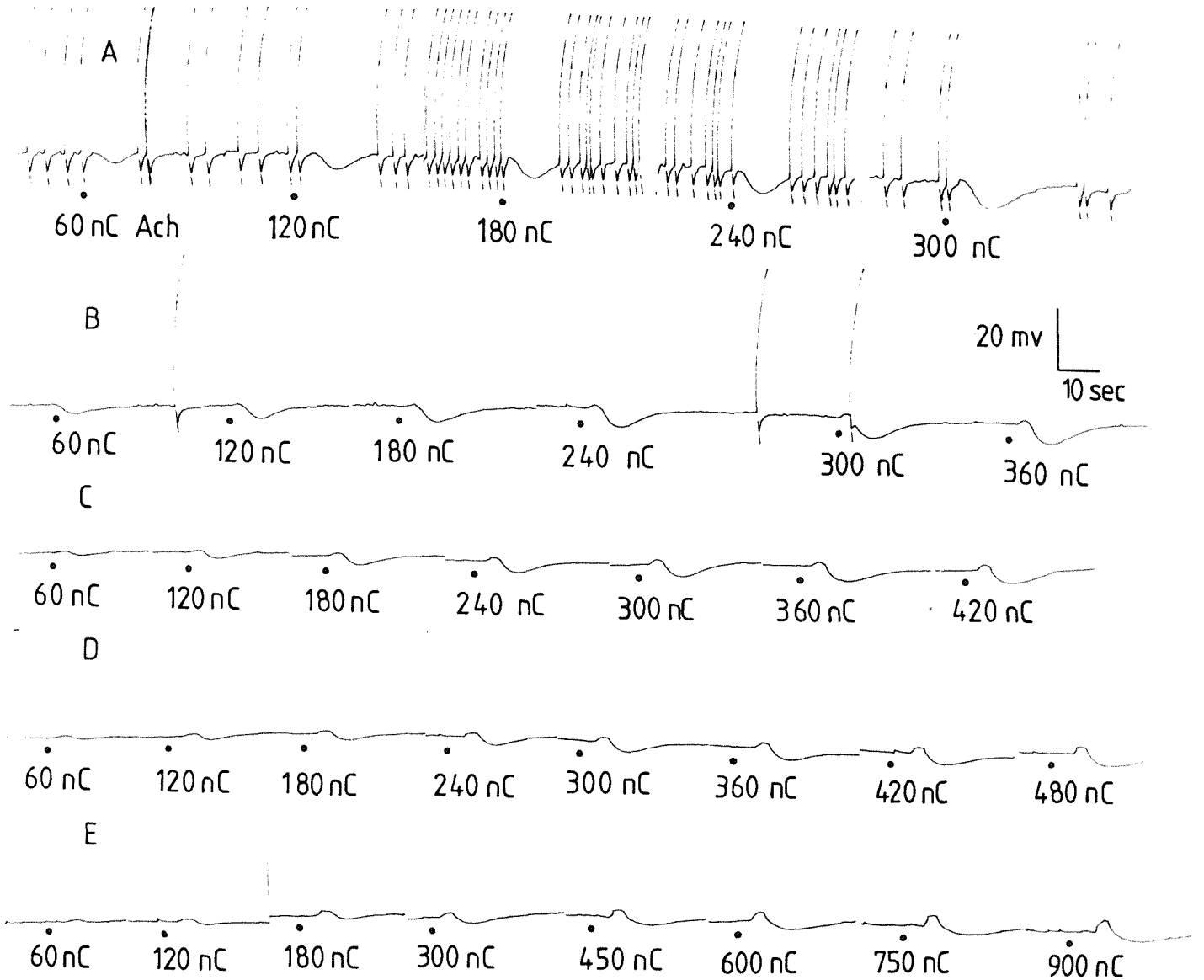

A
60 nC Ach
120 nC
180 nC
240 nC
300 nC
B
20 mv
10 sec
60 nC
120 nC
180 nC
240 nC
300 nC
360 nC
C
60 nC
120 nC
180 nC
240 nC
300 nC
360 nC
420 nC
D
60 nC
120 nC
180 nC
240 nC
300 nC
360 nC
420 nC
480 nC
E
60 nC
120 nC
180 nC
300 nC
450 nC
600 nC
750 nC
900 nC

