Regulation of Excitability of Molluscan Neurones by Convulsant Agents and Intracellular Messengers.

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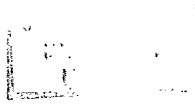

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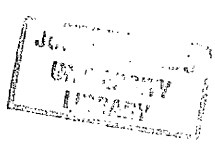

Abstract.

1) The aims of this study were to investigate the role of the intracellular second messengers, cyclic AMP and calcium ions in the generation of epileptiform activity, and to determine the cellular actions of the convulsant compound, pentylenetetrazol (PTZ). The isolated central nervous system of the pond snail Lymnaea stagnalis was used.

2) Two identified neurones, B1 and RPD1, showed opposing responses to PTZ. The response in B1 consisted of depolarisation, accompanied by bursts of action potentials and paroxysmal depolarising shift. RPD1 was inhibited in the presence of PTZ.

3) In both B1 and RPD1, the actions of PTZ appeared to be mediated by an increase in intracellular calcium ions. Extracellular calcium ions were not required to initiate a response to PTZ, but was needed to maintain the response.

4) The opposing effects of PTZ in the two cell types were due to separate calcium-activated membrane conductances; calcium-activated sodium conductance in B1, and calcium-activated potassium conductance in RPD1.

5) Intracellular iontophoresis of cyclic AMP into B1 and RPD1 induced a sodium-dependent, voltage-insensitive inward current, leading to depolarisation of the membrane.

6) Pentylenetetrazol had opposing effects on the amplitude of cAMP-induced current in the two cell types: amplitude was increased in B1 and decreased in RPD1.

7) Increased intracellular calcium ion concentration had no effect on cAMP-induced inward current, suggesting that PTZ's effects on the current were not mediated via calcium ions.

8) Pentylenetetrazol- and cAMP-stimulated sodium currents in B1 were shown to differ in their voltage-sensitivity and their susceptibility to blocking by pharmacological agents.

9) It is concluded that individual neurones may differ in their susceptibility to convulsant activity owing to possession of different intrinsic membrane conductances. The actions of PTZ appear to be largely due to increased intracellular calcium ion concentration though cAMP may have a role to play in some neurones.

Declaration.

I hereby declare that no part of this thesis has been submitted in support of an application for another degree or qualification at this or any other institute of learning.

Angus McL. Brown. October 1990

Education and Research Experience.

Since obtaining an Honours degree in Physiology at the University of Dundee in 1987, I have worked in the Department of Physiological Sciences at the University of Manchester. My time here has been spent under the supervision of Dr Cathy McCrohan.

Dedication.

I would like to dedicate this thesis to my mum, dad and brother. I greatly appreciate the sacrafices they have made for my education, and without their continual support and encouragement this thesis would not have been possible.

Acknowledgements.

I would like to thank my supervisor Cathy McCrohan firstly, for agreeing to be my supervisor, and secondly and more importantly, for her continual help, encouragement and advice over the last three years.

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I would lastly like to thank all the people who have made my stay in Manchester enjoyable. These include many students at the RNCM and my chums at the Medical School.

Abbreviations.

A	ampere
AMP	adenosine monophosphate
ATP	adenosine triphosphate
BTX	batrachotoxin
^{o}C	degrees centigrade
Ca^{2+}	calcium ion
cAMP	cyclic adenosine monophosphate
CBZ	carbamazepine
Cl^{-}	chloride ion
cGMP	cyclic guanosine monophosphate
CNS	central nervous system
CPG	central pattern generator
Co^{2+}	cobalt ion
CPTcAMP	chlorophenylthio-cyclic adenosine monophosphate
dB	decibel
D600	iproveratril
EEG	electroencephalogram
EGTA	ethyleneglycol-bis-(B-aminoethyl ether) N,N,N'-tetraacetic acid
E_K	potassium equilibrium potential
EPSP	excitatory post synaptic potential
et al.	and others
g	grammes
GABA	Y-aminobutyric acid
GDP	guanosine diphosphate
GTP	guanosine triphosphate

HEDTA	N-hydroxyethylethylenediamine-triacetic acid
Hz	Hertz
I_A	fast transient potassium current
IBMX	isobutylmethylxanthine
ILAE	International League against Epilepsy
$I_{K(Ca)}$	calcium dependent potassium current
$I_{K(V)}$	delayed rectifier potassium current
i.p.	intra peritoneal
IP_3	inositol 1,4,5-trisphosphate
i.v.	intra venous
k	kilo
K^+	potassium ion
KCl	potassium chloride
KOH	potassium hydroxide
l	litre
L-PIA	L-N6-phenylisopropyladenosine
m	milli
M	molar
M	mega
Mg^{2+}	magnesium ion
mins	minutes
MOPS	3-(N-morpholino) propane-sulphonic acid
n	number of replicates
n	nano
Na^+	sodium ion
NaOH	sodium hydroxide
NMDA	N-methyl-D-aspartate
n.s.	not significant

PHT	phenytoin
PTZ	pentylenetetrazol
PDS	paroxysmal depolarising shift
pH	$-\log_{10}$ hydrogen ion concentration
secs	seconds
SEM	standard error of the mean
TEA	tetraethylammonium
TMB-8	3,4,5-trimethoxybenzoic acid 8-(diethylamino)-octyl ester
TTX	tetrodotoxin
u	micro
V	volts
Vc	command potential
Vm	measured membrane potential
$<$	is less than

Chapter 1

INTRODUCTION

1.1 General Aims

The aim of this thesis was firstly, to investigate the role of second messengers in the generation of epileptiform activity, and secondly to investigate the mode of action of the convulsant drug, pentylenetetrazol, using a molluscan model.

A role for second messengers in the generation of epileptiform activity has been suspected for many years (Sattin, 1971; Lust, 1976), but their precise involvement remains unknown. Using molluscan neurones, Onozuka et al (1983) showed that an increase in intracellular cyclic AMP precedes the onset of epileptiform activity and as such cyclic AMP could be the initiator of the epileptiform activity. In mammalian tissue, Ferrendelli and Kinscherf (1977b) and Ferrendelli (1984) demonstrated that elevation of cyclic AMP levels was closely associated with seizure activity. Calcium ions have also been implicated in epileptogenesis. Heinemann et al (1977) showed that extracellular Ca^{2+} concentration decreased during seizure activity in cat cortical neurones. DeSarro et al (1988) demonstrated that prior application of Ca^{2+} channel antagonists prevented the induction of seizures into mice genetically susceptible to sound induced seizures. These results suggest that there is a Ca^{2+} influx into mammalian neurones during seizure activity.

A useful tool in the study of seizure activity is the convulsant drug pentylenetetrazol, which has been shown to elevate cyclic AMP levels in both mammalian (Ferrendelli and Kinscherf, 1977; Onozuka et al, 1989) and molluscan (Onozuka et al, 1983) preparations. Thus an understanding of the mode of action of pentylenetetrazol could yield useful information about the induction of epileptiform activity including a possible role of cyclic AMP.

In this study I have investigated the actions of pentylenetetrazol in two identified molluscan neurones which show different responses to pentylenetetrazol. The properties of a cyclic AMP-dependent ionic membrane current in both cell types was also studied to see if there was any connection between pentylenetetrazol's effects and this current. Finally a comparison of inward currents induced by cAMP and PTZ in an identified neurone was carried out.

This introductory chapter presents the background to the epileptic condition and its possible cellular basis. It then describes the models and compounds used in the study of epilepsy and ends with a detailed description of the model used in this study.

1.2 Epilepsy

Epilepsy is a brain disorder characterised by sudden, transient alterations in brain function leading to motor, sensory, autonomic or pyschic symptoms, often accompanied by unconsciousness (Sutherland, 1969). Epileptic attacks are recurrent, distinguishing them from isolated convulsions. It is estimated that 0.5 to 2% of the world's population suffers from this condition - up to 120 million people

worldwide. All types of epileptic attack start with abnormal paroxysmal discharges of neurones in the brain, which may spread locally or may travel via the neuronal axons to more distant groups of neurones (Sutherland, 1969). The site of origin of the discharge is called the epileptic focus. When the discharge remains confined to a small part of the cortex and is of short duration it may not lead to any obvious symptoms, but yet may be detected by electroencephalography (EEG; Cull, 1983). The EEG trace shows that epileptic attacks are accompanied by characteristic EEG patterns, and, as well as the type of seizure occurring, the area of discharge can be identified. If the motor system is involved there is a loss of voluntary power and this is usually accompanied by initial muscle contraction (the tonic phase), followed by powerful muscular jerks (the clonic phase; Jeavons and Aspinall, 1985). When a large part of the cortex or reticular activating system is involved the sufferer loses consciousness, but when the discharge is confined to sensory areas of the cortex the sufferer experiences hallucinations involving sight, hearing, smell, taste or altered sense of time (Jeavons and Aspinall, 1985).

Classification of epileptic seizures

The classification of epileptic seizures has been changed three times in the last ten years, leading to a great deal of confusion and misunderstanding. The International League Against Epilepsy (ILAE) have classified epileptic seizures in 1981, 1985 and 1989. I shall describe the 1981 classification as this is the most commonly used and understood, being used to the present day in most published material despite the two subsequent reclassifications. The 1985

classification (ILAE, 1985) used a system whereby the seizures were categorised by the syndrome, the syndrome being defined as a cluster of signs or symptoms occurring at the same time. Thus this new classification described such seizures as 'benign childhood epilepsy with centro-temporal spikes'. The 1989 classification (ILAE, 1989) used a system whereby the seizures were categorised on the grounds of their cause: idiopathic (primary) epilepsies of genetic origin; symptomatic (secondary) epilepsies of known origin (e.g. disorder of the CNS); and cryptogenic epilepsies presumed to be symptomatic but of unknown origin.

ILAE 1981 Classification of Epileptic Seizures (ILAE, 1981)

This system of classification separates epileptic seizures into two types, partial and generalised. The clinical and EEG data indicate that in partial seizures only one hemisphere of the brain is affected, whereas in generalised seizures both hemispheres of the brain are affected.

I. Partial (local / focal) seizures

Partial seizures may be classified into one of the three following groups.

1) Simple partial seizure. Consciousness is not impaired but there may be motor signs and somato-sensory or special sensory symptoms (simple hallucinations, tingling, light flashes, buzzing in ears, etc). Psychic symptoms indicating disturbances of higher cerebral function may occur. These include feelings of deja vu, distortion of time, and feelings of fear and anger. Autonomic symptoms include

pallor, sweating, flushing, pilo-erection and pupillary dilation.

2) Complex partial seizure. Consciousness is impaired but the seizure may start with simple partial seizure. There are two types: (a) simple partial seizure onset followed by loss of consciousness; (b) impairment of consciousness at beginning of seizure.

3) Partial seizures evolving to generalised seizures. These seizures can be subdivided into three types: (a) simple partial seizure evolving to generalised seizure; (b) complex partial seizure evolving to generalised seizure; (c) simple partial seizure evolving to complex partial seizure evolving to generalised seizure.

II. Generalised seizures

The first clinical changes indicate involvement of both cerebral hemispheres. Impairment of consciousness may be the initial manifestation. Both motor and EEG manifestations are bilateral indicating the involvement of both cerebral hemispheres. Generalised seizures can be classified into the following subclasses.

1) Absence seizures. Typical symptoms include the sudden cessation of ongoing activity; e.g. walking, talking. This is accompanied by a blank stare and inability to respond to external stimuli.

2) Tonic-clonic seizures. This is the most common type of generalised seizure and the one that most laymen associate with epilepsy. The sufferer loses consciousness accompanied by a sudden contraction of muscles (tonic phase). The sufferer falls to the ground and undergoes rhythmic jerks of the muscles (clonic phase).

3) Myoclonic seizures. Symptoms are single or multiple, brief, shock-like contractions which are localised to the face or trunk. The jerks may be rapidly repetitive or relatively isolated. Attacks occur

predominantly around the hour of going to sleep or waking.

4) Clonic seizures. This is a seizure lacking a tonic component and is characterised by repetitive clonic jerks.

5) Tonic seizures. Symptoms include violent muscular contraction which is either localised or affects a large mass of muscle.

6) Atonic seizures. Symptoms include a sudden dimunition of muscle tone which may lead to slackening of the jaw and head droop. The loss of consciousness is very brief.

III. Unclassified Epileptic Seizures

This category includes all the seizures which cannot be classified because of inadequate or incomplete data, and some that defy classification in the above categories. These include some neonatal seizures.

1.3 The cellular basis of epilepsy

Modern techniques have enabled workers to investigate in depth the cellular mechanisms underlying the generation of seizure activity. The earliest of these techniques to be employed was intra- and extracellular recording. In order for the cellular basis of epilepsy to be studied, it was first necessary to find a suitable model which was both simple to use, and which resembled the condition seen in humans. It was found that the most suitable model involved penicillin-induced seizures in mammalian (usually rats and mice) neocortex and hippocampus (Prince, 1978). After the first years of work several hypotheses concerning the mechanism of seizure generation were proposed (Prince, 1968; Dichter, 1969; Ayala, 1970;

Schwartzkroin, 1975) but these were so varied that the unifying theory so eagerly sought seemed remote. Given the evidence of recent work which has implicated a great many factors in the generation of seizures it has become apparent that it is very unlikely that a single mechanism is responsible for the generation of seizures.

The first major question to be addressed was what caused the seizure discharge by the neurones. Was it an intrinsic property possessed by neurones, which suddenly and inexplicably resulted in neurones generating seizure discharges, or was it a fault in the neuronal network? In the penicillin model it soon became clear that the majority of neurones involved in seizure discharge were normal (Dichter, 1989). This led to the conclusion that there must be two mechanisms involved in the generation of a seizure; firstly, the initial generation of seizure activity must only happen in a relatively small number of neurones, and secondly normal neurones must become affected by the seizure generating neurones.

For seizure-generating neurones to become excited they must become depolarised (Matsumoto and Ajmore-Marsan, 1964). This depolarising shift leads to bursts of action potentials and this is followed by a period of hyperpolarisation (Matsumoto and Ajmore-Marsan, 1964). There are varying theories about how the depolarisation occurs but one of the more feasible proposes that enhancement of excitatory post synaptic potentials (EPSP's) by voltage dependent cation currents is a major cause. There are five main ways in which this can occur (Dichter, 1989):

1) by removal of voltage dependent Mg^{2+} block of N-methyl-D-

aspartate (NMDA) receptors (Mayer and Westbrook, 1985);

2) by alterations in the space constants of dendrites of postsynapyic neurones;

3) by the removal of normal inhibitory mechanisms, thus resulting in increased activity;

4) by the potentiation of other neurotransmitter effects (Stanfield et al, 1985);

5) by an increase in the frequency of EPSP's.

In addition to these factors there are two other types of cation current which can cause depolarisation of a cell. The first are slowly activating Na^+ and Ca^{2+} currents (Connors, 1982) and the second a large transient Ca^{2+} current (Wong and Prince, 1978). Which of the five mechanisms are involved depends on the properties of the cell.

A period of hyperpolarisation occurs after the initial depolarisation causing an inhibition of the seizure activity and limiting the duration and frequency of the seizure discharge (Schwartzkroin and Prince, 1977). Under normal conditions there are at least seven separate mechanisms by which this hyperpolarisation can occur:

1) inhibitory post synaptic potentials (IPSP's) caused by the opening of K^+ channels (Alger, 1984);

2) the opening of Ca^{2+} dependent Cl^- channels ($I_{Cl(Ca)}$); Mayer, 1985);

3) the opening of Ca^{2+} dependent K^+ channels ($I_{K(Ca)}$); Meech, 1972);

4) the opening of voltage dependent K^+ channels (Hille, 1984);

5) the opening of GABA mediated Cl^- channels (DeLorenzo, 1988);

6) activation of the electrogenic sodium pump (Thomas, 1972);

7) the opening of second messenger activated Cl^- channels (Higashida and Brown, 1976).

As with the factors inducing depolarisation, the selection of the above methods of hyperpolarisation depends on the properties of the cell.

The description of the generation of seizure discharges so far has concentrated on the mechanisms occurring within single cells. A vital component in the generation of seizure discharges is how the depolarisation induced in one cell can spread to a large number of normal cells. Not only does the depolarisation have to spread to these cells; it must also spread synchronously. The work of Dichter and Spencer (1969) indicates that it is the activation of recurrent synaptic excitation which causes this. Dichter and Spencer (1969) demonstrated the existence of recurrent collaterals in rat hippocampus and neocortex. Ayala et al (1973) have shown that these collaterals act as a positive feedback mechanism and may act to induce the EPSP's which cause the initial depolarisation. The depolarisation of a large population of cells is vital in the generation of seizures. A major factor in the development of a full seizure is the replacement of hyperpolarisation by depolarisation. This may occur in a variety of ways.

1) Reduction of glycine and GABA induced synaptic inhibition. Glycine and GABA induced IPSP's are reduced by low frequency stimulation, exactly the behaviour seen during the depolarisation prior to seizure

activity (Barnes and Dichter, 1984).

2) Neurotransmitters may act to decrease the intracellular Ca^{2+} concentration resulting in a decreased $I_{K(Ca)}$ (Cole and Nicoll, 1984).

3) The increase in the extracellular K^{+} seen during the depolarisation would lead to changes in E_K which would tend to oppose any outward current (Kuffler, Nicholls and Martin, 1984).

4) The depolarisation induced by these above factors would release the voltage dependent Mg^{2+} block on N-methyl-D-aspartate receptors (Mayer and Westbrook, 1985) and would lead to augmentation of the depolarisation.

Clearly the cellular mechanisms underlying seizure activity are diverse. One factor which is apparent is that neuronal excitability must play a very important role in epileptiform behaviour. In the following section the role of second messengers in the control of nervous excitability is discussed.

1.4 Control of nervous excitability

The role of second messengers.

The major way in which neuronal excitability is controlled is by the action of neurotransmitters. Neurotransmitters are released from the presynaptic terminal of neurones and cross a synaptic gap to bind to post synaptic receptors. The binding of the neurotransmitter to the receptor induces a sequence of events which affects the cell's activity by altering membrane conductance to specific ions. There are two main ways in which this can be achieved: 1) the receptor can be directly coupled to an ion channel so that neurotransmitter binding

causes the ion channel to open; 2) the receptor is coupled to an enzyme system such that when the neurotransmitter binds, the enzyme converts a substrate to a product, known as a second messenger. With the exception of Ca^{2+}, a second messenger is a substance which is produced intracellularly by the action of an agonist at an external receptor site. The receptor site is coupled to an enzyme which acts to convert a substrate into the second messenger. The second messenger acts intracellularly to regulate specific biological and physiological processes, such as ion channel activation.

A number of second messengers have been identified, the best described being cAMP (Berridge, 1985), cGMP (Waldman and Murad, 1987), inositol 1,4,5- trisphosphate (Berridge, 1984) and Ca^{2+} (Tomlinson, 1985). The response of a neurone to an agonist depends on which second messenger system is activated. I will describe the cAMP and Ca^{2+} second messenger systems which are the two most relevant to this thesis.

Cyclic AMP

Production of cAMP is controlled by the membrane bound enzyme adenylate cyclase. Receptor sites are associated with adenylate cyclase and binding of neurotransmitters can either increase or decrease cAMP production depending on which receptor complex the agonist binds (Enna and Karbon, 1987). Receptors which stimulate adenylate cyclase activity include adrenergic (Bloom et al, 1982), dopamine D1 (Kebabian, 1979), serotonin (Cedar and Schwartz, 1972) and adenosine A2 (Daly et al, 1981). Receptors which inhibit adenylate cyclase activity include muscarinic cholinergic (Huginar,

1987) and adenosine A1 (Daly et al, 1981). Adenylate cyclase is associated with one of two guanine nucleotide binding proteins (G proteins). The G proteins cannot be active until GTP binds to them (Berridge, 1985). The binding of an agonist to a receptor site induces a conformational change in the G protein, making it susceptible to GTP (Berridge, 1985). This causes the G protein to bind to adenylate cyclase to either stimulate or inhibit depending on the G protein involved. The G protein-GTP complex is broken down by the hydrolysis of GTP to GDP which is catalysed by the enzyme GTPase (Berridge, 1985). Cyclic AMP is produced by the dephosphorylation of ATP to cAMP by the action of adenylate cyclase. Cyclic AMP is broken down to AMP by the action of the enzyme phosphodiesterase (Strada et al, 1984). Cyclic AMP produces its effects by binding to a cAMP-dependent protein kinase (Walter et al, 1977). The protein kinase is composed of 2 subunits, a receptor site and a catalytic subunit. When cAMP binds to the receptor it causes the regulatory subunit (which contains the receptor site) and the catalytic subunit to separate. The catalytic subunit, stable when attached to the regulatory site, is now active and acts to phosphorylate membrane proteins which produce a variety of actions; e.g. activation of ion channels, neurotransmitter release and synthesis (Krebs and Beavo, 1979).