Figure 51 Graphical representation of the data in Figure 50

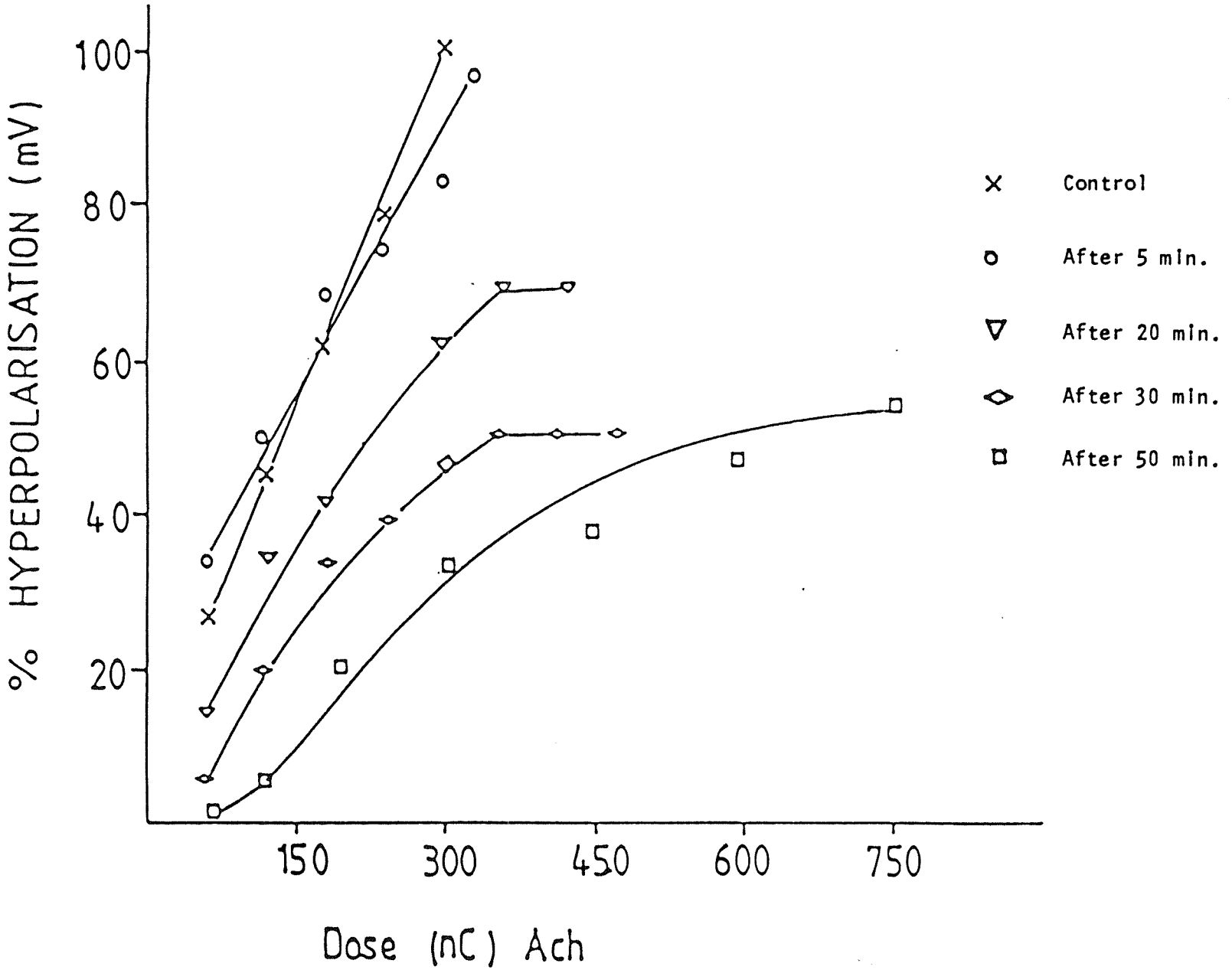
x. Control acetylcholine dose-response curve. The EC_{50} value for acetylcholine was 135nC.

o. Acetylcholine dose-response curve after exposure to 0.1μM tetracaine for 5 minutes. Tetracaine did not alter the curve. The EC_5 vlaue for acetylcholine was 120nC.

▽ Acetylcholine dose-response curve after exposure to 0.1μM tetracaine for 20 minutes. Tetracaine shifted the curve to the right and depressed the maximum. The EC_{50} vlaue for acetylcholine was 225nC.

◇ Actylcholine dose-respose curve after exposure to 0.1μM tetracaine for 30 minutes. Tetracaine shifed the curve to the right and depressed the maximum. The EC_{50} vlaue for acetylcholine was 360nC.

□ Acetylcholine dose-response curve after exposure to 0.1μM tetracaine for 50 minutes. The curve shifted to the right and depressed the maximum. The EC_{50} value for acetylcholine was 600nC.


% HYPERPOLARISATION (mV)
100
80
60
40
20
150
300
450
600
750
Dose (nC) Ach
Control
After 5 min.
After 20 min.
After 30 min.
After 50 min.

Dopamine & Tetracaine

The antagonist action of tetracaine on the dopamine receptor-K channel complex was investigated. The effect of 1μM tetracaine was tested in 5 preparations. In these experiments the blocking action of tetracaine on dopamine response was studied for comparison with the response to acetylcholine on the same neurone in terms of (a) increase in conductance and (b) graded blocking effect.

Figure(52 A,B) illustrates the response to standard control concentrations of acetylcholine and dopamine on cell F6. This neurone responds to acetylcholine with a sodium dependent depolarisation and to dopamine with a potassium mediated hyperpolarisation. The application of acetylcholine 0.1mM depolarised the membrane by 15mV and increased the membrane conductance by 0.09μS compared with the resting membrane conductance of 0.11 μS. Bath addition of 0.1mM dopamine caused a hyperpolarisation of 8mV and increased in conductance by 0.03 μS compared with the resting membrane 0.11μS.

The same concentrations of acetylcholine and dopamine were applied to the bath in the presence of 1 μM tetracaine. Tetracaine was left in contact with the preparation for 8 to 10 seconds. This compound hyperpolarised the membrane by 2mV but the membrane conductance was unchanged (figure 52, trace C_1). A block-effect relationship of 1 μM tetracaine to both responses of acetylcholine and dopamine was revealed as the amplitude of the acetylcholine depolarisation was reduced by 60% compared with the control.

The action potentials disappeared and the membrane conductance was decreased slightly by 0.08 μS, compared with

the resting conductance 0.11 µS figure 52,trace C_2. In contrast, the blocking effect of 1 µM tetracaine on the dopamine inhibitory response was not obviously altered. There was also no effect on the membrane conductance induced by dopamine figure 52 D_2. This effect of 1 µM tetracaine persisted after washout although higher doses of acetylcholine and dopamine would still give a response. For example, the response to 0.1mM acetylcholine was reduced by about 85% compared with the control and there was no change in the conductance of the membrane 40 minutes after the application of 1 µM tetracaine Figure 52, trace E. This contrasts with the inhibitory response to 0.1mM dopamine which was slightly larger compared to the control value, the membrane conductance was also increased by 0.06 µS compared with the resting conductance 0.11 µS Figure (52 F). However after a further 100 minutes the dopamine response was almost completely blocked figure (52, H_1). Higher concentrations of acetylcholine and dopamine were tested, 1mM acetylcholine depolarised the membrane by 9 mV and the membrane conductance slightly increased (figure 52 G). 1mM Dopamine caused a weak hyperpolarisation of 3 mV (trace H_2). The membrane conductance was unchanged. 10mM Acetylcholine depolarised the membrane by 9mV and increased the membrane conductance(figure 52, trace I).

Figure 52 (trace C_2,G,and I) shows that 1 µM tetracaine considerably prolongs both time to peak and the decay phase of acetylcholine induced depolarisation. These results indicate that the characteristics of the time course of the 1 µM tetracaine blocking effect on the excitatory response to acetylcholine and inhibitory response to dopamine are variable. The influence of tetracaine-induced blocking action on the excitatory response to acetylcholine was faster compared to that of dopamine. For example, 40 minutes

after the application of 1 μM tetracaine to the bath the depolarising response of 0.1mM acetylcholine was reduced by about 85% compared with the control(Figure 52, trace E). The hyperpolarising response of 0.1mM dopamine required a much longer time(100 minutes) to be blocked by about 88% compared the control(Figure 52 trace H_1).

Figure 53 shows a series of recordings demonstrating the action 0.01μM tetracaine as an antagonist of dopamine inhibitory responses. This concentration of tetracaine was left in the bath until the end of the experiment. Tetracaine hyperpolarised the membrane by 16 mV (trace B). Tetracaine was left for 5 minutes in the bath then the dopamine was ionophoretically reapplied. Trace (C) shows that tetracaine antagonised dopamine responses gradually with time. The antagonist action of tetracaine was quite clear after 30 minutes (trace D). It can also be seen that the action potential spikes declined when the response to dopamine was virtually blocked.

Figure 52 Shows the effect of 1μM tetracaine on the excitatory action to 0.1mM acetylcholine and the inhibitory response to 0.1mM dopamine. Tetracaine was washed out each time after either the reapplication of acetylcholine or reapplication of dopamine.

A & B Control responses to acetylcholine and dopamine. Acetylcholine depolarised the membrane by 15 mV and dopamine hyperpolarised the membrane by 8 mV.

C_1 The action of 1μM tetracaine on the cell membrane.