The cAMP signal is terminated by dephosphorylation of the proteins phosphorylated by the cAMP dependent protein kinase (Cohen, 1982). The enzyme phosphoprotein phosphatase stops the cAMP induced phosphorylation (Cohen, 1982). This enzyme is itself regulated by cAMP. When cAMP dependent kinase is activated it phosphorylates a phosphatase inhibitory protein which inhibits phosphoprotein

phosphatase which otherwise would immediately dephosphorylate and reverse the phosporylation reactions stimulated by cAMP (Cohen, 1982). When cAMP levels decrease the phosphoprotein phosphatase becomes active and terminates the action of cAMP on target proteins (Cohen, 1982).

Fig 1.1 summarises the processes described in this section.

$\underline{Ca^{2+}}$

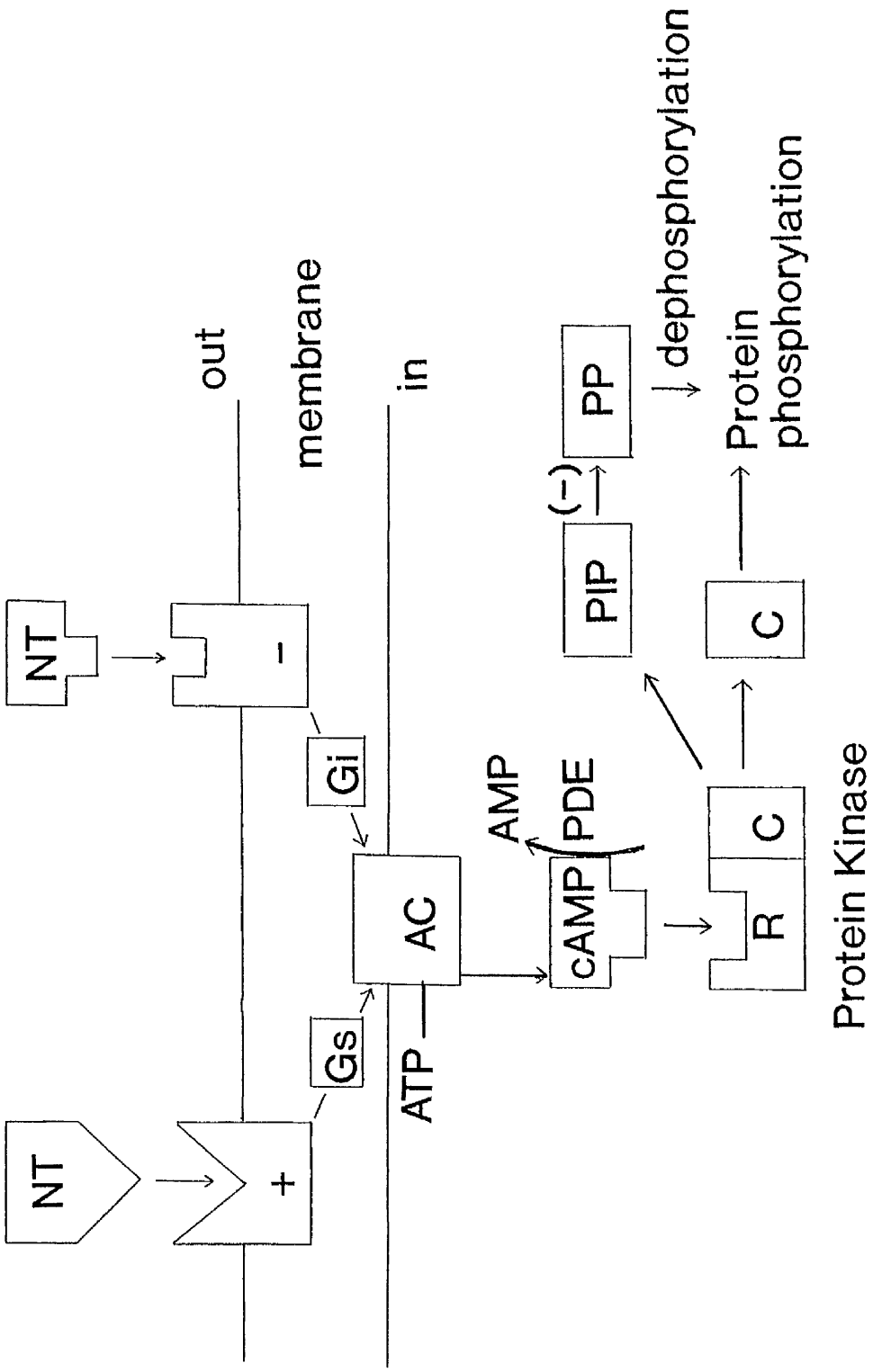
Calcium ions play a major role in modulating normal activity and function in the nervous system (Rasmussen, 1986). Among their most important roles is the modulation of synaptic activity (Katz and Miledi, 1967), stimulus secretion coupling (Katz and Miledi, 1970), and a role as a second messenger. Whereas cAMP is generated within the cell by the action of adenylate cyclase, Ca^{2+} can come from intra- or extracellular sources. Extracellular Ca^{2+} can enter the cell via voltage sensitive channels, whereas intracellular Ca^{2+} can be released from stores. The Ca^{2+} released is thought to come mainly from the endoplasmic reticulum in response to agonist stimulated membrane phosphoinositide hydrolysis (Berridge, 1984). In this process two active metabolites, inositol 1,4,5- trisphosphate and diacylglycerol are produced from the hydrolysis of phosphatidylinositol-4,5-bisphosphate (Berridge, 1984). Inositol 1,4,5- trisphosphate acts on the endoplasmic reticulum to release Ca^{2+} which can act as a second messenger in a variety of ways.

Calcium ions exert some of their effects via the calcium binding protein, calmodulin (Cheung, 1980; Klee et al, 1980). The binding of

Figure 1.1

Pathway of cAMP formation and degradation.

AC, adenylate cyclase; AMP, adenosine monophosphate; ATP, adenosine triphosphate; C, catalytic subunit; cAMP, cyclic AMP; Gi, inhibitory G protein; Gs, stimulatory G protein; PDE, phosphodiesterase; PIP, phosphatase inhibitory protein; PP, phosphoprotein phosphatase; R, regulatory subunit; NT, neurotransmitter.


NT
+
NT
−
out
membrane
in
Gs
Gi
AC
ATP
AMP
PDE
cAMP
R
C
C
PIP
(−)
PP
dephosphorylation
Protein
phosphorylation
Protein Kinase

Ca^{2+} to calmodulin forms the calcium/calmodulin complex which is capable of activating intracellular enzymes. The calcium/calmodulin complex binds to protein kinases leading to conformational changes in intracellular proteins and altered cellular function (Cheung, 1980). At least four calcium/calmodulin dependent protein kinases have been identified in the human brain (Nairn et al, 1985).

Calcium ions can also act as a second messenger without binding to calmodulin. Protein kinase C is found in many tissues but is found in particularly high concentrations in the brain (Walaas et al, 1983 a,b). Protein kinase C is activated at normal intracellular Ca^{2+} concentrations (about 100 nM) by diacylglycerol (Nishizuka, 1984). At higher intracellular Ca^{2+} concentrations (1 uM) protein kinase C is activated by phospholipid (Takai et al, 1979). The protein kinase C then acts on specific substrate proteins to induce conformational changes which can result in, among other things, ion channel activation.

Calcium ions are also capable of regulating at least three ion channels directly.

1) Calcium activated potassium channel ($I_{K(Ca)}$). Increased intracellular Ca^{2+} causes activation of an outward potassium current which causes a hyperpolarisation of the cell membrane (Meech, 1978).

2) Cation channel ($I_{cation(Ca)}$). Calcium activates a channel which is selective for both Na^+ and K^+ ions. The resulting current is inward and leads to depolarisation of the cell membrane (Kramer and Zucker, 1985).

3) Calcium channel ($I_{Ca(Ca)}$). Increased intracellular Ca^{2+} inactivates a calcium channel (Ewald and Levitan, 1987).

Activation of $I_{K(Ca)}$ results in a hyperpolarisation of the cell membrane. Activation of $I_{cation(Ca)}$ results in an inward current at resting potential causing depolarisation of the cell membrane. Inactivation of $I_{Ca(Ca)}$, along with the hyperpolarisation caused by $I_{K(Ca)}$ inhibits the calcium channel (Ewald and Levitan, 1987).

The role of second messengers in epilepsy

A link between epilepsy and second messengers has been suspected for many years. It has been demonstrated that there is an increase in cAMP levels in epileptic mammalian brains during seizures (Ferrendelli and Kinscherf, 1977b). The increase in cAMP in partial seizures in rats occurs only in the area of the brain where the discharge originates (Raabe et al, 1978). However the increase in cAMP occurs only after seizure onset, indicating that the cAMP accumulation may be a result of the seizure rather than the cause (Ferrendelli and Kinscherf, 1977b). It has been suggested that the cAMP increase is caused by noradrenaline via activation of adenylate cyclase (Folbergrova, 1984; Gross and Ferrendelli, 1982). Hattori et al (1986) showed that induction of seizure activity in rat cortex by the excitatory amino acid glutamate caused increased cAMP levels, but whether this occurred before or after the onset of seizure activity was not stated. Working on rat cerebral cortex, Onozuka et al (1989) have shown that injection of the convulsant agent, pentylenetetrazol, induced a threefold increase in the intracellular concentration of cAMP. Qualitatively similar results were obtained by Ferrendelli and

Kinscherf (1977b) who demonstrated that pentylenetetrazol increased the concentration of cAMP in a range of brain areas in rat. In the Japanese land snail Euhadra peliomphala, pentylenetetrazol induced convulsant activity accompanied by an increase in intracellular cAMP levels (Onozuka et al, 1983). This increase in cAMP occurred prior to seizure onset and was thus thought to play a role in the generation of seizure activity. Thus it appears that agents which induce convulsant activity cause increases in the intracellular cAMP concentration in both mammalian and molluscan preparations, but whether this increase is the cause of the activity or the result of it is not yet certain.

Because of Ca^{2+}'s role as a second messenger, it can be assumed that alterations in normal function of Ca^{2+}-regulated processes could underlie some of the changes in neuronal excitability in seizure disorders. Accumulating evidence suggests that dysfunction of Ca^{2+}-regulated enzymatic processes or Ca^{2+} ion channels may underlie seizure activity (DeLorenzo, 1986).

1.5 Experimental models used in the study of epileptiform activity

Clinical studies

Patients suffering from epilepsy take part in a clinical trial by consent. The trial monitors a drug's ability to reduce the number of seizures experienced by the patient. The number of seizures before and after a specified drug regime is recorded to gauge the anticonvulsant properties of the test drug (Mattson et al, 1985; Rodin, 1987; Wilder and Rangel, 1987). The major disadvantage with

this kind of study is the lack of willing suitable volunteers suffering from the appropriate epileptic condition. Trials are expensive, labour intensive and subject to human error, such as the patient forgetting to take the drug, taking the wrong dose, taking it at the wrong time, etc. These studies are non-invasive and the only information they provide is whether a drug is capable of reducing seizure frequency. Such trials do not give any information of about the test drug's mode of action.

Whole animal studies

These models involve the induction of epileptiform activity in an animal (usually rats and mice) by a variety of means listed below. A test drug is monitored to gauge its anticonvulsant effect on the induced seizures.

1) Kindling

This method involves the repeated administration of initially subconvulsive electrical stimuli via implanted bipolar electrodes in a designated area of the brain. The initial stimulus often elicits a partial seizure and subsequent stimulations induce the development of generalised seizures. When this stage is reached the animal is said to be kindled, and can be left for up to a year, and will still respond with a generalised seizure to one of the first two electrical stimuli administered. The amygdala region is often chosen for stimulation because of its sensitivity to electrical stimulus, but other regions such as the globus pallidus, caudate-putamen and hippocampus are also used. This procedure has been used by Barraco et al (1984) and Minabe et al (1987).

2) Electric shock

Wong and Rahwan (1989) have used this method to induce seizures in mice. A current of 50 mA delivered for 0.2 seconds via corneal electrodes induces repeatable seizures.

3) Audiogenic seizures

Some strains of mice are susceptible to sound induced seizures; e.g. the Swiss albino RB strain (Maitre et al, 1974) and the DBA/2 (DeSarro et al, 1988). Sounds of 100 dB at a frequency of 7500 Hz induce seizures in these mice.

4) Chemically induced seizures

Injections of proconvulsant compounds such as pentylenetetrazol (Speckmann and Caspers, 1978; Oyama, 1987; Diehl et al, 1984), penicillin (Schwartzkroin and Prince, 1977), bicuculline (DeLorenzo, 1988) and picrotoxin (Alger and Nicoll, 1980) induce seizures in rats, mice, guinea pigs and rabbits. The actions of these drugs have been fairly well documented and show that they do not share the same mode of action.

Whole animal studies usually set out to investigate a putative anticonvulsant's ability to inhibit experimentally-induced seizures. These studies only indicate whether a drug is capable of inhibiting seizures; they do not indicate a specific mode of action.

In vitro models

Two areas of the mammalian CNS have been used for in vitro studies of epileptiform activity; the hippocampus and the neocortex.

Hippocampal slices. Transverse sections (400-500 μm thick) of

hippocampus are taken and mounted in a superfusion bath. Tetanic current pulses delivered via stimulating electrodes in the stratum pyramidale of the CA3 region induce seizure discharge from pyramidal neurones (Higashima, 1988).

Neocortical slices. Slices of neocortex 500 μM thick, containing the cerebral cortex and corpus callosum, are mounted in a superfusion chamber. Seizures can be induced in one of two ways. The first is to omit Mg^{2+} from the saline which relieves the voltage dependent block of NMDA receptors and results in seizure activity (Jones, 1989). The second uses tungsten stimulating electrodes in the white matter near the cingulate region, where both evoked and spontaneous potentials can be monitored via recording electrodes (Aram et al, 1989).

In both these slice preparations the test drug is applied to the superfusion bath and its ability to inhibit seizure discharge can be assessed.

The molluscan CNS

The molluscan CNS has been chosen as a model by many groups of workers for the following reasons. The context of simplicity must be taken into account when comparing the CNS's of mammals and molluscs. The human CNS contains 10^{14} neurones (Rozsa, 1984) whereas the CNS of the freshwater pond snail Lymnaea stagnalis, for example, contains between 10,000 (Rozsa, 1984) and 15,000 (Bogerd et al, 1991). Thus the CNS of snails is many times more 'simple' than the mammalian CNS, and is therefore advantageous for experimental purposes. In the CNS of many molluscs (e.g. Helix pomatia, Lymnaea stagnalis, Euhadra peliomphala, Aplysia californica and Pleurobranchaea californica)

individual neurones are identifiable. Each cell type always occurs in the same position in the CNS and thus identification is easy. Since the neurones do not all have the same properties it is no surprise that the response of different cells to drugs varies. Sugaya et al (1973) have identified three different cellular responses to pentylenetetrazol (PTZ) in Euhadra peliomphala. This indicates that a cell's response to a compound depends on its endogenous properties; e.g. receptor type and density on the cell's surface, intracellular second messenger systems, and type and density of ion channels possessed by the cell. Thus molluscan neurones cannot be treated as a homologous population but must be treated as individual entities with their own unique properties.

1.6 Pentylenetetrazol as a convulsant agent

The compound pentylenetetrazol (PTZ), also known as pentytetrazole, leptozol and metrazol, is membrane permeable and has the ability to induce convulsant activity in both mammalian and molluscan preparations. Injection of PTZ into an animal can result in generalised tonic/clonic seizures if the correct concentration is used (50 mg/kg in mice, J Wilden, personal communication). Another way in which PTZ can induce seizures is by kindling. This is similar to the electrical kindling described in Section 1.5, but instead of an electrical stimulus, subconvulsive doses of PTZ are used. Diehl et al (1984) used male Wistar rats which were injected with 30 mg/kg PTZ. Successive daily injections of PTZ induced seizures such that, after 20 injections, doses ineffective after the first injection induced a generalised tonic seizure. Thus PTZ can induce seizures in whole animals, but to gain a better understanding of PTZ's effects

identified regions of the brain, or isolated neurones must be used. In cultured dorsal root ganglion cells Speckmann and Casper (1978) demonstrated a depolarisation of the cell's resting membrane potential in response to 5-20 mM PTZ, confirming PTZ's ability to excite these cells. Bingmann and Speckmann (1986) have demonstrated, using guinea pig hippocampal CA3 neurones, that PTZ (2-10 mM) induced an initial hyperpolarisation followed by a persistent paroxysmal depolarising shift (PDS). This PDS was dependent upon extracellular Ca^{2+}, indicating that influx of Ca^{2+} may be intimately linked with PTZ's effects. However a different mode of action for PTZ has been proposed by Oyama (1987) who has shown that PTZ (30 mg/kg i.v.) reduced the amplitude and duration of the I_A current in isolated rabbit nodose ganglion cells. The I_A current is involved with the control of neuronal excitability and firing patterns (Connor and Stevens, 1971); a reduction in this current leads to increased neuronal excitability. Another possible mode of action for PTZ involves cAMP. Onozuka et al (1989) demonstrated that intraperitoneal injection of PTZ (100 mg/kg) induced a 3 fold increase in cAMP concentrations in rat cerebral cortex. This injection of PTZ also caused the release of Ca^{2+} from the endoplasmic reticulum. There is no dispute about PTZ's ability to induce epileptiform activity in a variety of mammalian preparations but a common mode of action has not yet been elucidated.

The effects of PTZ on molluscan neurones has also been investigated. As previously stated the advantage of the molluscan CNS is that it contains identifiable neurones so that the same cell can be used, compared to mammalian preparations where work is carried out on

regions of the brain or isolated cells from a particular area where the cells may not all be the same.

In 1973, Sugaya et al first showed that neurones of the Japanese land snail Euhadra peliomphala show heterogeneous responses to PTZ (10-100 mM). Three cell types were characterised; D, H and I cells. D cells were depolarised by acetyl choline and showed a large depolarisation and bursting activity in response to PTZ. H cells were hyperpolarised by acetyl choline and showed a moderate degree of excitation in response to PTZ. I cells were insensitive to acetyl choline and were practically insensitive to PTZ. In 1978, Sugaya and Onozuka showed that the calcium ions within D neurones became concentrated near the cell membrane following application of PTZ. Later, Sugaya et al (1978) demonstrated that Ca^{2+} was released from intracellular stores and migrated to the inner surface of the cell membrane, where binding induced conformational changes and activated ion channels. In 1983, Onozuka et al showed that cAMP was involved in PTZ's effects. By assaying for cAMP, adenylate cyclase and protein kinase they concluded that PTZ acted by binding to adenylate cyclase, resulting in an increase in cAMP levels. This cAMP was thought to cause the release of Ca^{2+} from intracellular lysosome-like granules (Sugaya and Onozuka, 1978). The Ca^{2+} could then act at the internal side of the cell membrane to phosphorylate membrane proteins and activate ion channels leading to epileptiform activity. By 1986, Onozuka et al had identified that Ca^{2+}/calmodulin-dependent protein kinase was activated in the presence of PTZ, and that it phosphorylated two proteins of known molecular weight. Sugaya et al (1987) showed that PTZ-sensitive neurones responded to PTZ in two consecutive ways.

Firstly the neurones exhibited bursting activity as previously described. This was followed by a previously unreported hyperpolarisation and cessation of bursting activity. This was thought to be due to the released Ca^{2+} increasing in concentration and activating Ca^{2+}-dependent potassium channels (Sugaya et al, 1988).

Using Aplysia neurones, Hartung and Hermann (1987) showed that PTZ blocked voltage-dependent Na^{+} and Ca^{2+} currents and also inhibited the delayed rectifier current $I_{K(V)}$. Pentylenetetrazol increased the Ca^{2+} activated potassium current $I_{K(Ca)}$ and induced an inward current. These effects did not occur when PTZ was injected intracellularly, suggesting an extracellular site of action. These results explain the neuronal activity seen after application of 100 mM PTZ. The depolarisation was induced by the inward current and was sustained due to inhibition of the delayed rectifier. The Ca^{2+} dependent potassium current was subsequently activated, possibly by the release of intracellularly stored Ca^{2+}, and resulted in the cell membrane repolarising (Hartung and Hermann, 1987). Walden et al (1988) demonstrated, using identified neurones of Helix, that PTZ (20-80 mM) induced a non-specific inward current followed by activation of a calcium-dependent potassium current which resulted in repolarisation of the cell.