C_2 In the presence of 1μM tetracaine the depolarising response to acetylcholine was reduced by about 50% of control.

D_1 After about 15 minutes wash and reapplication of 1μM tetracaine. Tetracaine did not changed either the membrane conductance or the membrane potential.

D_2 In the presence of 1μM tetracaine the inhibitory response to 0.1mM DA only slightly changed though this was about 20 minutes after the application of tetracaine in C_1.

E Following removal of tetracaine the excitatory responses to 0.1mM acetylcholine was reduced by about 80% of control. This was 40 minutes after the application of tetracaine in C_1

F In the absence of tetracaine. The inhibitory response to 0.1mM was largely unchanged though it was 60 minutes after the application of tetracaine in C_1.

G	Following washing and in the absence of tetracaine a high concentration of acetylcholine (1mM) was applied. The excitatory response to 1mM acetylcholine was greatly reduced compared to control. This was about 80 minutes after the application of tetracaine in C_1. This concentration did not overcome the blocking effect of tetracaine.
H_1 & H_2	After washing for about 15 minutes in the absence of tetracaine. The inhibitory response to 0.1mM dopamine was almost abolished and subsequent higher dose of 1mM dopamine did not induce a clear response. This was 100 minutes following the application of tetracaine in C_1.
I	After washing for about 15 minutes a higher dose of acetylcholine (10mM) depolarised the membrane by about 9 mV. This was about 120 minutes after the application of tetracaine in C_1.

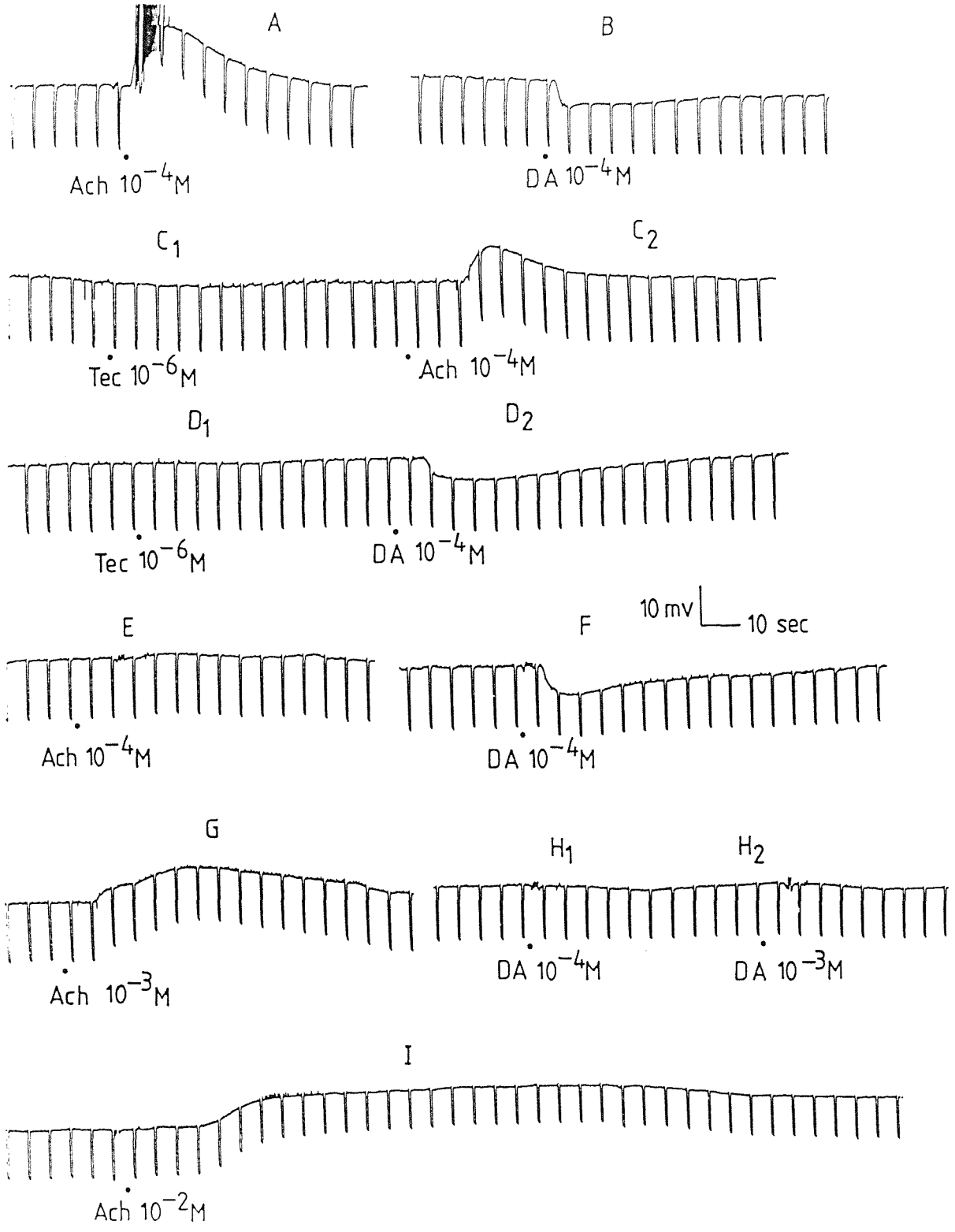

A
B
Ach 10^{-4}M
DA 10^{-4}M
C_1
C_2
Tec 10^{-6}M
Ach 10^{-4}M
D_1
D_2
Tec 10^{-6}M
DA 10^{-4}M
10 mv
10 sec
E
F
Ach 10^{-4}M
DA 10^{-4}M
G
H_1
H_2
Ach 10^{-3}M
DA 10^{-4}M
DA 10^{-3}M
I
Ach 10^{-2}M

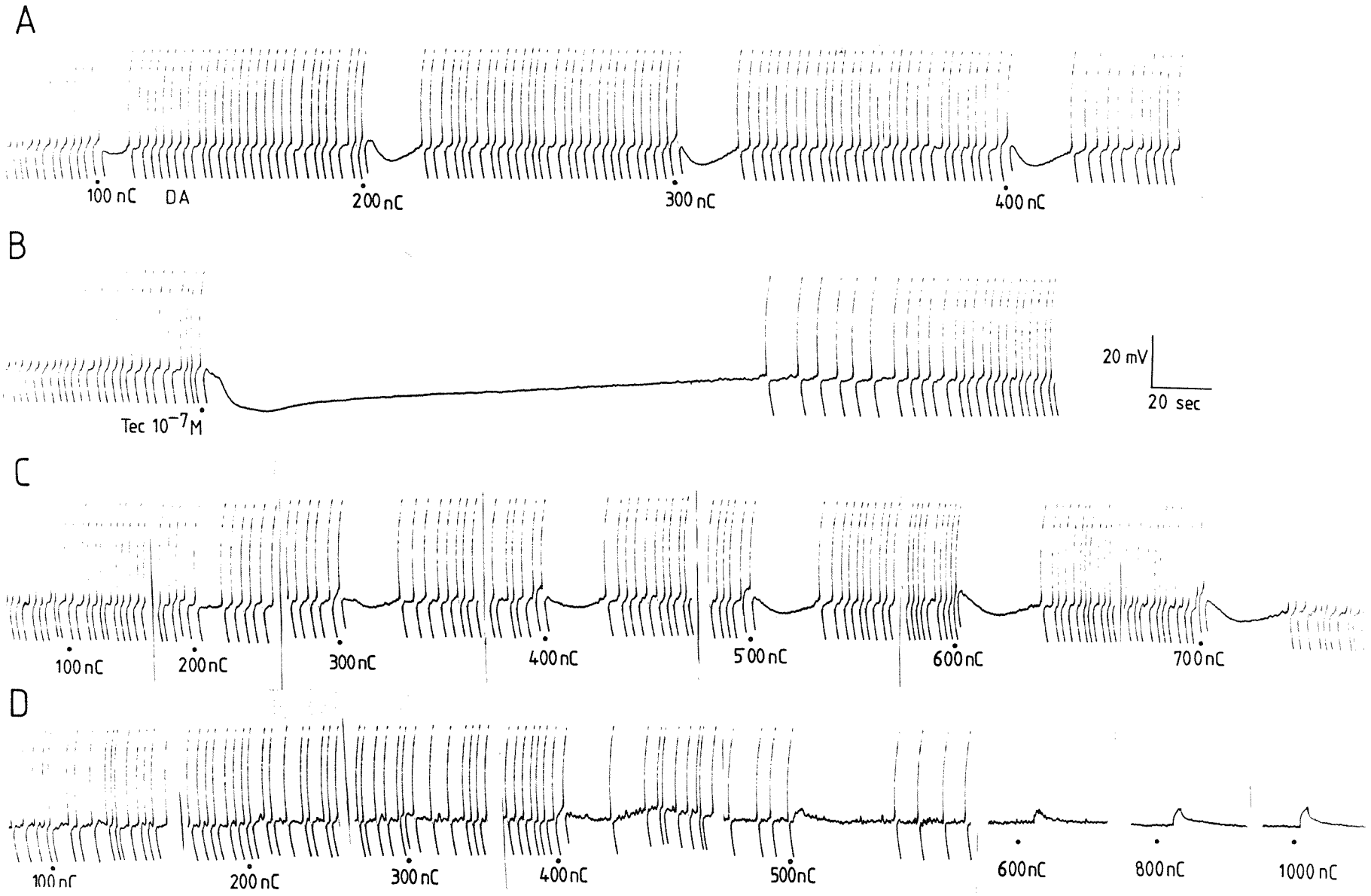
Figure 53 The effect of tetracaine on the hyperpolarisation responses to ionophoretic application of dopamine on neurone F-6. Tetracaine was left in the bath until the end of the experiment.