Working on identified neurones of Lymnaea, McCrohan and Gillette (1988b) described a PTZ-induced slow inward current which was carried by Na^{+}. A similar Na^{+} current was induced by intracellular injection of cyclic AMP (McCrohan and Gillette, 1988a). McCrohan and

Gillette (1988b) suggested that PTZ activated this current by acting as a phosphodiesterase inhibitor, since isobutyl methylxanthine (IBMX - a phosphodiesterase inhibitor) mimicked PTZ's ability to induce epileptiform activity. They also showed that PTZ increased the amplitude of the cAMP-induced, sodium dependent inward current, reinforcing the hypothesis that PTZ may produce its epileptiform effects by increasing cAMP concentrations.

From the evidence reviewed above, it is clear that the mechanism of action of PTZ has yet to be fully elucidated. Since PTZ is in widespread use in studies of the ionic basis of convulsant activity, further information concerning its actions would be of considerable value.

1.7 Antiepileptic drugs used in animal models of epilepsy

There are four main classes of anticonvulsant drug used in the study of epileptiform activity in animal models. These are classified by their mode of action. The benzodiazepines, carbamazepine and phenytoin are also in clinical use.

1) GABA enhancing compounds

GABA is the major inhibitory neurotransmitter in the mammalian CNS, high concentrations being found in the neocortex, hippocampus and other forebrain structures. GABA acts via GABA-A receptors to open Cl^- channels, leading to hyperpolarisation of the cell membrane. GABA can also act at GABA-B receptors to open K^+ channels pre or postsynaptically. An overwhelming body of data (DeLorenzo, 1988) indicates that the major inhibitory action of GABA in the CNS is the

ability to regulate Cl^- channel permeability. The largest group of anticonvulsant drugs to act at the GABA receptor are the benzodiazepines. The mode of action of benzodiazepines was little understood until Squires and Braestrup (1977) demonstrated the high affinity binding of [^{3}H]-diazepam to rat brain homogenate, indicating the presence of benzodiazepine receptors in the CNS. Olsen et al, (1986) showed the localisation of benzodiazepine receptors at the GABA / Cl^- ionophore. The binding of benzodiazepines to the benzodiazepine receptor enhances binding of GABA to the GABA receptor, resulting in an increased Cl^- current and hyperpolarisation. There is a third site present in the GABA receptor complex. Picrotoxin binding to this site inhibits GABA binding and reduces the Cl^- current (Olsen et al, 1986). Barbiturates binding to this site potentiate benzodiazepine binding and thus indirectly increase the flow of Cl^- (Twyman et al, 1988).

2) NMDA receptor antagonists

The possible role of excitatory amino acids in epilepsy was first shown by Hayashi (1951), who reported that injection of sodium glutamate and aspartate into the cerebral cortex of dogs and primates induced generalised seizures. Excitatory amino acids found in the mammalian CNS include glutamate, aspartate, quinolinic acid and homocysteate. All of these compounds induce convulsions when injected directly into dog brains (Johnston, 1973; Stone and Javid, 1983). The development of dicarboxylic amino acid analogues, which act as agonists or antagonists, enabled the study of the postsynaptic receptor site at which glutamate and aspartate exert their actions. Experimental evidence suggests the existence of three types of glutamate receptor named after their preferred exogenous agonists;

NMDA, kainate and quisqualate (Watkins and Evans, 1981). Glutamate activates all three receptor types whereas aspartate appears to activate only NMDA receptors. Most routine central EPSP's are generated by non NMDA receptors (McDermott and Dale, 1987); in physiological conditions NMDA receptors are blocked by Mg^{2+} in a voltage dependent manner. At resting potential the NMDA receptors are blocked but as the cell depolarises the block is removed and the receptor becomes receptive to NMDA agonists (Mayer and Westbrook, 1986). It has been postulated that the NMDA receptor may be involved in epileptiform activity (Dingledine et al, 1986; Artola and Singer, 1987; Mody et al, 1988). A number of analogues of the endogenous excitatory amino acids have been shown to have antagonistic properties (Perkins et al, 1981; McLennan and Liu, 1982). These compounds have potent anticonvulsant effects on NMDA-induced epileptiform activity.

3) Calcium channel antagonists

Controlling Ca^{2+} entry into the cell is the first major step in regulating the effect of Ca^{2+}-mediated seizure activity. Three types of voltage gated Ca^{2+} channel have been described in mammalian CNS (Tsien et al, 1988); the T, L and N channels. T channel conductance is small, its duration transient and its inactivation rapid. L channel conductance is large, its duration long lasting and its inactivation slow. N channel parameters are intermediate between the T and L values. Ca^{2+} channel antagonists have been shown to reduce seizure activity (Ashton and Wauquier, 1985). Ethosuximide has been shown to inhibit T channels in thalamic neurones (Coulter et al, 1988) and in primary afferent neurones (Gross et al, 1989) at therapeutically relevant concentrations. Phenytoin (see later),

inhibited binding of [^{3}H]nitrendipine (a compound which binds to Ca^{2+} channels in brain membranes with high affinity) to Ca^{2+} channels in the drug's therapeutic concentration range (Greenberg et al, 1984). DeSarro et al (1988) have shown that the Ca^{2+} channel antagonists flunarizine and dihydropyridine are potent anticonvulsants in the DBA/2 strain of mice (susceptible to sound induced seizures). However the general picture of Ca^{2+} channel antagonists is that they are not used clinically due to unconvincing results.

4) Other anticonvulsant agents

a) Carbamazepine. Carbamazepine (CBZ) or Tegretol is a tricyclic immunostilbene derivative, related structurally to the antidepressant imipramine and the anticonvulsant phenytoin (Schauf et al, 1974). Carbamazepine is used to treat generalised tonic/clonic seizures but not generalised absence seizures. It is also used in the treatment of trigeminal neuralgia (Rasmussen and Riishede, 1970) and glossopharyngeal neuralgia (Saviolo and Fiasconase, 1987). Schauf et al (1974) showed that CBZ induced a voltage-dependent block of Na^+ channels in axons of the marine worm Myxicola. Carbamazepine (20 μM) inhibited the Na^+ current by 40% at a membrane potential of -120 mV and 95% at -45 mV. The therapeutic serum concentration of CBZ is in the same range as used in these experiments. In rat brain synaptosomes, the IC_{50} for inhibition of binding of [^{3}H] batrachotoxin Aα benzoate (BTX) to the receptor site responsible for activation of Na^+ channels (Catterall et al, 1981) by CBZ was 131 μM (Willow and Catterall,1982). Willow et al, (1983) showed that 40 μM CBZ inhibited BTX activated influx of Na^+ into cultured neuroblastoma cells. These findings suggest that CBZ's mode of action

is to interact with the receptor site responsible for the activation of fast Na^+ channels and thus reduce Na^+ current.

Recently however it has been suggested that part of CBZ's mode of action may be mediated via adenosine receptors. In 1982, Skerrit showed that 400 uM CBZ inhibited binding of the adenosine analogue L-N6-phenylisopropyladenosine, indicating that CBZ may itself bind to adenosine receptors. Follow up studies (Skerrit, 1983 a,b) supplied additional evidence that CBZ inhibited L-N6-phenylisopropyladenosine binding and also showed that CBZ mimicked the effects of the adenosine receptor antagonist theophylline on electrically stimulated guinea pig ileum. Marangos et al (1983) showed that CBZ inhibited binding of the adenosine agonist, [^{3}H] diethylphenylxanthine and the adenosine antagonist [^{3}H] cyclohexyladenosine to adenosine receptors. Studies by Wier et al (1984) also showed that CBZ inhibited adenosine agonist and antagonist binding. Adenosine receptors have been divided into three subclasses (Schwabe, 1985), the A1, A2 and P sites. Adenosine binds with high affinity (nM) to the A1 site and with low affinity at the A2 site (uM). Adenosine receptors are coupled to the enzyme system adenylate cyclase which produces cAMP by dephosphorylating adenosine triphosphate (ATP). Activation of the A1 receptor by agonist binding inhibits adenylate cyclase activity causing a reduction in cAMP levels, while A2 receptor agonist binding stimulates adenylate cyclase activity and causes an increase in cAMP levels. The P site is internal and mediates the inhibitory effects of high concentrations of adenosine.

A number of studies have shown that CBZ binds to the A1 receptor

(Fujiwara et al, 1986; Gasser et al, 1988; Daval et al, 1989; Clark and Post, 1989), thus inhibiting cAMP production. These studies indicate two important things. Firstly, that at least part of CBZ's anticonvulsant effects may be via adenosine A1 receptors, and secondly, that adenosine may be an endogenous anticonvulsant. It has been known for some time that adenosine depresses the activity of central neurones in vivo (Dragunow et al, 1984). In behavioural tests, adenosine and its derivatives have been shown to have sedative, hypnotic and anticonvulsant activity (Barraco et al, 1983, 1984; Dunwiddie and Worth, 1982; Haulica, 1973; Maitre et al, 1974; Radulovacki et al, 1982). Agents which inhibit the adenosine activating enzyme, adenosine deaminase, such as deoxyformycin and erythro-9-(2-hypoxy-3-nonyl) adenine, have sedative effects in rats and mice (Radulovacki et al, 1982; Mendelson et al, 1983). Inhibitors of adenosine uptake such as nitrobenzylthioinosine and papaverine depress locomotor activity in mice (Crawley et al, 1983). The adenosine receptor antagonist caffeine has behaviourally stimulant and proconvulsant activities in mice (Marangos, 1984). This evidence suggests that adenosine is involved in the regulation of CNS excitability.

b) Phenytoin. Matsuki et al, (1984) showed that 100 μM phenytoin (PHT) blocked sodium channels by increasing the number of channels in the inactivated state and by delaying the transition from inactivated to closed. In a similar study, Courtney and Etter (1983) showed that PHT selectively blocked the inactive form of closed sodium channels thus inducing anticonvulsant activity. McLean and McDonald (1983) showed that PHT at 1-2 μg/ml (therapeutic level) limited the ability of mouse spinal cord neurones to sustain high frequency repetitive

firing of action potentials. It was thought that PHT slowed the recovery of sodium channels from inactivation. Sugaya et al (1983) showed in molluscan neurones that PHT inhibited the changes in intracellular protein induced by the convulsant drug pentylenetetrazol, indicating a possible further mode of action of PHT. Ferrendelli and Kinscherf (1977a) showed that PHT inhibited Ca^{2+} influx into cells of mouse brain slices by blocking voltage gated Ca^{2+} channels. They also showed that PHT inhibits cAMP and cGMP accumulation activated by Na^{+} influx, the initiating step for the accumulation of these second messengers. These results indicate that PHT has a whole spectrum of possible anticonvulsant modes of action and may exert its effects by employing one or several of the actions described above. It seems likely that its mode of action is determined by the preparation, as not all will contain the necessary target sites described. The wide range of modes of action of anticonvulsants, described above, adds weight to the view that epileptogenesis itself may have multiple origins.

1.8 The experimental model used in this study

The phylum mollusca is the second largest in the animal kingdom, including about 100,000 species. Although there is a great diversity in the phylum, molluscs can generally be regarded as originating from a group of bilaterally symmetrical animals. They have a well developed head, containing the sensory organs, which is attached to the muscular foot, used for locomotion (Kershaw, 1983).

The class Gastropoda is the largest molluscan class with about 75,000

living species and is divided into three subclasses; prosobranchia, opisthobranchia and pulmonata. The main two subclasses used in electrophysiological studies are the opisthobranchs and the pulmonates. The opisthobranchs are a marine subclass which have a reduced shell size or no shell at all. They are commonly called the sea slugs and include Aplysia, Tritonia, Pleurobranchaea, Archidoris and Anisodoris. The pulmonates have the body cavity modified to form a lung and are subdivided into two superorders, the basmmatrophora (aquatic) and stylommatophora (terrestrial). Lymnaea and Planorbis belong to the former and Helix and Limax belong to the latter. There are both terrestrial and aquatic snails, but only terrestrial slugs. In gastropods the body and CNS have become asymmetrical due to a process called torsion. This involves the visceral mass and mantle being rotated through 180^{o} (McCrohan and Winlow, 1985). The snail's shell is also asymmetrical and its function is to protect the animal. (For a general review of the gastropod molluscs see Kershaw 1983).

The gastropod molluscan CNS is particularly suitable for electrophysiological studies as the neurones are easily visible on the surface of the ganglia and it is relatively easy to place microelectrodes into identified cells. The mollusc used in this study was Lymnaea stagnalis, a freshwater snail. The CNS of Lymnaea is estimated to contain between 10,000 and 15,000 neurones contained within 11 discrete ganglia, 5 paired and 1 unpaired. This asymmetry has been produced by torsion (McCrohan and Winlow, 1985). In the CNS torsion has led to the fusing of some primary ganglia; e.g. the right parietal ganglia and the supraintestinal ganglia have fused to form

the modern right parietal ganglion, and the visceral ganglia and the subintestinal ganglia are fused to form the modern visceral ganglion.

A large volume of work has been carried out on the Lymnaea CNS. This work includes mapping of the neurones (Benjamin et al, 1979; Benjamin and Winlow, 1981; Khennak and McCrohan, 1988; Kiss and Salanki, 1977; Winlow and Benjamin, 1976), and investigations of the effect of drugs (Fowler and Partridge, 1984; Girdlestone et al, 1989; McCrohan and Gillette, 1988b), age (Janse et al, 1986), metal ions (Audesirk and Audesirk, 1983) and sensory input (Janse et al, 1988) on neuronal activity. The synaptic connections of identified neurones of the buccal ganglia have been investigated (Rose and Benjamin, 1981 a,b; Elliott and Benjamin, 1985 a,b) as have the effects of peripheral oxygen concentration (Janse et al, 1985). Recently a large volume of work on neurotransmitter pathways and function has been carried out, and is still in progress (Audesirk, 1989; McCaman, 1985; Tuersley and McCrohan, 1988).

In an investigation into the cellular mechanisms underlying epilepsy an important question is whether a cell's inherent properties determine its susceptibility to seizures? To study this question, identified neurones must be used. In the present work a preliminary study was carried out to examine PTZ's effects on a variety of identified neurone types in the Lymnaea CNS. The majority of cells exhibited increased activity of varying degrees in the presence of 40 mM PTZ. One cell type however, was inhibited by PTZ. This cell was the right parietal dorsal 1 (RPD1) cell, and this is the first molluscan neurone to be described in which PTZ is purely inhibitory. McCrohan and Gillette (1988b) had already described the excitatory

(convulsant) actions of PTZ in buccal neurones, including the identified motoneurone, B1. A comparison of PTZ's effects on the B1 cell and the RPD1 cell therefore offered an excellent opportunity to investigate PTZ's mode of action on identified neurones which show different responses to PTZ.

The B1 neurone.

The paired B1 neurones occur symmetrically in the buccal ganglia of Lymnaea (Figs 1.2; 1.3). The morphology of the cell was first described by Benjamin et al (1979) who showed that it had the largest cell body on the dorsal surface of the buccal ganglia, about 100 um in diameter. The cell sends axonal projections to both right and left dorsobuccal nerves. The axonal projection divides to send one branch to the salivary glands and the other branch to the gut (Benjamin et al, 1979). It has been shown that the B1 cells are electrotonically coupled, probably at the buccal commissure where the axons appear to be in very close proximity to each other (Benjamin et al, 1979).

The B1 neurone is involved in the control of feeding activity of the snail. The feeding pattern is generated by a network of central pattern generating interneurones, the N1, N2 and N3 interneurones (Elliott and Benjamin, 1985). The B1 cell is a motoneurone which becomes active during the N1 phase of feeding. It is thought to innervate the salivary glands (Benjamin et al, 1979) and its function may be to stimulate the glands during the initial stages of feeding behaviour.

Figure 1.2

Dorsal view of the central nervous system of Lymnaea. Nerves numbered after Slade et al (1981). A, anterior; al, anterior lobe; brn, buccal retractor nerve; cc, cerebral commissure; dbn, dorsobuccal nerve; L, left; lbn, laterobuccal nerve; ldb lateral dorsal body; mdb, medial dorsal body; P, posterior; pbn, postbuccal nerve; pjn, posterior jugalis nerve; R, right; st, statocyst.

1. cerebrobuccal connective
2. superior labial nerve
3. median labial n.
4. penis n.
5. tentacle n.
6. optic n.
7. nuchal n.
8. left parietal n.
9. cutaneous pallial n.
10. intestinal n.
11. anal n.
12. genital n.
13. right internal parietal n.
14. right external parietal n.
15. inferior cervical n.
16. superior cervical n.
17. columellar n.
18. superior pedal n.
19. inferior pedal n.
20. median pedal n.
21. dorsal pedal commissure
22. ventral pedal commissure
23. medial columellar n.
24. cerebropedal connective
25. pedal pleural connective

Cell types

B1, buccal 1 cell; RPD1, right parietal dorsal 1 cell.

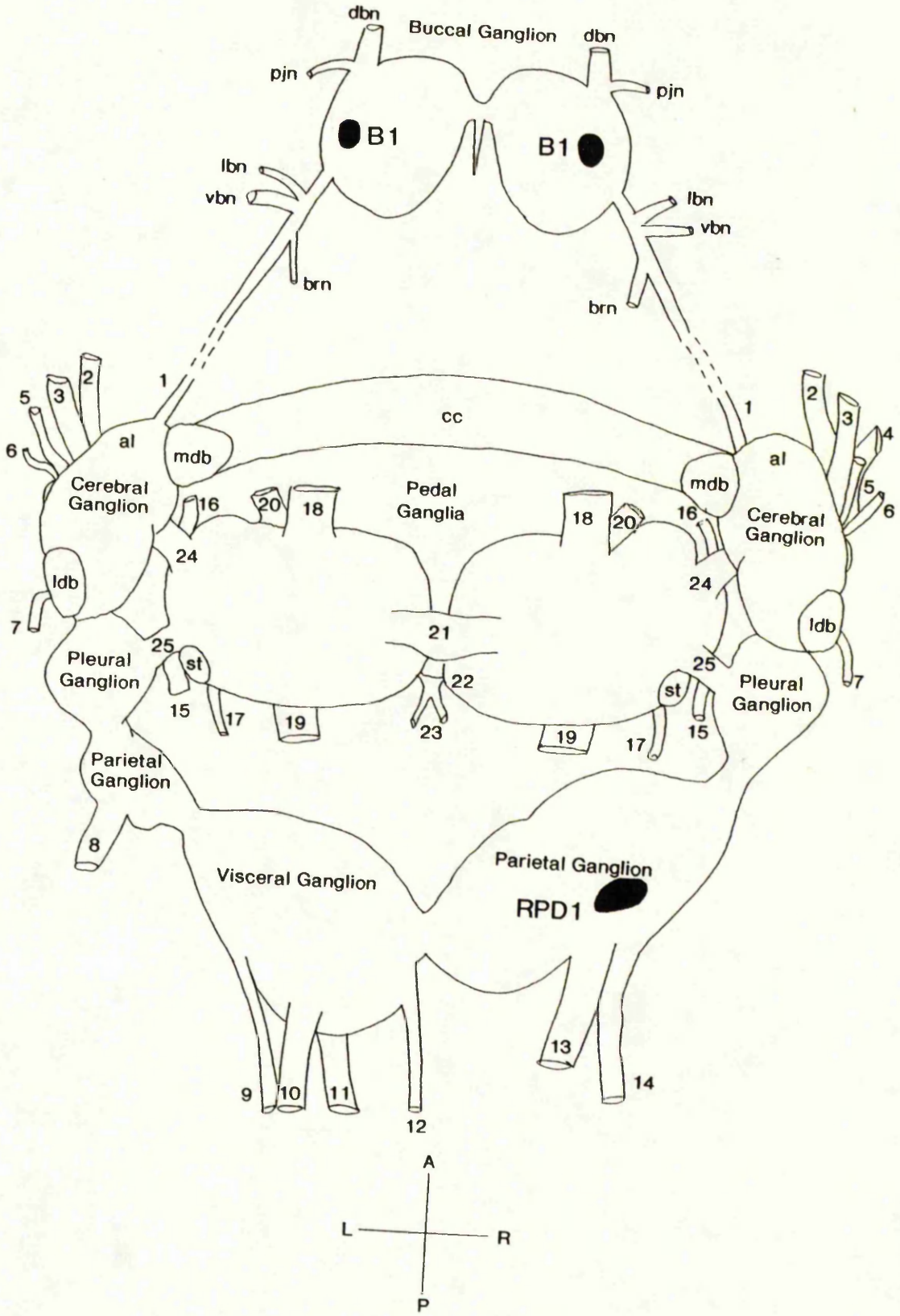
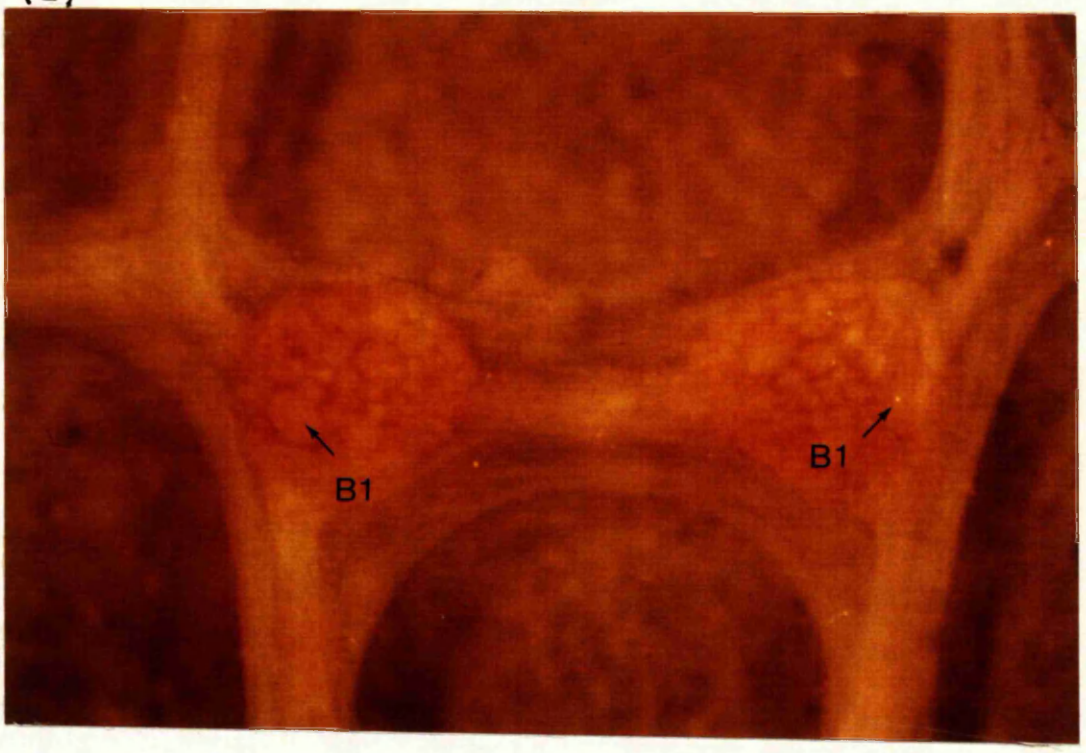