A Control inhibitory responses to a series of ionophoretic doses of dopamine.

B Bath application of 0.1μM tetracaine hyperpolarised the membrane by about 16 mV. Although tetracaine induced a long inhibitory response the membrane potential returned to its pretreatment value.

C In the presence of 0.01μM tetracaine (as a final concentration) for 5 minutes there is a clear reduction in dopamine inhibitory responses.

D In the presence of 0.01μM tetracaine for 30 minutes, the inhibitory responses to dopamine were greatly reduced with time.


A
100 nC
DA
200 nC
300 nC
400 nC
B
Tec 10^{-7}M
20 mV
20 sec
C
100 nC
200 nC
300 nC
400 nC
500 nC
600 nC
700 nC
D
100 nC
200 nC
300 nC
400 nC
500 nC
600 nC
800 nC
1000 nC

DISCUSSION

Procaine has been extensively studied at the level of the cholinergic receptor of the neuromuscular junction where an acetylcholine antagonist effect has been demonstrated. The antagonist effect of local anaesthetics on motor end-plate is shown as a decrease in amplitude of end-plate potentials of frog muscle (Maeno,1966; Kordas, 1970). Maeno assumed that the procaine effect on the e.p.p. is mostly on the sodium pathway and as result procaine changes the amplitude and time course of the sodium conductance which is increased by transmitter action. He also observed a transient phase dominated by increased potassium conductance, followed by a phase consisting of a small but sustained increase in sodium conductance. In this study procaine has both agonist and antagonist actions on the inhibitory receptor of acetylcholine. The antagonist effect was due to a reduction in size of the inhibitory response to acetylcholine. In terms of agonist action procaine mimics the action of acetylcholine by hyperpolarising the cell membrane and increasing the input conductance.

The results on *Helix* neurones show that the local anaesthetic tetracaine has both agonist and antagonist actions. At high concentrations tetracaine mimics the action of acetylcholine on D-cells by depolarising the membrane and increasing the conductance but is less potent than acetylcholine. At low concentrations of tetracaine the responses to ionophoretically applied acetylcholine on both D and H neurones were reduced. This reduction was time-dependent and irreversible.

Helix cells were less sensitive to procaine and tetracaine than to acetylcholine. Some neurones failed to respond to either local anaesthetic although these cells always respond to acetylcholine. These results were consistent with those obtained by Israel and Meunier (1979). They reported that procaine was active on only about two-thirds of *Helix* neurones. However these authors claimed that procaine activated the cholinergic receptor on *Helix* neurones. They considered that procaine is a structural analogue of acetylcholine (Gage, 1976). In addition their results indicated that procaine depolarisation is due to the activation of acetylcholine receptors since the ionic (sodium dependent) electrical (voltage dependent) and pharmacological properties (d-tubocurarine and atropine blockade) are similar.

Since procaine is a lipophilic compound and is also water soluble then it is possible that there are dissimilarities in the lipid environment surrounding the acetylcholine receptors which to some extent limits the movement of procaine. Hence it may reach its site of action via a different path way to that of acetylcholine. This could result in possible differential sensitivities on *Helix* neurones to procaine and acetylcholine. In the paper of Kerkut et,al (1975), it was shown that most cells respond to acetylcholine. If the conclusion of Israel and Meunier (1979) is correct that the receptor and ion channel protein are not homogenous for all cells then procaine might only activate some acetylcholine receptors or ion channels and this would explain why many cells are insensitive to this compound. Furthermore, a study on the interaction of cholinergic ligands with nicotinic receptor protein from *Torpedo marmorata* showed that the local anaesthetic binding site is different from the acetylcholine-binding site (Cohen

et,al 1974). They suggest a specific location for the site-inside the channel.