dbn
Buccal Ganglion
dbn
pjn
pjn
B1
B1
lbn
vbn
lbn
vbn
brn
brn
cc
al
mdb
Cerebral Ganglion
Pedal Ganglia
mdb
al
Cerebral Ganglion
ldb
ldb
Pleural Ganglion
Pleural Ganglion
st
st
Parietal Ganglion
Visceral Ganglion
Parietal Ganglion
RPD1
A
L
R
P

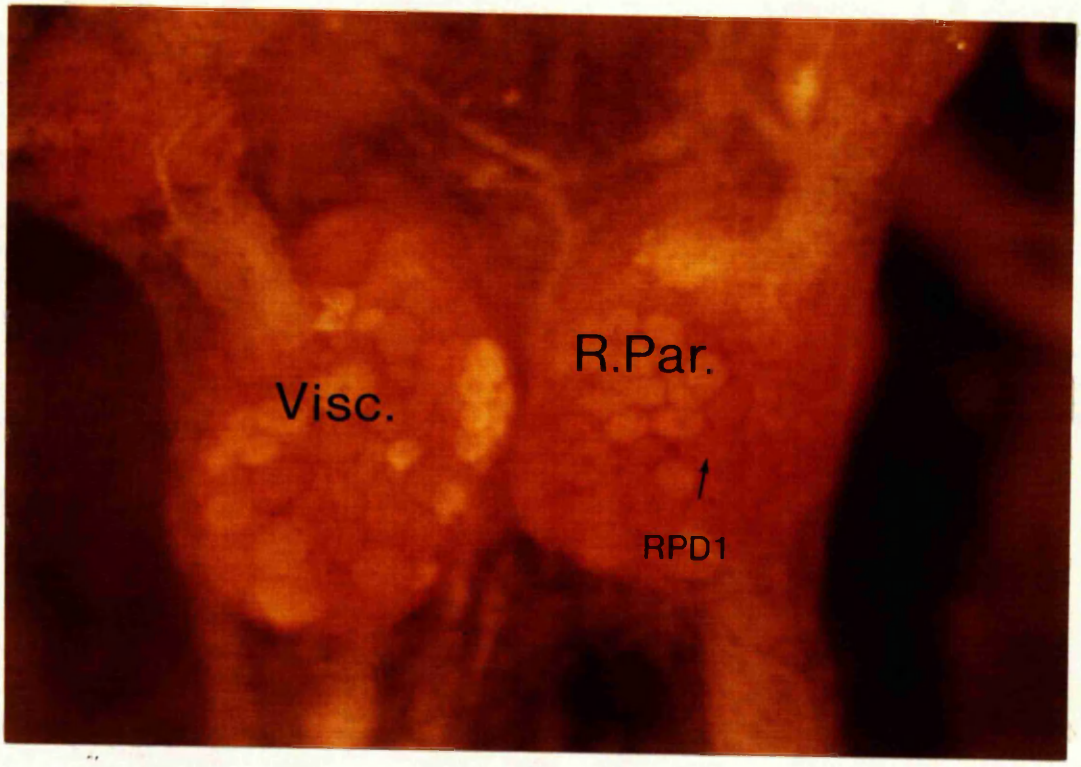
Fig 1.3. (a) Photograph of the B1 neurone in the paired buccal ganglia. The cell occurs symmetrically in the right and left buccal ganglia. (b) The RPD1 neurone is located in the right parietal ganglion.

(a)



200 µm

(b)



Anterior

The RPD1 neurone

This neurone is located on the dorsal surface of the right parietal ganglion (Figs 1.2; 1.3). Its diameter varies from 120 - 150 um making it one of the largest neurones in the CNS (Benjamin and Winlow, 1981). It sends axonal branches down the internal and external parietal nerves but its main axonal branch is to the right pedal ganglion via the right pleural ganglion (Benjamin and Winlow, 1981). Kiss and Salanki (1977) showed that the RPD1 cell (they called it the P13 cell) is affected by stimulation of the intestinal and anal nerves, but it has no direct axonal projections to these nerves. They also showed that the RPD1 cell sends axonal projections down the right internal parietal neurone but described no projections to the right pedal ganglion.

The RPD1 cell responds to electrical stimulation of the mantle edge, pneumostome area and lung wall (Janse et al, 1985). However its function is still unknown.

1.9 Aims of the project

The aim of this study was to investigate the mode of action of PTZ, and also to study the role of second messengers (Ca^{2+} and cAMP) in the generation of epileptiform activity in single neurones. Pentylenetetrazol is a convulsant drug which induces epileptiform activity in humans, rats, mice and molluscs. The fact that it is able to do this in the wide variety of species and cell types suggests that its seizure-inducing action may be fundamental to a wide range of cell types. An understanding of its mode of action could therefore provide an insight into the general principles governing the

endogenous generation of seizures during epilepsy.

The molluscan CNS was chosen for this study because of the large body of work which has previously been carried out and due to its large, accessible and identifiable neurones. The B1 and RPD1 cells were chosen as the B1 cell exhibited characteristic epileptiform activity in response to PTZ whereas the RPD1 cell exhibited inhibition of activity in the presence of PTZ.

A cAMP-dependent inward membrane current was also studied in both cell types. The reason for this was that it had been proposed that PTZ may act to increase intracellular cAMP levels in the B1 cell (McCrohan and Gillette, 1988b). If this were the case a comparison of the characteristics of this current in the B1 and RPD1 cells might prove useful in determining the mechanisms underlying the opposing effects of PTZ in the two cell types.

CHAPTER 2

MATERIALS AND METHODS

2.1 Lymnaea stagnalis

Specimens of the pond snail Lymnaea stagnalis were obtained from the animal suppliers, Blades Biological, Cowden, Kent and Sciento, Salford, Greater Manchester. The snails weighed between 2 and 6 grammes and were kept in aerated tap water at room temperature (20-24 ^{o}C) in five gallon plastic tanks fitted with undergravel filters. Lighting was provided on a 12 hour per day cycle by a single fluorescent tube placed above each tank. The snails were fed mainly with lettuce but were occasionally fed with a preparation (Tetramin) intended for gold fish when lettuce was difficult to obtain.

2.2 Preparation of the CNS

All experiments were performed on the isolated central nervous system (CNS) with all the ganglia present and all the major nerves attached (Fig 1.2). The cerebral commissure was cut to obtain a greater degree of stability when the CNS was pinned down. The remaining interganglionic commissures and connectives were left intact. Cutting the cerebral commissure did not appear to affect the properties of the cells recorded.

The CNS was pinned by its nerves to a Sylgard (Dow-Corning)-lined black 2 ml watch glass. The black watch glass reduced light reflection and increased the contrast between the ganglia and the

dish. After the CNS had been pinned down using fine insect pins, the external sheath covering the ganglia to be studied was carefully removed using fine watchmakers forceps. Small lumps of protease powder (type XIV, Sigma) were placed on the inner ganglionic sheath for between 5 and 10 minutes, followed by thorough washing with saline. This caused softening of the sheath and enabled it to be removed by careful teasing with fine forceps. All experiments were carried out at room temperature which varied between 20 and 24 ^{o}C. The watch glass contained 1 ml of saline for all experiments.

2.3 Physiological salines

Experiments were carried out in saline buffered with 3-(N-Morpholino) propane-sulphonic acid (MOPS, BDH). Normal saline had the following composition in mM (McCrohan and Gillette, 1988a).

NaCl	60
KCl	1.6
$CaCl_2$	4
$MgCl_2$	1.5
MOPS	10

One litre of saline was made up at a time and adjusted to pH 7.5 by the addition of 1 M NaOH. Sodium free saline had NaCl substituted by equimolar arginine hydrochloride and pH adjusted using 1 M KOH. High calcium saline had the calcium concentration increased to 12 mM; no correction was made for the increase in osmolarity. Low calcium saline (1 uM calcium) was made following the directions of Evans and Marty (1986) and consisted of the following in mM.

NaCl	60
KCl	1.6
$CaCl_2$	12
$MgCl_2$	1.5
MOPS	10
HEDTA	40

N-hydroxyethylethylenediamine-triacetic acid (HEDTA, SIGMA) was used as the calcium buffer rather than EGTA as it has been shown to have greater calcium buffering properties (Evans and Marty, 1986).

2.4 Drugs

Drugs were dissolved in normal saline to give stock solutions so that addition of 0.2 ml stock solution to the 1 ml of saline in the Sylgard-lined dish gave the desired final drug concentration. The exceptions to this protocol were experiments carried out in zero sodium and low or high calcium salines. In these instances the drug was dissolved in the corresponding saline prior to addition to the bath. Some drugs were not soluble enough in water to be dissolved in saline. Iproveratril (D600) was one such drug. In this instance the drug was dissolved in 100% ethanol and then diluted in saline until a 5% ethanol solution was obtained. Addition of 0.2 mls of this solution to the Sylgard lined dish resulted in the desired concentration of the drug in a 1% ethanol solution. Control studies showed that 1% ethanol did not significantly affect neuronal activity. Carbamazepine (CBZ) was also very insoluble in water but could not be treated in this way as it precipitated out of solution when the ethanol solution was added to the saline. It was found that

the best way to obtain the desired concentration of CBZ was to dissolve the CBZ in 100% ethanol warmed to about 40 ^{o}C. A drop of this solution (approximate volume 0.01 ml) was directly added to the bath, resulting in a bath concentration of 1% ethanol without precipitation. (see Table 2.1 for a summary of all drugs used in this study).

2.5 Electrophysiological Recordings

2.5.1 Single electrode; membrane potential recording

Intracellular recordings were made from identified neurones using glass microelectrodes filled with 3 M potassium acetate, filtered through a 0.22 um Millipore filter (Millipore SA, 67120 Molsheim, France). Microelectrodes were pulled on a Scientific and Research Instruments Ltd vertical pipette-puller from 1.2 mm diameter, fibre-filled capillary tubing (Clark Electromedical Instruments, Pangbourne, Reading). Microelectrodes had resistances of between 20 and 30 Mohms. Prior to recording, the tip of the microelectrode was brushed carefully against the tip of a pair of fine forceps. This resulted in the tip being broken and the resistance decreasing to 5 to 15 Mohms. This type of broken-back microelectrode was found to produce clean and easy impalements. Immediately prior to use, the electrode tip was dipped in black waterproof ink (Rotring drawing ink K, etching, 17 black) to facilitate the viewing of the tip under the microscope. The microelectrode was fitted to an electrode holder comprising a silver wire, which contacted the electrolyte, and a 2mm plug which was inserted into the head stage of a Dagan 8500 voltage clamp system. The Dagan 8500 is a two electrode voltage clamp system

Table 2.1

Drugs	Supplier	Presumed action
Amiloride	SIGMA	Na^+ channel blocker
Adenosine 5'-monophosphate (AMP)	SIGMA	Product of cAMP breakdown
Cyclic adenosine 3'5'-monophosphate (cAMP)	SIGMA	Intracellular second messenger
Carbamazepine (CBZ)	SIGMA	Na^+ channel blocker and suspected adenosine A2 receptor agonist
8-(4-Chlorophenylthio)-adenosine 3'5'-cyclic monophosphate (CPTcAMP)	SIGMA	Membrane permeable cAMP analogue
Ethyleneglycol-bis-(B-amino ethyl ether) N,N N'-tetra acetic acid (EGTA)	SIGMA	Ca^{2+} chelator
Guanosine 3'5'-cyclic monophosphate (cGMP)	SIGMA	Intracellular second messenger
Iproveratril (D600)	KNOLL	Ca^{2+} and $I_{K(Ca)}$ channel blocker
3-Isobutyl-1-methylxanthine (IBMX)	SIGMA	Phosphodiesterase inhibitor
Pentylenetetrazol (PTZ)	SIGMA	Induces convulsant activity
Quinidine	SIGMA	$I_{K(Ca)}$ channel blocker
Tetrodotoxin (TTX)	SIGMA	Fast Na^+ channel blocker
Tetraethylammonium bromide (TEA)	SIGMA	Non specific K^+ channel blocker

3,4,5-trimethoxybenzoic acid 8-(diethylamino)-octyl ester (TMB-8)	SIGMA	Blocker of Ca^{2+} from intracellular stores

with two separate electrode preamplifiers, one used for voltage sensing and the other primarily for current passing. The microelectrode was connected to the former which was arranged as a standard intracellular microelectrode amplifier with unity gain, driven guard, bridge balance, capacity compensation and selectable high frequency cut-out. A frequency response of DC to 10 KHz was used. Recorded signals were amplified a further ten times using an additional amplifier (CFP 8120), whose frequency response was DC to 4 KHz. The output was viewed on a storage oscilloscope (Tektronix 5111) and recorded on video tape using a two channel pulse code modulating system (Medical System Corps) feeding a domestic video recorder (National Panasonic NVG12). Permanent records were made using a 2 channel ink jet recorder (Gould 2200). A silver wire indifferent electrode was placed in the bath and connected to the virtual ground (chamber) socket of the Dagan 8500. Mains interference (50 Hz) was eliminated by enclosing the preparation area within a 5 mm wire mesh Faraday cage. The preparation was lit using a fibre optic cold light source.

2.5.2 Two electrode voltage clamp

The voltage clamp system consists of three components; a voltage sensing electrode connected as described in 2.5.1, a current passing electrode and a feedback system which compares the measured membrane potential (Vm) with a desired command potential (Vc) set by the experimenter. The current passing electrode was connected to the second preamplifier of the Dagan 8500, with the output amplified a further 10 times by a CFP 8121 amplifier before being connected to the second channel of the oscilloscope. Once a cell had been

penetrated with both microelectrodes, the Dagan 8500 was set to operate in voltage clamp mode. This entailed setting a command potential (Vc) and switching the system to clamp mode 3 to produce a fairly 'tight' clamp. A voltage clamp uses the difference between the voltages (Vm and Vc) as an error signal which is amplified to drive the actual membrane voltage by appropriate current injection such that Vm equals Vc and the error signal approaches zero. The signal that is measured in clamping is the current required to be passed to maintain Vc. The gain or amplification of the error signal feedback was increased until the current trace started to oscillate at which point the gain was slightly reduced to provide a cell tightly clamped at Vc. Unless otherwise stated the Vc was set at -60 mV which was close to resting potential for most cells. (For an overview of voltage clamping see Hille, 1984).

2.5.3 Steady-State I-V curves

If a cell is voltage clamped and Vc swept very slowly in a ramp-like fashion over a range of voltages and plotted against the measured current, then a steady-state I-V curve is produced. The shape of this curve reflects the sum of all the steady state conductance pathways that are active at each voltage and as such can give information about voltage dependent conductances. The effects of drugs on I-V curves can yield information about which conductances are being affected by the drug. The steady-state I-V curve only provides information about steady-state (i.e. long lasting) conductances, since fast, inactivating conductances (eg I_{Na} underlying action potentials) are quickly inactivated. For my experimental purposes the neurone was voltage clamped as described in 2.5.2. A function

generator (Feedback FG601) was connected to the clamp input of the Dagan 8500, such that, while in voltage clamp mode, the command potential could be altered continually in a ramp-like fashion. In this case Vc was altered by 2 mV per second over the membrane potential range of Vc $\pm$ 50 mV. As Vc was -60 mV this caused the membrane potential to be varied over the range of -10 mV to -110 mV.

2.5.4 Iontophoresis of cyclic AMP

Cyclic AMP was iontophoresed intracellularly during both voltage and current recording, both procedures using the same microelectrode configuration. Intracellular iontophoresis of cAMP allows the experimenter to control directly the amount of cAMP that passes into the cell, and provided that the electrode resistance does not change, the same iontophoretic current reliably iontophoreses the same amount of cAMP each time and thus the cell's responses to injected intracellular cAMP are repeatable. The voltage sensing microelectrode was prepared as described in 2.5.1. The current microelectrode was prepared from double-barrelled 1.5 mm fibre-filled glass capillary tubing (Clark Electromedical Instruments, Pangbourne, Reading). Using a dentist's drill, a hole about 5 mm long was drilled into one side of the microelectrode. This allowed the cAMP solution to be injected into one barrel without contaminating the other barrel. It was essential that the glass was clean, so it was placed in detergent-containing boiling water for 15 minutes and then rinsed in cold distilled water. It was then placed in an oven at 100 ^{o}C overnight to dry it thoroughly. To further ensure that there was no crossmixing of the solutions in the two barrels, the top of the cAMP containing barrel was sealed using high strength epoxy adhesive before filling

with cAMP. The "drilled" barrel contained 0.2 M adenosine 3'5'-cyclic monophosphate (cAMP) and 20 mM Tris buffer adjusted to pH 7.5 with 1 M KOH and the other barrel was filled with 3 M potassium acetate. The double-barrelled microelectrode was mounted in the electrode holder with silver wire connecting the potassium acetate containing barrel to the current injection probe of the Dagan 8500. A second silver wire was passed into the cAMP containing barrel via the drilled hole in the side. This was connected to a stimulator (Grass S48) via a stimulus isolation unit (Grass SIU5). Current pulses of 5 second duration and 1 - 5 μA were passed to iontophorese the cAMP. The current return was the second barrel of the microelectrode and as such no current crossed the cell membrane. For cAMP iontophoresis during voltage recording, both electrodes were inserted into the cell and identical voltage registrations were seen. The cAMP was iontophoresed by passing a current between the two barrels of the double barrelled microelectrode. The response of the membrane potential to the cAMP was recorded. For cAMP iontophoresis under voltage clamp the cell was voltage clamped as described in 2.5.2 and cAMP iontophoresed as described above, and the current response was recorded. In experiments where adenosine 5'-monophosphate (AMP) and guanosine 3'5'-cyclic monophosphate (cGMP) were iontophoresed the procedure was exactly the same as described above.

2.5.5 Pressure injection of pharmacological agents

During either current or voltage recording, a further, independent, microelectrode was used to inject compounds into the neurone using pressure ejection. The effect produced on the current or membrane potential could then be recorded. The pressure ejection

microelectrode was filled with a solution containing the compound to be injected, making sure that there were no air bubbles. The electrode was prepared such that it had a tip resistance of less than 5 Mohms after being broken back. It was then connected to a 20 cm length of polythene tubing which was in turn attached to a 60 ml plastic syringe. When viewed down a microscope (x20) it could be seen that depression of the plunger of the syringe caused drops of solution to be expelled from the tip of the microelectrode. Depression of the plunger of the syringe by the same amount produced roughly the same sized drop. Compounds which were pressure injected were calcium ions, ethyleneglycol-bis-(B-aminoethyl ether) N,N,N',N'-tetraacetic acid (EGTA), potassium chloride and pentylenetetrazol. The amount of the compound injected could be calculated if the concentration of the compound in solution and the diameter of the sphere of solution injected were known. The diameter of the sphere was measured using a calibrated eyepiece. The pressure ejection electrode was connected to a separate microelectrode amplifier via a microelectrode holder containing 2 M KCl, and its signal was monitored on a third channel of the oscilloscope, but not recorded permanently. Once the neurone had been impaled the compound was injected by applying pressure to the syringe plunger. Depression of the plunger by the same amount resulted in roughly the same sized response in the neurone (eg repeated injection of calcium ions induced a similar level of depolarisation and bursting activity in the B1 cell) and it was assumed that roughly the same amount of compound was being injected. Control injections of 0.1 M KCl induced no significant effects. The Ca^{2+} solution injected contained 0.1 M $CaCl_2$ / 0.1 M KCl in distilled water. The PTZ solution contained 7

M PTZ and 0.1 M KCl. The EGTA solution contained 250 mM EGTA and 250 mM MOPS in distilled water adjusted to pH 7.5 by the addition of 1 M KCl.

The effect on membrane potential of intracellular pressure injection of compounds was investigated by penetrating the neurone with two electrodes, a voltage sensing electrode as described in 2.5.1 and a pressure injection electrode as described above. For pressure injection into a voltage clamped cell, the cell was penetrated with 3 electrodes, the pressure injection electrode and 2 others to voltage clamp the cell as described in 2.5.2.

2.5.6. Simultaneous injection of cAMP and Ca^{2+} under voltage clamp. A neurone was impaled with three microelectrodes; a single and a double-barrelled microelectrode as described in 2.5.4., and an additional calcium containing microelectrode as described in 2.5.5. The purpose of this experiment was to investigate the effect of increased intracellular Ca^{2+} concentration on the neurone's response to iontophoresis of cAMP. The neurone was voltage clamped at -60mV and control pulses of cyclic AMP injected to ensure that the current response was the same for repeated pulses of cyclic AMP. Calcium ions were then intracellularly pressure injected until a current response of between 1 and 2 nA was obtained. This was used as a baseline against which the cAMP induced current was measured. Cyclic AMP iontophoresis was then superimposed on the calcium injection.

CHAPTER 3

RESULTS

Section I

Mode of action of PTZ in B1 and RPD1 cells of Lymnaea.

Introduction

In molluscan neurones there have previously been shown to be two types of response to the convulsant agent pentylenetetrazol (PTZ). Firstly, the neurone may be insensitive to PTZ and show no response. An example of this type of neurone is the I cell of the Japanese land snail Euhadra peliomphala (Sugaya et al, 1973). Secondly, the neurone may become excited by PTZ. This excitation usually consists of depolarisation of the cell membrane accompanied by superimposed bursts of action potentials. At higher concentrations of PTZ paroxysmal depolarising shift (PDS) may develop. Neurones displaying this type of behaviour include the D and H cells of Euhadra (Sugaya et al, 1973), the B1, B2 and B3 neurones of Helix (Speckman and Caspers, 1978), the LP1 6 neurone of Tritonia (Partridge, 1975) and the B1, B2 and B4 neurones of Lymnaea (McCrohan and Gillette, 1988b).

This study was initiated by completing a survey of the effect of PTZ on a variety of identified neurones in Lymnaea. The majority of cells examined displayed excitation in the presence of PTZ although a few cell types appeared to be insensitive to PTZ. The B1 cell was chosen for further study as it displayed classic epileptiform activity in the presence of PTZ and a considerable body of work had already been

carried out on this cell, including its response to PTZ (McCrohan and Gillette, 1988b). In the course of the survey, one cell type, RPD1, was found to be inhibited by PTZ. This was the first description of a cell whose activity was inhibited by PTZ. This in itself made it worthy of further study, but a comparison of the effects of PTZ on cells whose responses were opposite also gave an excellent opportunity to investigate PTZ's mode of action. Onozuka et al (1983) have suggested that PTZ's mode of action in the D neurone of the Japanese land snail Euhadra is mediated by release of intracellularly stored Ca^{2+}. This possibility was investigated for the B1 and RPD1 neurones.

Results

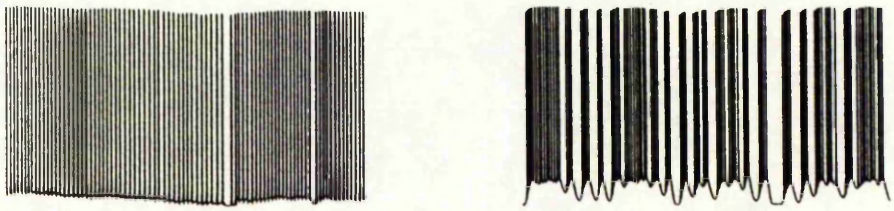
Responses of identified neurones to PTZ.

The response to PTZ of various cell types was examined. Figure 3.1 illustrates the response of cells in the right parietal ganglion. In A group cells, 20 mM PTZ induced bursting activity (Fig 3.1a). This consisted of a depolarisation of the cell membrane by up to 15mV accompanied by bursts of action potentials (n=27). In B group cells, 20 mM PTZ induced bursting activity, including depolarisation of the cell membrane by up to 10mV (Fig 3.1b, n=15). In C group cells, 20 mM PTZ induced a paroxysmal depolarising shift (PDS - see later for detailed description). The onset of the PDS was very rapid and depolarised the cell membrane by up to 45mV (Fig 3.1c, n=11).

In the RPD2 cell of the right parietal ganglion, 20 mM PTZ induced bursts of double or triple spikes. The afterhyperpolarisation following action potentials was abolished and the cell membrane

Fig 3.1. Effect of PTZ on the A group, B group and C cells of the right parietal ganglion. (a) Application of 20 mM PTZ induced bursting activity in an A group cell. (b) In a B group cell 20 mM PTZ also induced bursting activity. (c) In a C cell 20 mM PTZ induced a very rapid paroxysmal depolarising shift.

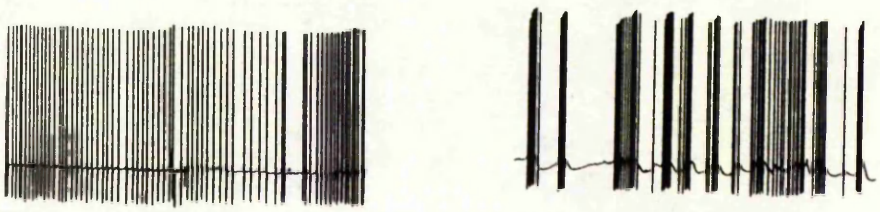
(a) A gp



Control

20 mM PTZ
2 mins

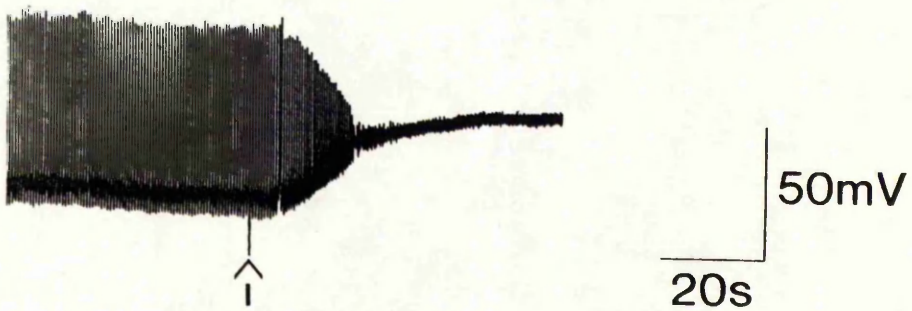
(b) B gp



Control

20 mM PTZ
4 mins

(c) C cell



20 mM PTZ

depolarised by up to 8mV (Fig 3.2a, n=18). A concentration of 20 mM PTZ induced bursting activity in the VD1 cell of the visceral ganglion. This consisted of depolarisation of the cell membrane by between 15 and 20mV accompanied by bursts of action potentials, interrupted by brief periods of repolarisation (Fig 3.2b, n=6). The effect of PTZ on the visceral yellow cells was to induce a PDS, but this took a considerable time to occur, up to 4 minutes in all cases (Fig 3.2c, n=3).

Responses of B1 and RPD1 to PTZ.

In the B1 cell, bath application of PTZ led to dose-dependent bursting activity. The B1 cell is usually quiescent with a resting membrane potential of about -60mV. A concentration of 20 mM PTZ led to sustained depolarisation of the cell membrane by 15.6 $\pm$ 3.7mV (mean $\pm$ SEM; n=27) accompanied by bursts of action potentials (Fig 3.3a). The bursts of action potentials were fairly evenly spaced and interrupted by periods of repolarisation of the cell membrane; however, this repolarisation did not return the membrane potential to control levels. The bursting activity lasted for as long as PTZ was present in the bath, and was reversible when PTZ was washed out (not shown). A higher concentration of PTZ (40 mM) induced a paroxysmal depolarising shift (PDS) in the B1 cell. This type of response is similar to that seen in mammalian neurones during epileptiform activity (Dichter, 1989). The PDS consisted of depolarisation of the cell membrane to a plateau 36.4 $\pm$ 8.7mV above resting potential (Fig 3.3b, n=19). At the beginning of the PDS, high frequency action potential firing was observed. However as the plateau was reached, action potential amplitude decreased until generation of action

Fig 3.2. Effect of PTZ on RPD2. VD1 and yellow cells. (a) In an RPD2 cell 20 mM PTZ induced double and triple bursts of action potentials. (b) In the VD1 cell 20 mM PTZ induced bursts of action potentials which were fairly regular in frequency. (c) Pentylenetetrazol (20 mM) induced a slow paroxysmal depolarising shift in the yellow cell.

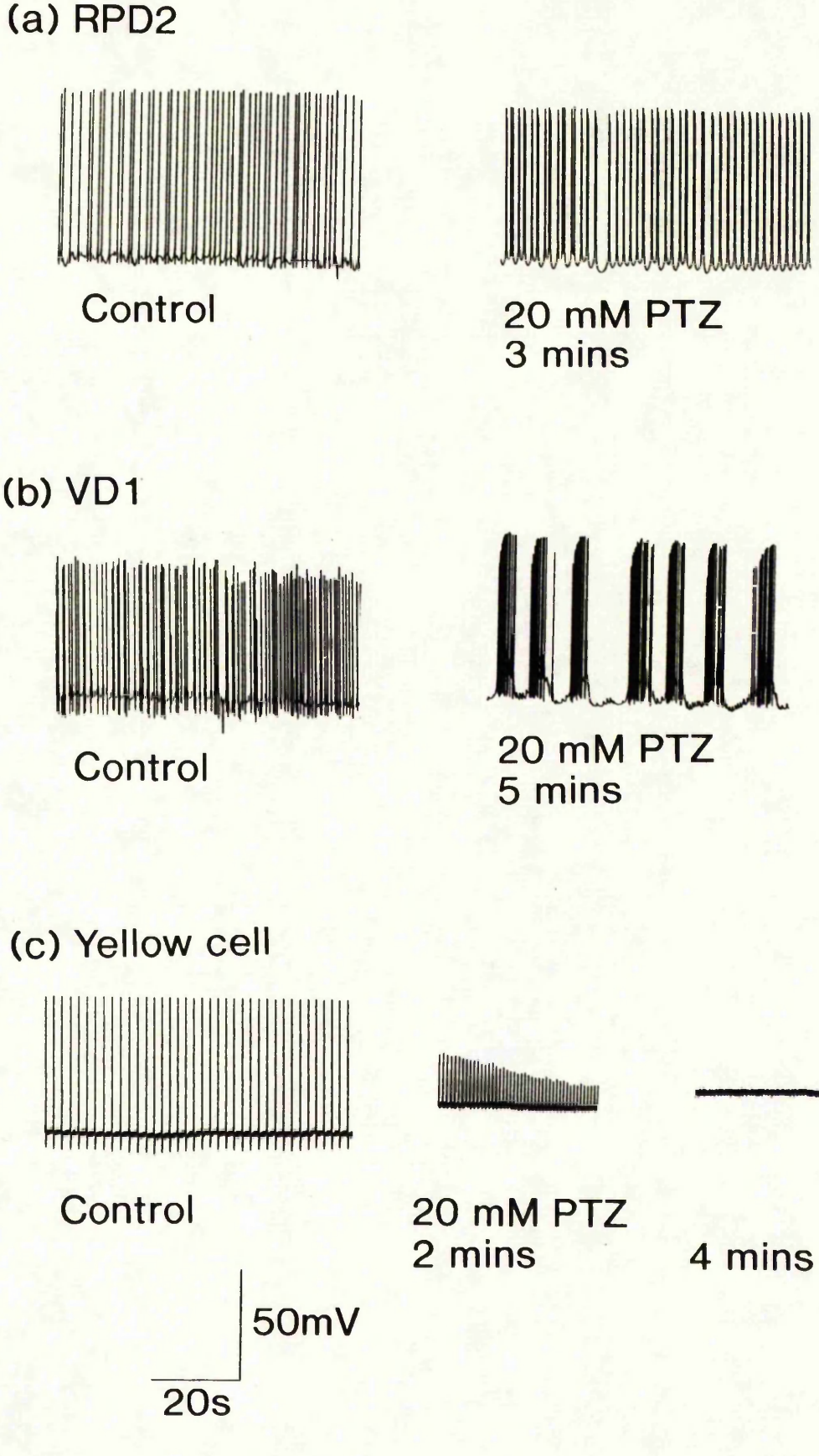
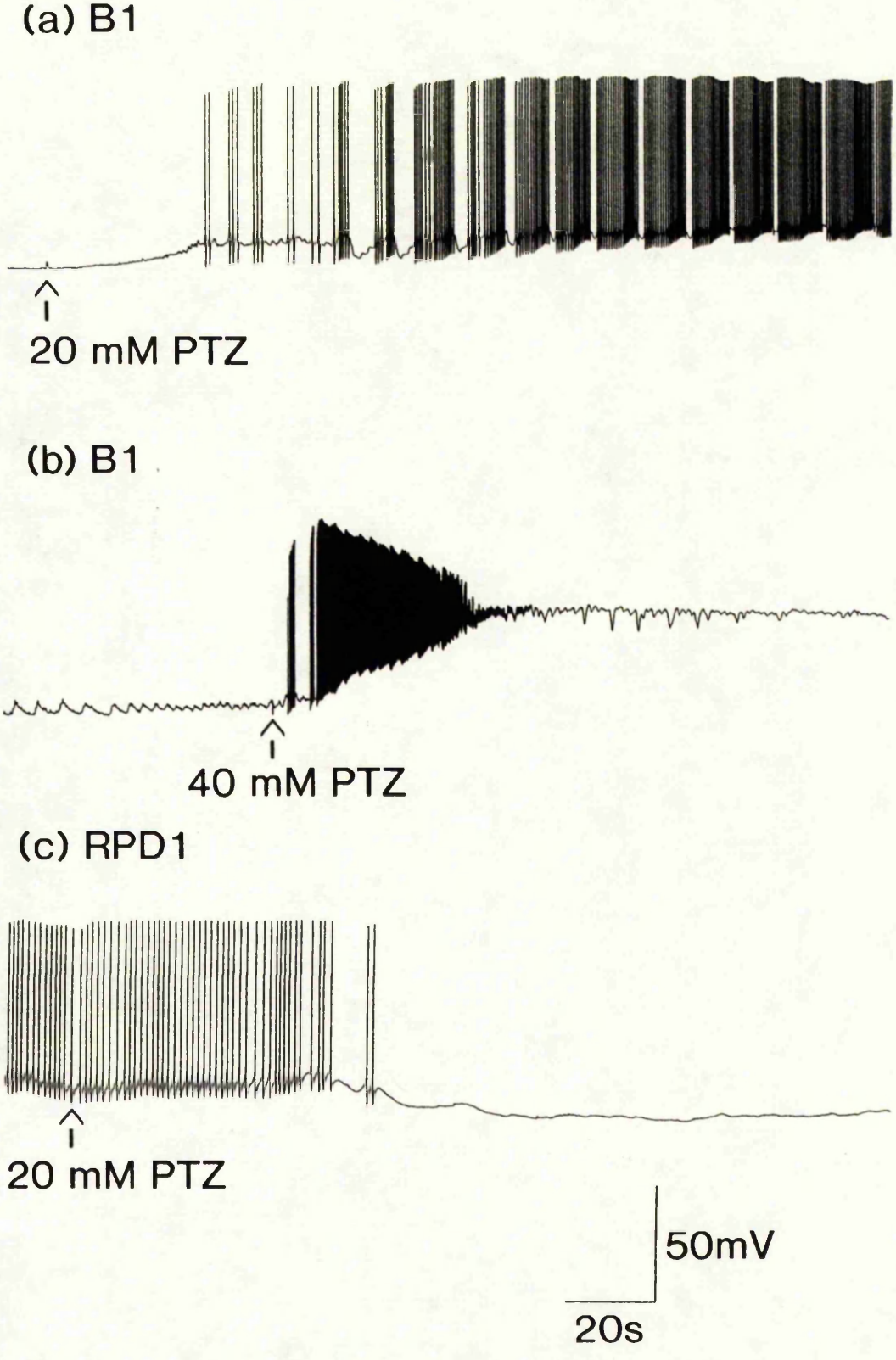

(a) RPD2
Control
20 mM PTZ
3 mins
(b) VD1
Control
20 mM PTZ
5 mins
(c) Yellow cell
Control
20 mM PTZ
2 mins
4 mins
50mV
20s

Fig 3.3. Effect of PTZ on B1 and RPD1 cells. (a) In the presence of 20 mM PTZ, the B1 cell exhibited bursting activity which consisted of depolarisation of the cell membrane accompanied by superimposed bursts of action potentials. (b) A slow depolarising shift developed on application of 40 mM PTZ to a B1 cell. (c) In the RPD1 cell, application of 20 mM PTZ inhibited the cell's endogenous pacemaker activity and induced an hyperpolarisation of the cell membrane. The arrows mark the onset of PTZ application.


(a) B1
20 mM PTZ
(b) B1
40 mM PTZ
(c) RPD1
20 mM PTZ
50mV
20s

potentials ceased altogether. In some cells the plateau was punctuated by brief (< 2 sec) hyperpolarisations, indicating synaptic inputs from other cells. Spontaneous repolarisation to the original resting potential, as has been described for many molluscan neurones treated with PTZ (e.g. visceral and parietal neurones of Lymnaea; Doerner et al, 1982), was never observed in B1 neurones, and the plateau was maintained indefinitely. This suggested that any mechanism for terminating or interrupting the PDS (e.g. a Ca^{2+}-dependent K^{+} channel) was absent in B1, or not sufficiently strong to be effective.

In the RPD1 cell, application of PTZ led to inhibition of spontaneous firing activity and hyperpolarisation of the cell membrane (Fig 3.3c). This effect was dose dependent, 20 mM PTZ being the lowest concentration which would produce these effects. A concentration of 20 mM PTZ led to hyperpolarisation of the cell membrane by 12.7 $\pm$ 2.7 mV (n=44). Higher concentrations of PTZ caused a decrease in the time for inhibition to begin, and produced a greater hyperpolarisation of the cell membrane (not shown).