In this study low concentrations of tetracaine blocked the excitatory and the inhibitory responses to ionophoretically applied acetylcholine. This blocking effect differentiated between these two response types. The excitatory response was blocked faster than the inhibitory response. The blocking rate increased with time shifting the acetylcholine dose response curve to the right. It is also observed that the time tetracaine was in the bath did not induce a progressive blocking action against acetylcholine responses unless acetylcholine was applied ionophoretically. These data indicate that tetracaine blocks the open channel which is activated by acetylcholine but not the closed channel. Strichartz (1976) reported evidence from voltage clamp studies on single nerve fibres that indicated anaesthetic molecules interact with sodium channels directly from the inner surface of the nerve membrane which opened during membrane depolarisation preventing the normal sodium ion flux. This author also indicated that molecules can dissociate from open channels but not from channels that remain closed when the nerve is kept at rest.

It was interesting that tetracaine not only blocked the excitatory responses (Na mediated) and the inhibitory response (chloride mediated) to acetylcholine but also blocked the inhibitory response to dopamine (potassium mediated). In later experiments combinations of dopamine and acetylcholine were used in the presence of tetracaine. Tetracaine prolonged the time to peak of the excitatory response of acetylcholine and it is possible that this action is due to the effect of tetracaine on other conductances besides sodium conductance which are activated

during excitation. Furthermore, the acetylcholine excitatory response requires approximately half the time for a reduction to about 80% of control compared to the inhibitory response of dopamine to be reduced to the same extent by the same concentration of tetracaine. These experiments give further evidence that tetracaine is not selective for sodium conductance although it blocks sodium conductance first, then potassium and chloride. It therefore, seems that the active site of tetracaine on transmitter gated channels is not selective. Neher and Steinbach (1978) reported on the mechanism of local anaesthetic action based on suggestions by Adams (1976,1977) for barbiturates and procaine which postulated that the drug molecule like other ions acts inside the membrane to occupy the ion channels for several msec (or tens or even hundreds of msec in the case of QX-314) and thereby blocks the channel to other ions.

On leech neurones, *Macrobdella docora*, Johansen and Kleinhaus (1987) demonstrated that procaine and strychnine produced changes in the shape of action potentials, for example, prolonged the duration of action potentials and reduced the amplitude. These changes are compatible with the assumption that procaine and strychnine blocked not only the increase in sodium permeability, but also the increase in K-permeability associated with the action potentials.

The generation of the action potentials of snail neurones could be spontaneous or could be synaptically driving from another synapse. It was shown in chapter four that d-tubocurarine blocked the inhibitory and the excitatory responses to acetylcholine but did not block the generation of action potentials. This may indicate that the action potentials are generated from the recording soma.

This study was intended to investigate whether or not tetracaine blocks the action potentials. Tetracaine in increasing concentrations failed to block the action potentials after 40 minutes whereas the bursting which is associated with the action potentials disappeared after 7 minutes following its application. Since the bursting induced higher depolarisation compared with the action potentials therefore it is very likely that the bursting is linked to a sodium transmitter gated channel rather than to the voltage gated channel. This may be explained in that tetracaine blocked the bursting because the bursting induced enough depolarisation which opened a large number of sodium transmitter gated channels compared with the action potentials which are generate at the steady state of the cell membrane.

In these experiments the neurones were at normal membrane potential. These data suggest that the membrane potential needs enough depolarisation in order to block the sodium channels. These data are in agreement with observations of Country (1975) that use-dependent block with QX-314 requires the channel to be opened and it develops only when depolarisation pulses are large enough to open channels.

CHAPTER 7

GENERAL DISCUSSION AND CONCLUSION

Our knowledge of the neurophysiology and neuropharmacology of annelid central nervous system is largely confined to studies on leech since there has been relatively little work on earthworm central neurones. The neurones in the earthworm are relatively small and also difficult to record from and to identified. However, it is likely that there are similarities between the organization of the leech and earthworm nervous systems. For example, both contain excitatory motoneurones which are probably cholinergic. Leech ganglia can synthesise relatively large amounts of acetylcholine (Sargent 1977) and choline acetyltransferase has been identified in specific leech excitatory motoneurones, for example, the longitudinal motoneurone (L cell), the annulus erector motoneurone (AE cell), the heart excitor motoneurone (HE cell) and eleven other motoneurones (Wallace 1981). The inhibitory motoneurones contain no detectable choline acetyltransferase activity. Sensory neurones have also been extensively studies on the leech (Nicholls and Baylor) and do not appear to be cholinergic. Choline acetyltransferase activity has been identified in the central nervous system of *Nereis* (Marsden et al 1981). In contrast to the situation in the leech, little is known regarding the electrophysiology of the earthworm central nervous system. It would be interesting to develop a preparation whereby recordings could be made from single neurones and then study nerve-muscle events in detail. Use could be made of current neurochemical and immunocytochemical techniques to aid in the localization of the motoneurone. Leech boy wall muscle

acetylcholine receptors are largely nicotinic though there is evidence for two possible subtypes (Flacke and Yeoh 1968b). The earthworm body wall muscle acetylcholine receptors studied in this thesis are sensitive to α-bungarotoxin and so have a nicotinic component though nicotine is not very active on them. Tubocurarine is also an antagonist against acetylcholine on both leech and earthworm muscle which suggests similarities with vertebrate nicotinic receptors. The sensitivity of the earthworm preparation to acetylcholine is low in the presence of physostigmine compared to the situation in the leech and the reason for this is not immediately apparent.