Experiments were carried to investigate PTZ's mode of action in the two cell types, B1 and RPD1. The first of these experiments was to identify PTZ's site of action - whether it was acting extracellularly, intracellularly, or both. Intracellular pressure injection of PTZ, resulting in a calculated intracellular concentration of about 40 mM, produced no lasting effect in either cell type. In the B1 cell PTZ injection induced a transient burst of action potentials and a depolarisation of the cell membrane, but

these effects ceased as soon as the injection of PTZ was stopped (Fig 3.4a, n=3). In the RPD1 cell intracellular injection of PTZ resulted in a short-lived increase in firing activity but this rapidly died away (Fig 3.4b, n=3). In both cell types, after pressure injection had ceased the cells returned to their normal pattern of firing activity. The brief effects induced by PTZ suggest that it may be acting both extracellularly and intracellularly. However it should be noted that the response of RPD1 to injection of PTZ was opposite in polarity to that seen following extracellular application.

Role of extracellular Ca^{2+} ions in PTZ-induced effects.

Since extracellular Ca^{2+} ions have been implicated in the generation of seizures in mammalian preparations (Heinemann et al, 1977), the next line of study was to examine the dependence of PTZ's effects on extracellular Ca^{2+}. Application of PTZ was carried out in saline which had the Ca^{2+} concentration buffered to 1 uM by HEDTA, a Ca^{2+} chelator. Initially a saline with as little Ca^{2+} as possible was sought, but it was found that cells displayed low resting potentials (about -20mV) and were incapable of firing action potentials in saline which contained less than 1 uM Ca^{2+}. It appears therefore that cells need a basal level of Ca^{2+} in order to function normally. Saline containing Ca^{2+} buffered to 1 μM was termed 'low Ca^{2+} saline'. In low Ca^{2+} saline, PTZ produced responses in the B1 and RPD1 cells which were qualitatively indistinguishable from those seen in normal saline. In the B1 cell 20 mM PTZ caused depolarisation of the cell membrane by 22.8 $\pm$ 0.9mV, accompanied by bursts of action potentials (Fig 3.5a, n=5). In the RPD1 cell 20 mM PTZ caused hyperpolarisation of the cell membrane by

Fig 3.4. Effect of intracellular pressure injection of PTZ. (a) In the B1 cell a transient depolarisation of the cell membrane with a very short period of action potential firing occurred. These effects lasted only as long as the PTZ was being injected into the cell. (b) The RPD1 cell exhibited increased activity during the period of PTZ injection but this effect stopped on cessation of PTZ injection. The bars indicate periods of PTZ injection. See p.88 for control.

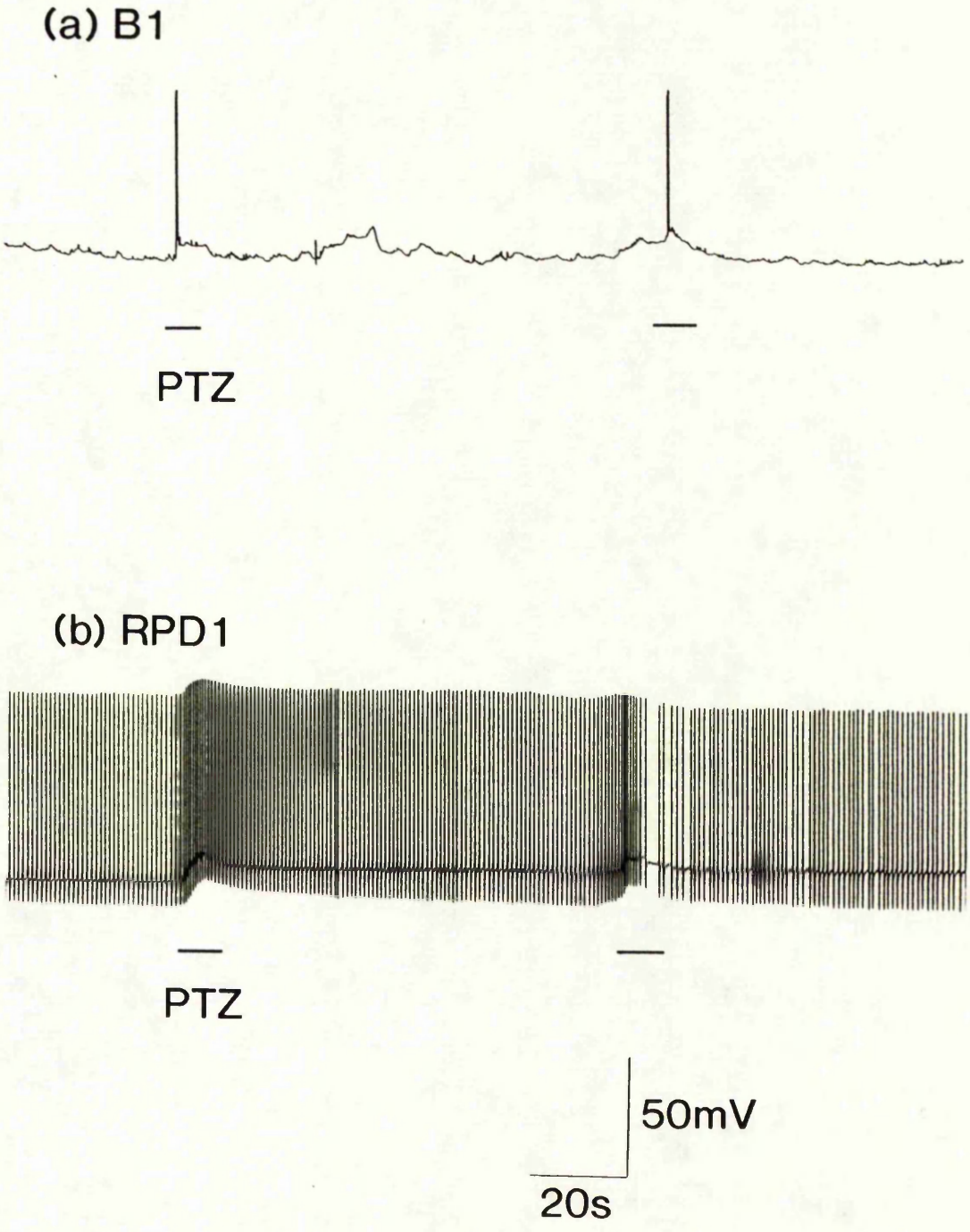
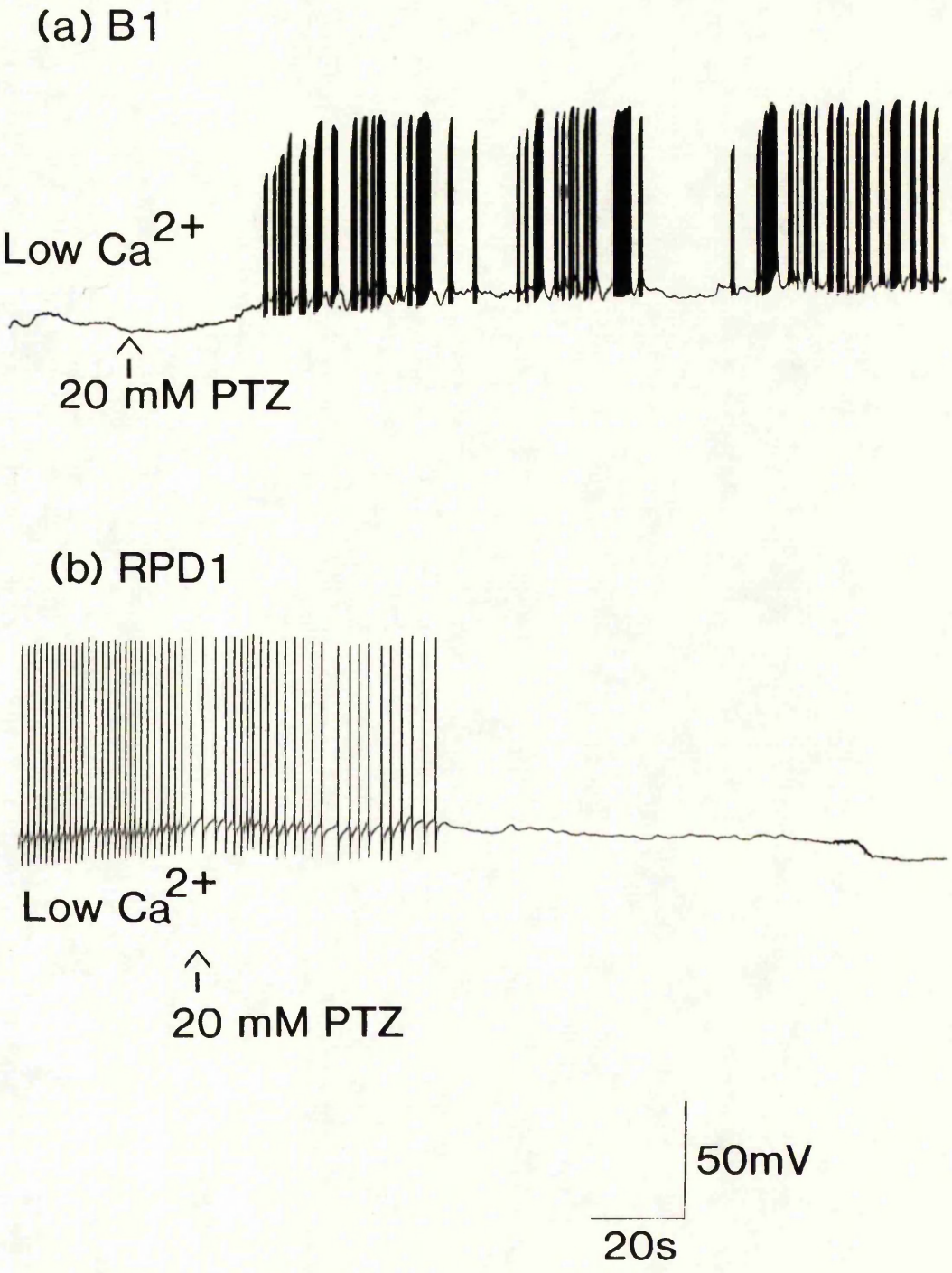

(a) B1
PTZ
(b) RPD1
PTZ
50mV
20s

Fig 3.5. Effects of PTZ in low Ca^{2+} saline. (a) In the B1 cell 20 mM PTZ induced a depolarisation of the cell membrane accompanied by superimposed bursts of action potentials. The bursts of action potentials were interspersed with periods of repolarisation. (b) In the RPD1 cell 20 mM PTZ inhibited the cell's inherent firing activity and caused hyperpolarisation of the cell membrane. The arrows mark the onset of PTZ application.

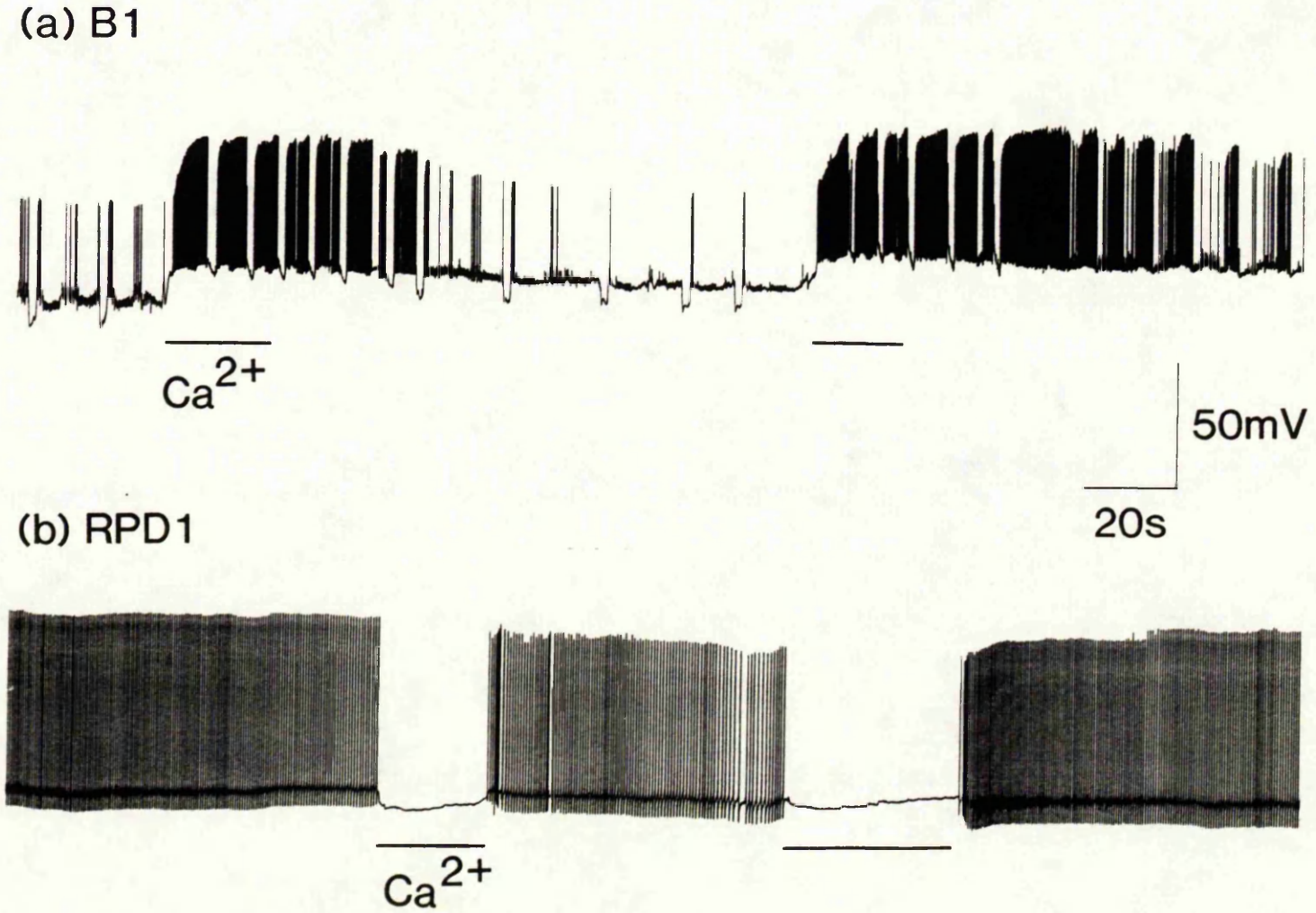

(a) B1
Low Ca^{2+}
20 mM PTZ
(b) RPD1
Low Ca^{2+}
20 mM PTZ
50mV
20s

13.7 ± 1.0mV, and inhibition of the cell's endogenous pacemaker activity (Fig 3.5b, n=5). The depolarisation produced by 20 mM PTZ in the B1 cell was greater than that seen in normal saline, suggesting that extracellular Ca^{2+} may partially inhibit PTZ-induced depolarisation in the B1 cell. The effect of PTZ on the RPD1 cell produced a similar magnitude of hyperpolarisation to that seen in normal saline indicating that extracellular Ca^{2+} does not affect PTZ-induced actions in the RPD1 cell. Thus it would appear that normal levels of extracellular Ca^{2+} are not necessary for PTZ to produce its effects in both cell types, and indeed extracellular Ca^{2+} may inhibit PTZ-induced depolarisation in the B1 cell.

Role of intracellular Ca^{2+} ions in PTZ-induced effects.

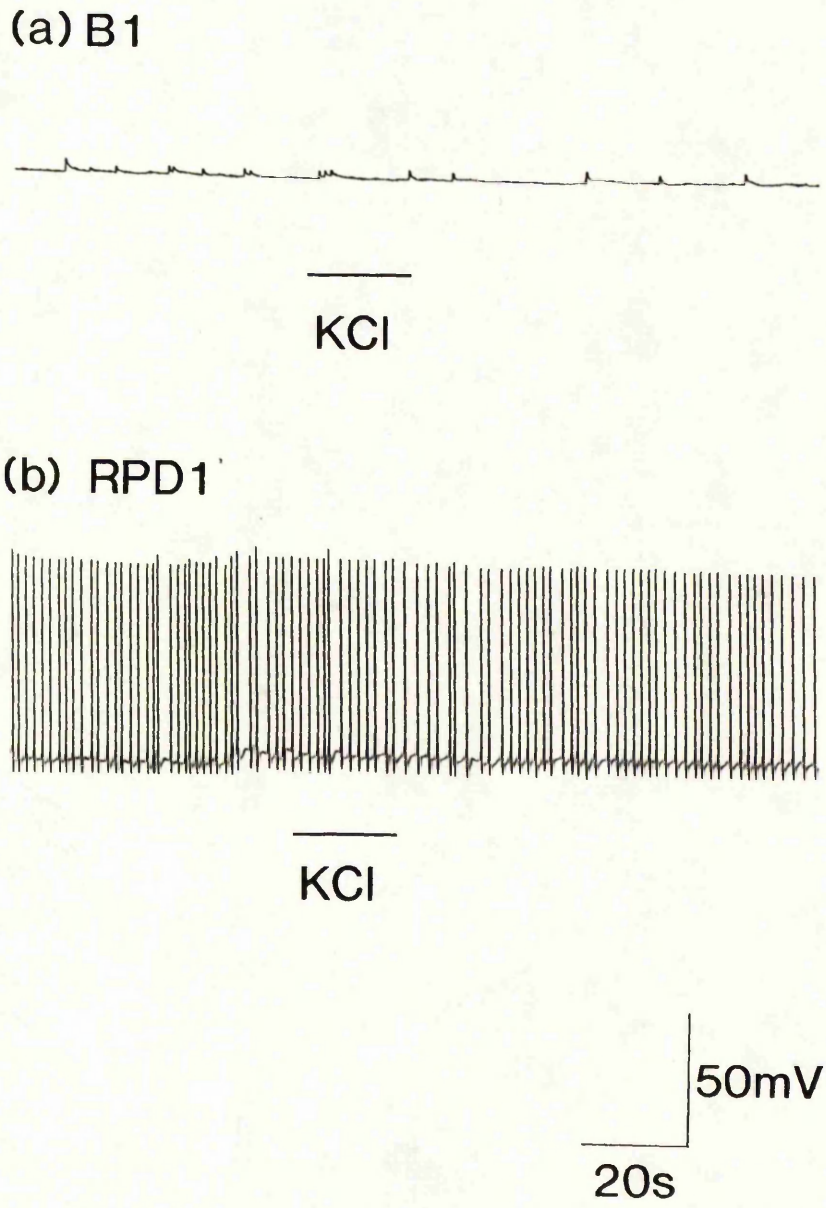
The effect of raising intracellular Ca^{2+} concentration was studied, since elevated intracellular Ca^{2+} levels have been reported in both mammalian and molluscan cells during epileptogenesis (Onozuka et al, 1989). Intracellular pressure injection of Ca^{2+} in pulses of 10 to 40 seconds duration led to responses in both cell types that were qualitatively similar to those induced by bath application of 20 mM PTZ. In the B1 cell, Ca^{2+} injection led to a depolarisation of the cell membrane of between 5 and 15mV, accompanied by bursts of action potentials (Fig 3.6a, n=8). In the RPD1 cell, intracellular pressure injection of Ca^{2+} led to an inhibition of the cell's pacemaker activity, and an hyperpolarisation of the cell membrane (Fig 3.6b, n=5). The response in RPD1 lasted only as long as the period of Ca^{2+} injection. The response in B1 was more long lasting, continuing for up to 300 seconds after the end of Ca^{2+} injection. Injection of KCl had no effect on either cell type (Fig 3.7a,b).

Fig 3.6. Effect of intracellular pressure injection of Ca^{2+} into the B1 and RPD1 cells. (a) In the B1 cell, injection of Ca^{2+} induced a depolarisation of the cell membrane which was accompanied by bursts of action potentials. This effect outlasted the period of Ca^{2+} injection. (b) Pressure injection of Ca^{2+} into the RPD1 cell resulted in an inhibition of the cell's inherent firing activity and caused a hyperpolarisation of the cell membrane. This stopped as soon as Ca^{2+} injection had stopped. The bars indicate periods of Ca^{2+} injection.


(a) B1
Ca2+
50mV
20s
(b) RPD1
Ca2+

0·1M

Fig 3.7. Control experiments were carried out in which KCl was pressure injected into the B1 and RPD1 cells. Intracellular pressure injection of KCl produced no apparent effect in either the B1 cell (a) or the RPD1 cell (b). The bars indicate periods of pressure injection.


(a) B1
KCl
(b) RPD1
KCl
50mV
20s

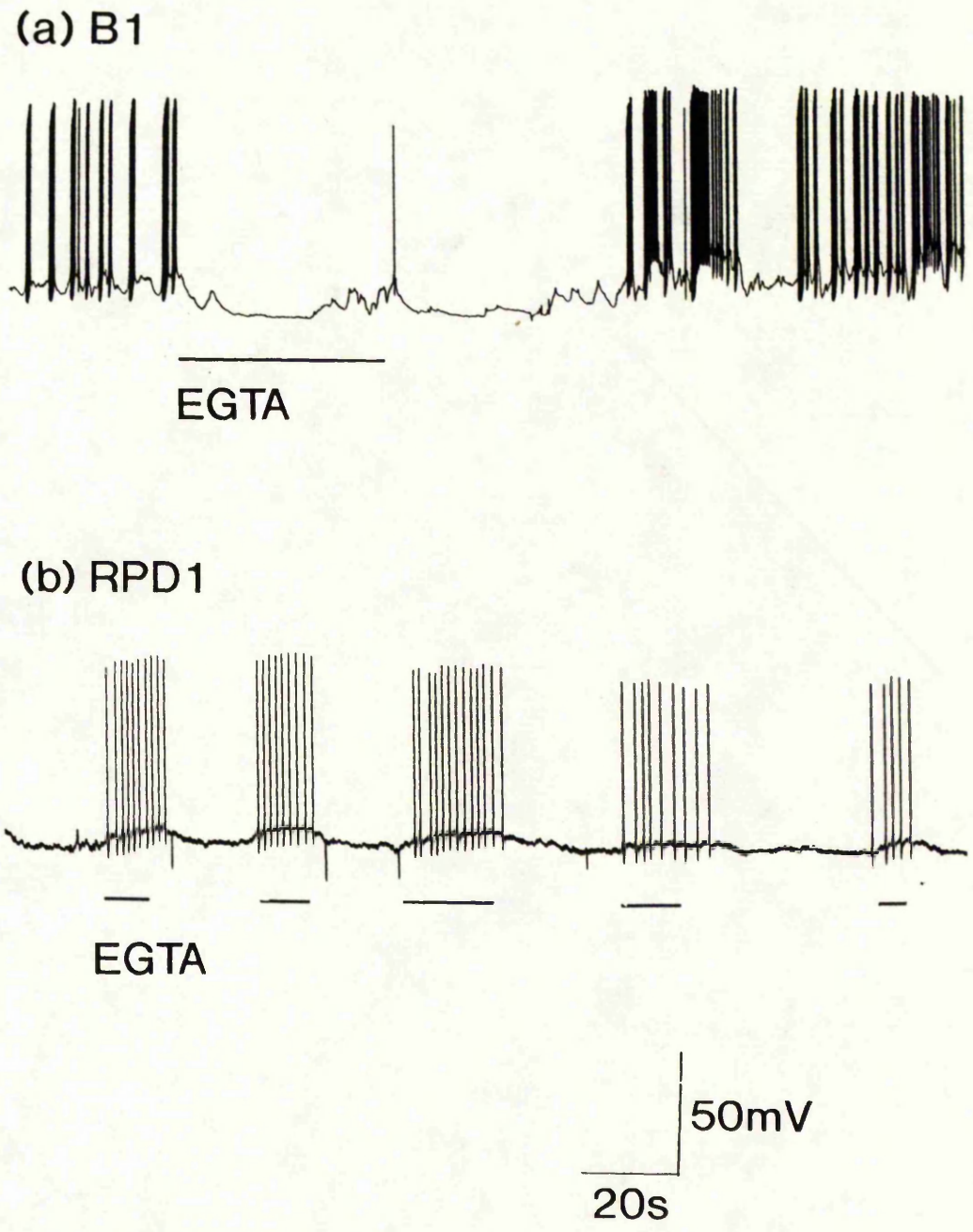
These results indicate that intracellular injection of Ca^{2+} produces apparently the same effects as extracellular application of PTZ, raising the possibility that PTZ may be acting in both cell types via an increase in intracellular Ca^{2+} concentration. To follow this line of study, the next step was to reduce intracellular Ca^{2+} levels during PTZ-induced effects in both cell types.

In both types of neurone, intracellular pressure injection of EGTA, the Ca^{2+} chelator, abolished PTZ-induced effects. In the B1 cell EGTA injection led to an hyperpolarisation of the cell membrane and cessation of bursting activity which had been induced in the cell by previous application of 20 mM PTZ (Fig 3.8a, n=5). In the RPD1 cell, EGTA injection led to depolarisation of the cell membrane and reintroduced action potential firing in a cell pretreated with PTZ (Fig 3.8b, n=6). In both cell types the response to EGTA injection outlasted the duration of EGTA injection. These results reinforce the findings illustrated in Fig 3.6, that increased intracellular Ca^{2+} concentration may underlie at least part of PTZ's action. If this is the case, where does this Ca^{2+} come from? There are two possible sources, release of Ca^{2+} from intracellular stores, and Ca^{2+} influx across the neuronal membrane. To investigate the first of these possibilities the compound TMB-8 was used.

The compound 3,4,5-trimethoxybenzoic acid 8-(diethylamino)-octyl ester (TMB-8) has been reported to block the release of Ca^{2+} from intracellular stores in rat pancreatic acini (Ikeda et al, 1984). Application of TMB-8 inhibited PTZ-induced effects in both cell types, with a delay of about 10 minutes. In the B1 cell, PTZ-induced

to 1 mM cell concentration.

Fig 3.8. Effect of intracellular pressure injection of $EGTA_n$ in B1 and RPD1 cells pretreated with 20 mM PTZ. (a) Application of 20 mM PTZ to the B1 cell induced bursting activity. Subsequent injection of EGTA resulted in a suppression of this bursting activity and repolarisation of the cell membrane. The effect continued for up to a minute after the injection of EGTA had stopped. (b) In an RPD1 cell which had been silenced by application of 20 mM PTZ, intracellular pressure injection of EGTA resulted in depolarisation of the cell membrane and restoration of firing activity. The bars indicate periods of intracellular EGTA pressure injection.



bursting activity was abolished by 0.2 mM TMB-8 and the membrane potential repolarised to control levels (Fig 3.9a, n=5). In the RPD1 cell, spontaneous firing activity was restored by 0.2 mM TMB-8 in a cell previously treated with PTZ, but the pattern of firing was less regular than before treatment with PTZ (Fig 3.9b, n=5). The reversal of PTZ's effects by TMB-8 suggests that PTZ may act via the release of intracellularly stored Ca^{2+}.

The effect on PTZ's effects of blocking Ca^{2+} influx from the extracellular medium was investigated by adding 2 mM $CoCl_2$ to normal saline. It has been reported by Byerly et al (1986) that addition of divalent cations in the mM concentration range inhibits Ca^{2+} currents in Lymnaea neurones. In the presence of 2 mM Co^{2+} ions, bath application of 20 mM PTZ induced depolarisation of the cell membrane accompanied by bursts of action potentials. This effect, however, was short lived and died away within 10 minutes (Fig 3.10a, n=5). This result indicates that influx of Ca^{2+} is necessary for PTZ-induced bursting activity to be maintained indefinitely in the B1 cell. In the RPD1 cell 20 mM PTZ induced hyperpolarisation of the cell membrane and inhibited the cell's endogenous firing activity. This response however, was sustained (Fig 3.10b, n=5). There is therefore a discrepancy in the effect of blocking Ca^{2+} influx in B1 compared to RPD1. In the B1 cell the results imply that extracellular Ca^{2+} is not necessary for PTZ to initiate bursting activity, but that it is needed for the maintenance of the effect. An explanation for this is that PTZ causes the release of Ca^{2+} from intracellular stores, which induces bursting activity in the cell. Once these stores are depleted they are re-filled by Ca^{2+} influx

Fig 3.9. Effects of a blocker of intracellular Ca^{2+} release on PTZ-induced effects in the B1 and RPD1 cells. (a) The normally quiescent B1 cell developed bursting activity in the presence of 20 mM PTZ as previously described. Subsequent application of 0.2 mM TMB-8 resulted in an inhibition of this bursting activity and repolarisation of the cell membrane. (b) In an RPD1 cell silenced by PTZ, inherent firing activity was restored by 0.2 mM TMB-8. This reversal of PTZ-induced effects took about 10 minutes in both cell types.

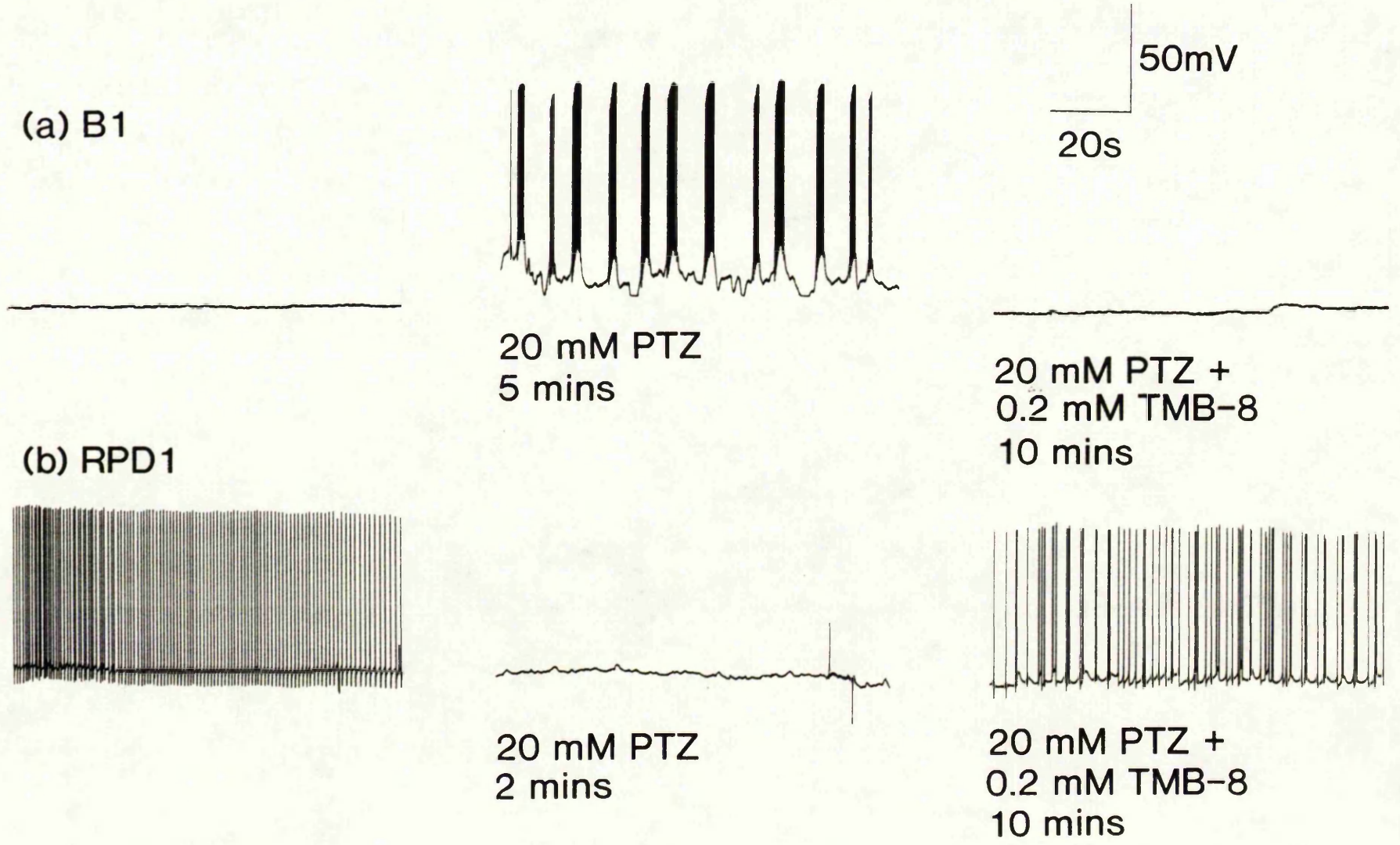
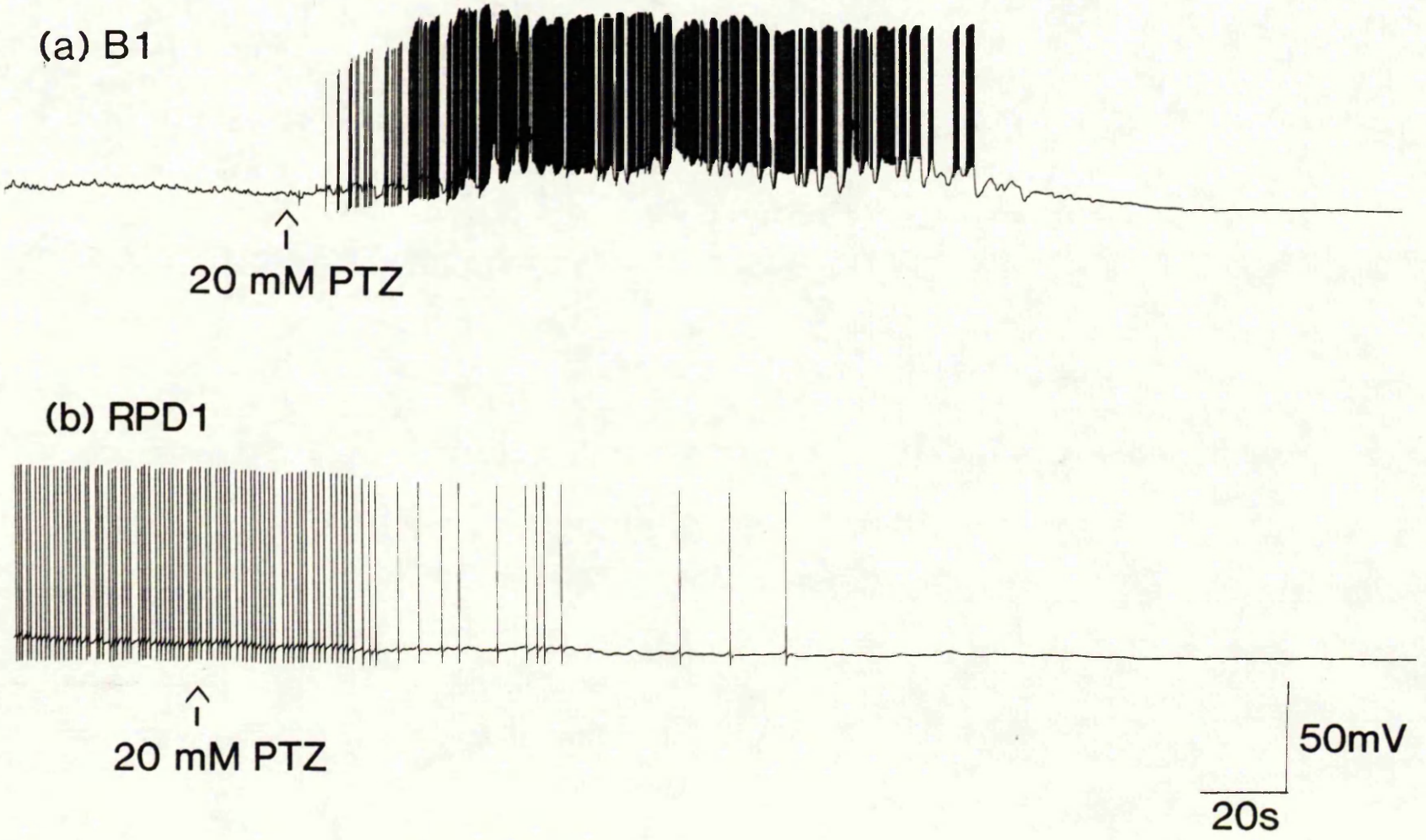

50mV
20s
(a) B1
20 mM PTZ
5 mins
20 mM PTZ +
0.2 mM TMB-8
10 mins
(b) RPD1
20 mM PTZ
2 mins
20 mM PTZ +
0.2 mM TMB-8
10 mins

Fig 3.10. Effects of PTZ on the B1 and RPD1 cells in saline containing 2 mM $CoCl_2$. (a) In the B1 cell, 20 mM PTZ induced a short lived depolarisation of the cell membrane accompanied by bursts of action potentials. This effect however did not persist and the membrane repolarised and bursts of action potentials ceased after a period of less than 10 minutes. (b) In the RPD1 cell 20 mM PTZ induced a membrane hyperpolarisation and inhibited the cell's inherent pacemaker activity. This effect was long lasting and did not reverse. The arrows mark PTZ application.

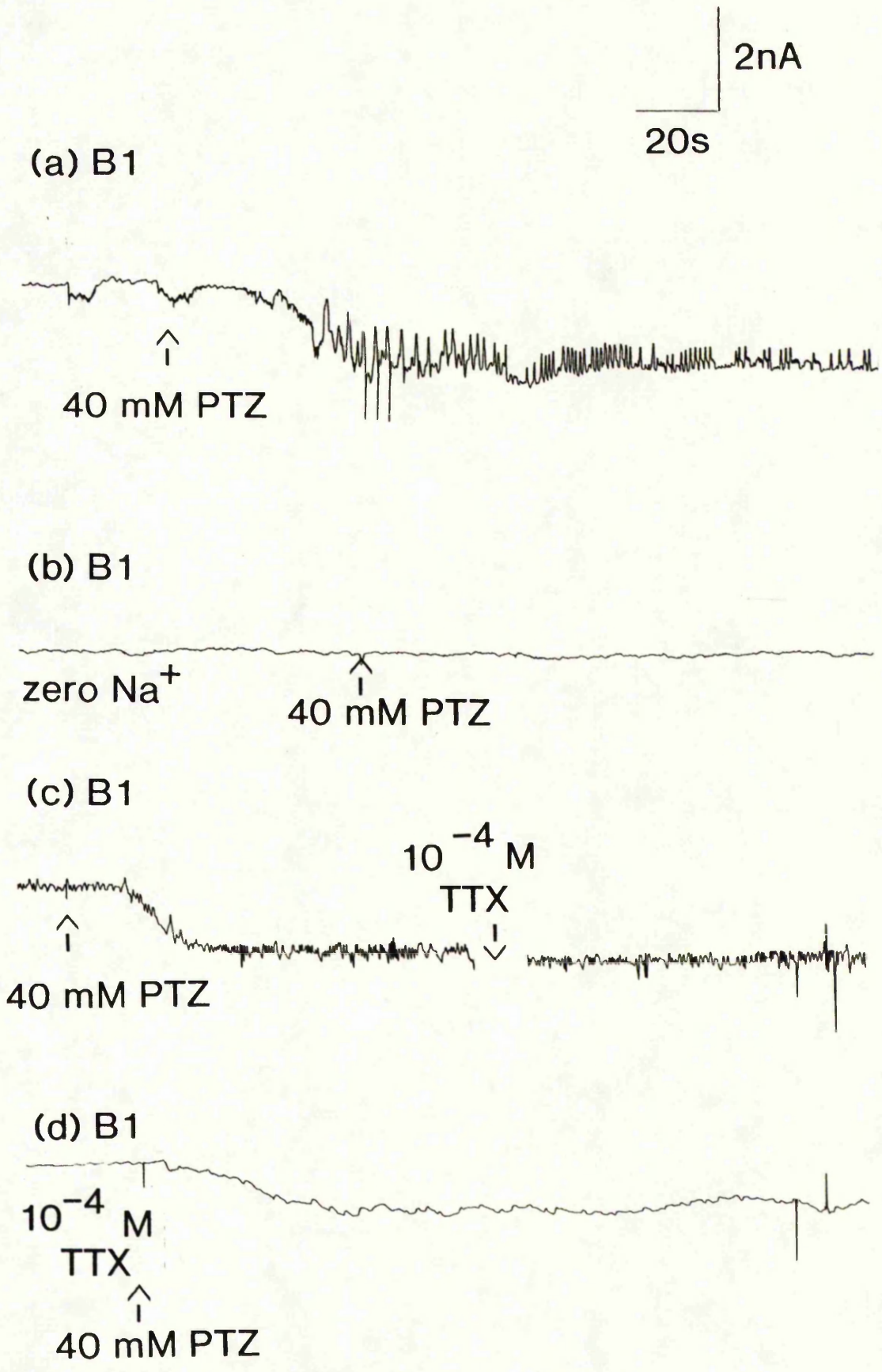

(a) B1
20 mM PTZ
(b) RPD1
20 mM PTZ
50mV
20s

from the extracellular medium. If this influx is blocked (in this case by Co^{2+}) then the stores are unable to refill and the bursting activity stops. An alternative explanation must be sought however for the effects in the RPD1 cell.