The precise mechanism and site of action of anthelmintic compounds is often not fully understood. The snail brain provides a useful screening model for investigating which receptors can be modified following the application of compounds such as anthelmintics. The work described in this thesis shows that certain anthelmintics can act on snail acetylcholine receptors and supports the findings for interactions at *Ascaris* acetylcholine receptors (Harrow and Gratin 1985). These *Ascaris* receptors are similar to vertebrate ganglionic nicotinic receptors (Lewis et al 1980) and so it is interesting that in the present study anthelmintics such as pyrantel are antagonised by ganglion blockers when tested on snail acetylcholine receptors. However, from a comparison of potencies of anthelmintics on *Ascaris* peripheral and snail central acetylcholine receptors clear differences are revealed, suggesting that there are differences in the preferred requirements of the receptor or additional proteins around

the receptor.

It would be interesting to examine the action of anthelmintics more precisely by applying them ionophoretically and studying their responses under voltage clamp. This is particularly true for hycanthone which might block activated channels and this could best be studied when acetylcholine is applied ionophoretically. It would also be worth testing this compound against cholinergic synaptic events in the snail. While evidence clearly shows an interaction with acetylcholine receptors it would also be worth looking for possible interactions with other receptor systems.

Snail central neurones possess a range of glutamate receptors (Piggott et al 1975) and glutamate is likely to be a central transmitter in *Helix* (Judge et al 1977). There have been many structure activity studies using glutamate analogues and eight were tested in the presented study. Of these only the L- and D-isomers of thio-glutamic acid possesed glutamate-like activity and both compounds were approximately equiactive with L-glutamate. The receptor did not appear to discriminate between the two isomers of the thio analogue. This finding is interesting since often with invertebrate preparations, the introduction of a thio group reduces potency. For example, thiomuscimol is far less active than muscimol on cells excited by GABA while piperidine-4-sulphonic acid is an antagonist, devoid of any GABA-like activity (Vehovszky et al 1989).

Excitatory compounds, such as acetylcholine, when

applied to snail neurones often exhibit an afterhyperpolarising phase following the initial depolarisation and this afterhyperpolarisation might be associated with an electrogenic pump. A similar afterhyperpolarisation was observed following the application of L-glutamate in chloride free saline where in normal saline L-glutamate hyperpolarises the membrane potential through an increase in chloride conductance. Although the evidence is not complete this afterhyperpolarisation might be associated with a pump system. Since it is reduced in sodium free saline it might be associated with the entry of sodium ions when chloride ions leave the cell. Alternatively it could be associated with calcium entry which in turn might activate a potassium current although in potassium free saline the afterhyperpolarisation is not enhanced. It would be interesting to test both ions which interfere with calcium entry such as cobalt or manganese and compounds such as 4-aminopyridine and tetraethylammonium which interfere with potassium ion movement. If this afterhyperpolarisation could clearly be shown to be electrogenic then it would be an interesting observation.

Local anaesthetics are another important group of compounds where their mechanism of action is not fully understood. The snail brain can provide a model system for studying the possible interaction between local anaesthetics and either receptors or ionophores or a mixture of both. Interestingly these compounds can not only interact with transmitter receptors such as acetylcholine, but can also activate ion channels associated with the receptors.

However, the receptors are not particularly sensitive to local anaesthetics and require fairly high concentrations which in turn lead to desensitisation and rapid fade of the response when applied to the bath. This might be overcome by applying the local anaesthetic either ionophoretically or by pressure ejection. A far wider survey should be made of local anaesthetics on different receptor types to obtain information regarding the structural requirements of local anaesthetics for optimal receptor activation. Some preliminary studies using dopamine receptor responses demonstrate that local anaesthetics also interact with potassium ion channels and also the present work shows that these compounds can interact with receptors linked to ion channels for chloride, sodium and potassium. The critical factor is the concentration of local anaesthetic required to cause these different actions and the time for onset of the response following application of the compounds. These areas could form the basis of future research.

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