Membrane conductances induced by PTZ in B1.

The responses to PTZ in B1 and RPD1 are likely to be produced by activation of specific conductance pathways across the neuronal membrane. In the next series of experiments the currents induced by PTZ were studied under voltage clamp conditions. In the B1 cell, held under voltage clamp at a potential of -60mV, application of PTZ led to a slow inward current. The time taken for the current to appear varied from 20 to 60 seconds after the onset of PTZ application, and the time taken for the current to reach its peak amplitude varied from 60 to 120 seconds. Application of 40 mM PTZ induced an inward current of 3.13 $\pm$ 1.02 nA (Fig 3.11a, n=7). This current lasted for as long as PTZ was present in the bath. Synaptic currents were superimposed on the current induced by PTZ, suggesting increased activity in presynaptic cells. When this experiment was repeated in zero Na^+ saline, 40 mM PTZ induced no inward current (Fig 3.11b, n=5), indicating that the inward current induced by PTZ is either directly carried by Na^+, or at least dependent on extracellular Na^+. The PTZ induced inward current in B1 was found to be insensitive to tetrodotoxin (TTX). Application of 10^{-4} M TTX had no effect on the PTZ-induced inward current (Fig 3.11c, n=3). Furthermore prior application of 10^{-4} M TTX did not prevent PTZ from inducing an inward current in the B1 cell (Fig 3.11d, n=3). This distinguishes the current from the fast TTX-sensitive Na^+ current

Fig 3.11. Characteristics of the inward current induced by PTZ in the B1 cell under two electrode voltage clamp; holding potential -60mV. (a) Application of 40 mM PTZ induced an inward current in the B1 cell. (b) In zero Na^{2+} saline (sodium chloride substituted by equimolar arginine hydrochloride), application of 40 mM PTZ was unable to induce an inward current. (c) The inward current induced by 40 mM PTZ was insensitive to 0.1 mM TTX. (d) Prior application of 0.1 mM TTX did not prevent 40 mM PTZ from inducing an inward current. The arrows mark the onset of drug application.


2nA
20s
(a) B1
40 mM PTZ
(b) B1
zero Na^+
40 mM PTZ
(c) B1
10^{-4} M
TTX
40 mM PTZ
(d) B1
10^{-4} M
TTX
40 mM PTZ

associated with the rising phase of the action potential (Narahashi et al, 1964).

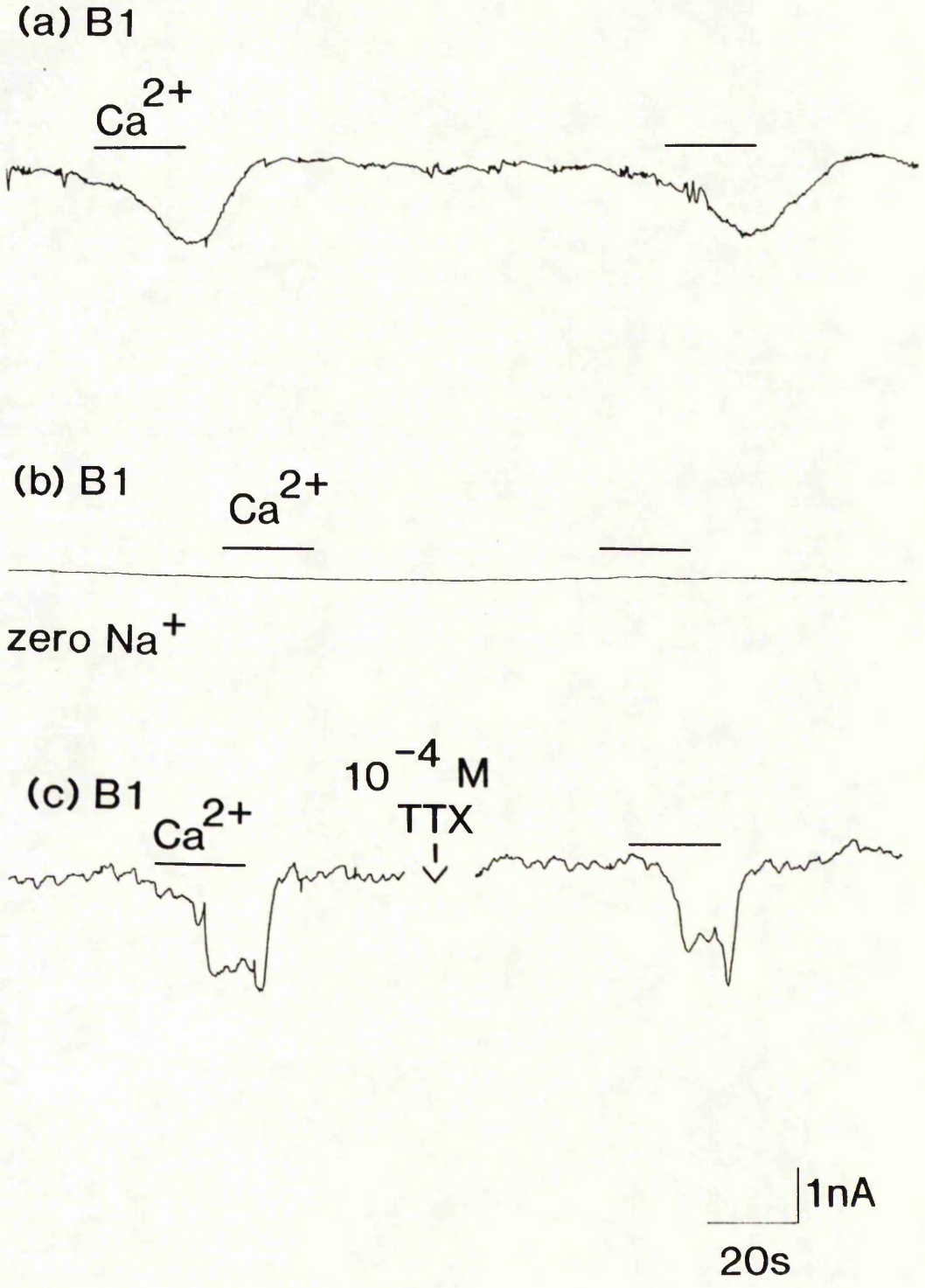
Membrane conductances induced by Ca^{2+} injection in B1.

Since Ca^{2+} injection mimicked PTZ's effects on firing activity, the current induced in B1 by injection of Ca^{2+} ions was studied to compare it with the current induced by PTZ. Intracellular pressure injection of Ca^{2+} into the B1 cell induced a repeatable slow inward current. The amplitude of the current produced depended on the amount of Ca^{2+} injected and was in the range 1.5 to 2.5 nA. This current was transient and started to decay soon after the injection of Ca^{2+} had ceased (Fig 3.12a, n=5). When this experiment was repeated in zero Na^{+} saline, intracellular Ca^{2+} injection did not induce an inward current (Fig 3.12b, n=5). This indicates that the inward current induced by Ca^{2+} injection was probably carried by Na^{+}. Like the PTZ-induced inward current, the current induced by Ca^{2+} injection was insensitive to 10^{-4} M TTX (Fig 3.12c, n=5). These results indicate similarities between the currents induced by PTZ and Ca^{2+}. They are both inward, dependent on Na^{+}, and TTX-insensitive. This provides fairly strong evidence that they are in fact the same current, supporting the hypothesis that PTZ acts in the B1 cell via an increase in intracellular Ca^{2+}.

Membrane conductances induced by PTZ in RPD1.

In the RPD1 cell the most likely cause of the hyperpolarisation induced by PTZ is an efflux of K^{+}. To investigate this possibility a range of K^{+} channel blockers was used to see whether they inhibited PTZ-induced responses in the RPD1 cell. Inhibitors of K^{+}

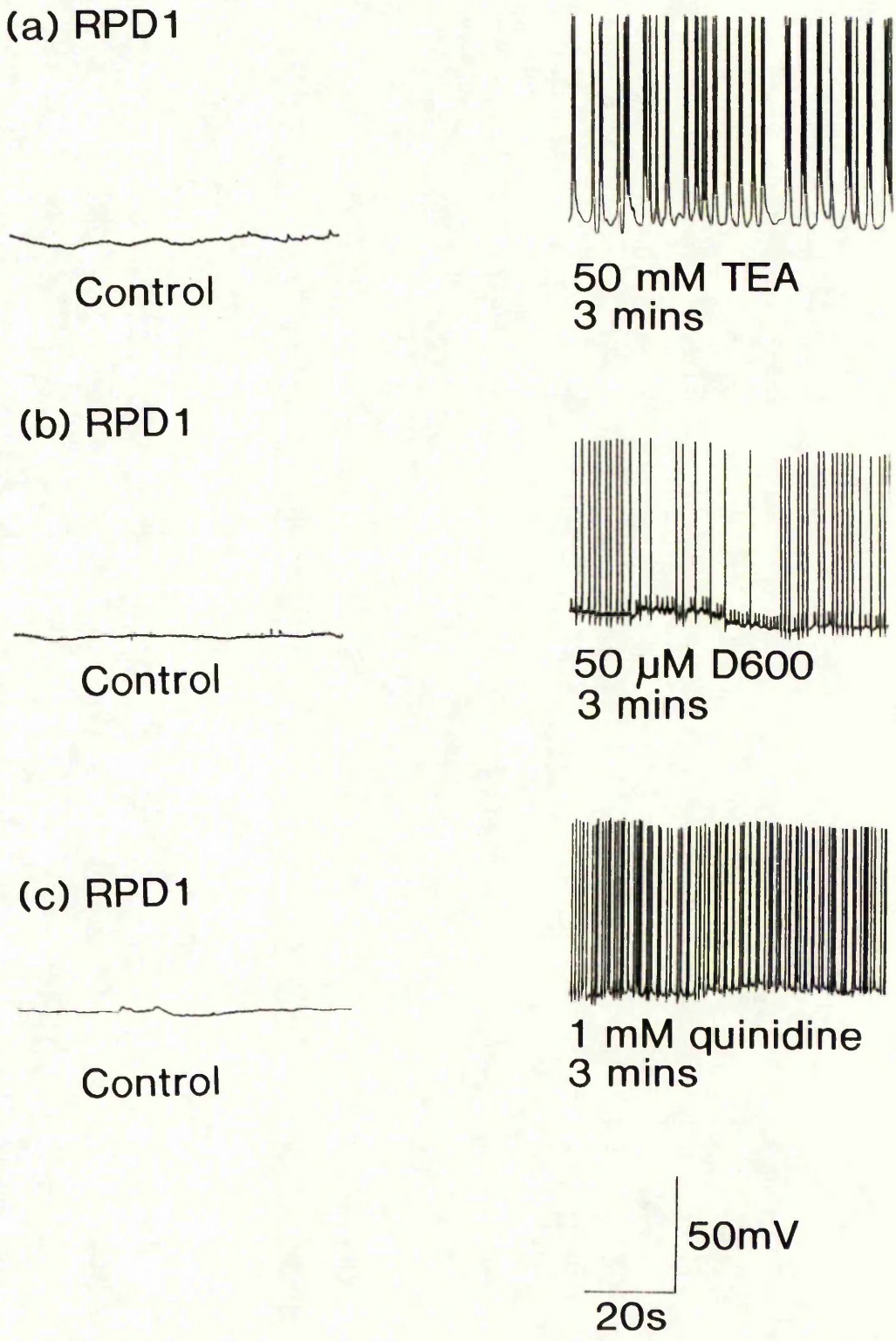
Fig 3.12. (a) Intracellular pressure injection of Ca^{2+} into a voltage clamped B1 cell induced a repeatable inward current. The cell was clamped at a holding potential of -60mV. This current began to decay soon after the injection of Ca^{2+} had ceased. (b) Injection of Ca^{2+} did not induce an inward current in zero sodium saline. (c) The inward current induced by Ca^{2+} injection was insensitive to 0.1 mM TTX. The arrows indicate drug application and the bars indicate Ca^{2+} injection.


(a) B1
Ca2+
(b) B1
Ca2+
zero Na+
(c) B1
Ca2+
10−4 M
TTX
1nA
20s

channels, and more specifically inhibitors of Ca^{2+}-dependent K^+ channel, abolished PTZ-induced effects in the RPD1 cell. The non-specific K^+ channel blocker tetraethylammonium (TEA) restored firing activity in cells previously treated with PTZ (Fig 3.13a, n=5), with a delay of between 1.5 and 2 minutes. As would be expected of a compound which blocks the delayed rectifier (voltage dependent K^+ channel) the action potential duration was greatly increased, some action potentials lasting as long as 1 second. Iproveratril, or D600, is a derivative of the Ca^{2+} channel antagonist verapamil (Gola and Ducreux, 1985). It has been reported to specifically block the Ca^{2+}-dependent K^+ channel in molluscan neurones at a concentration of 50 µM (Gola and Ducreux, 1985). Application of 50 µM D600 to an RPD1 cell previously treated with PTZ resulted in depolarisation and generation of action potentials (Fig 3.13b, n=5) after 4 and 6 minutes, although the action potential frequency did not return to its control level. This suggests that the hyperpolarisation induced by PTZ in the RPD1 cell is indeed via an efflux of K^+ through a Ca^{2+}-dependent K^+ channel. Additional inhibitors of the Ca^{2+}-dependent K^+ channel were studied to try to confirm this finding.

Quinidine has been reported to inhibit the Ca^{2+} dependent K^+ channel in molluscan neurones (Hermann and Gorman, 1984). Application of 1 mM quinidine caused a reversal of PTZ-induced effects in the RPD1 cell after 2 minutes. The cell depolarised and action potential firing was restored to its original level (Fig 3.13c, n=6). Thus inhibitors of the Ca^{2+}-dependent K^+ channel block PTZ-induced hyperpolarisation in the RPD1 cell. To look at this in more detail

Fig 3.13. Effect of K^+ channel blockers on PTZ-induced inhibition in the RPD1 cell. In all three recordings the RPD1 cell was initially inhibited by application of 20 mM PTZ. (a) The inhibition induced by PTZ was reversed by 50 mM TEA. Action potentials were prolonged (b) The compound D600 caused reversal of PTZ's effects in the RPD1 cell leading to reintroduction of action potential firing. (c) Quinidine caused a reintroduction of action potential in the RPD1 cell.


(a) RPD1
Control
50 mM TEA
3 mins
(b) RPD1
Control
50 µM D600
3 mins
(c) RPD1
Control
1 mM quinidine
3 mins
50mV
20s

the currents induced by PTZ and Ca^{2+} were studied under voltage clamp.

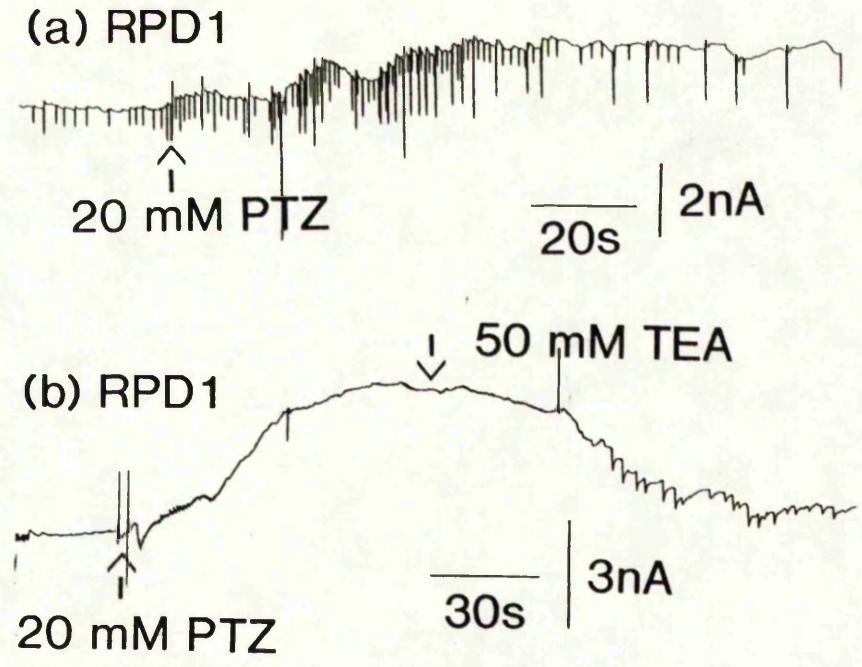
Application of 20 mM PTZ to a voltage clamped RPD1 cell, held at -60 mV, induced a slow outward current of 2.02 $\pm$ 0.47 nA in amplitude (Fig 3.14a, n=3). The current took between 30 and 60 seconds to appear and the time taken to reach its peak amplitude varied between 60 and 120 seconds. The current continued for as long as PTZ was present and was reversible on washout of PTZ (not shown). This outward current was abolished by treatment with 50 mM TEA (Fig 3.14b, n=3), indicating that it was carried by K^+. Prior application of 50 µM D600 prevented PTZ from inducing an outward current (Fig 3.14c, n=3), as did prior application of 1 mM quinidine (Fig 3.14d, n=3). This strongly suggests that the current induced by PTZ is a Ca^{2+}-dependent K^+ current.

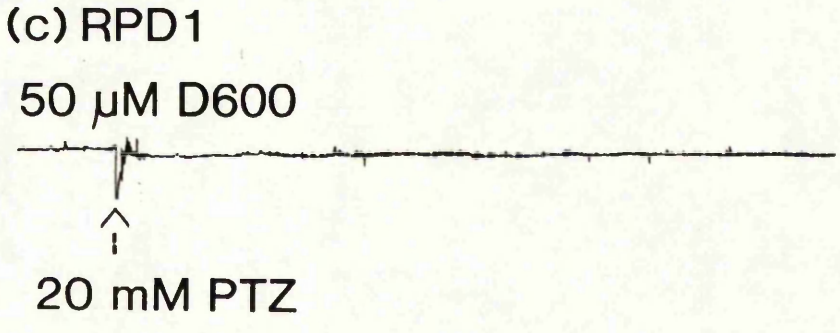
It could be argued that application of D600 and quinidine may cause an inward current which would tend to oppose the outward current induced by PTZ, and thus make it appear that these inhibitors were directly blocking a K^+ through Ca^{2+}-dependent K^+ channels. However application of D600 (Fig 3.15a, n=3) and quinidine (Fig 3.15b, n=3) did not induce an inward current; therefore this possibility can be discounted.

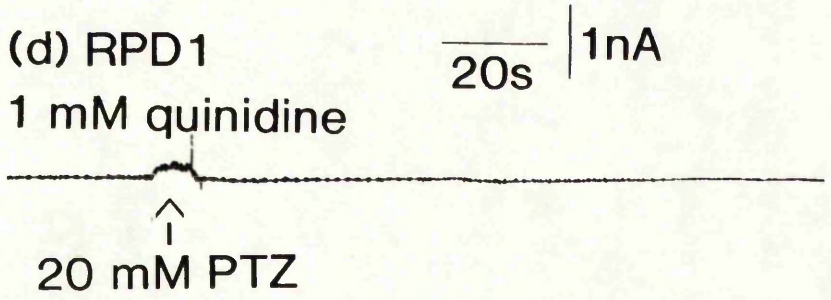
Membrane currents induced by Ca^{2+} injection in RPD1.

Intracellular pressure injection of Ca^{2+} induced a repeatable slow outward current in RPD1. The current amplitude depended upon the amount of Ca^{2+} injected but varied between 1 and 2 nA, and decayed

Fig 3.14. Effects of PTZ application on the RPD1 cell under voltage clamp (holding potential -60mV). (a) Application of 20 mM PTZ induced an outward current in RPD1. (b) The outward current induced by PTZ was inhibited by subsequent addition of 50 mM TEA, suggesting that this PTZ-induced outward current is caused by an efflux of K^+ through K^+ channels. (c) Prior application of 50 μM D600 prevented PTZ from inducing an outward current, as did prior application of 1 mM quinidine (d). The arrow marks the onset of drug application.


(a) RPD1
20 mM PTZ
20s
2nA
50 mM TEA
(b) RPD1
30s
3nA
20 mM PTZ


(c) RPD1
50 µM D600
20 mM PTZ


(d) RPD1
20s
1nA
1 mM quinidine
20 mM PTZ

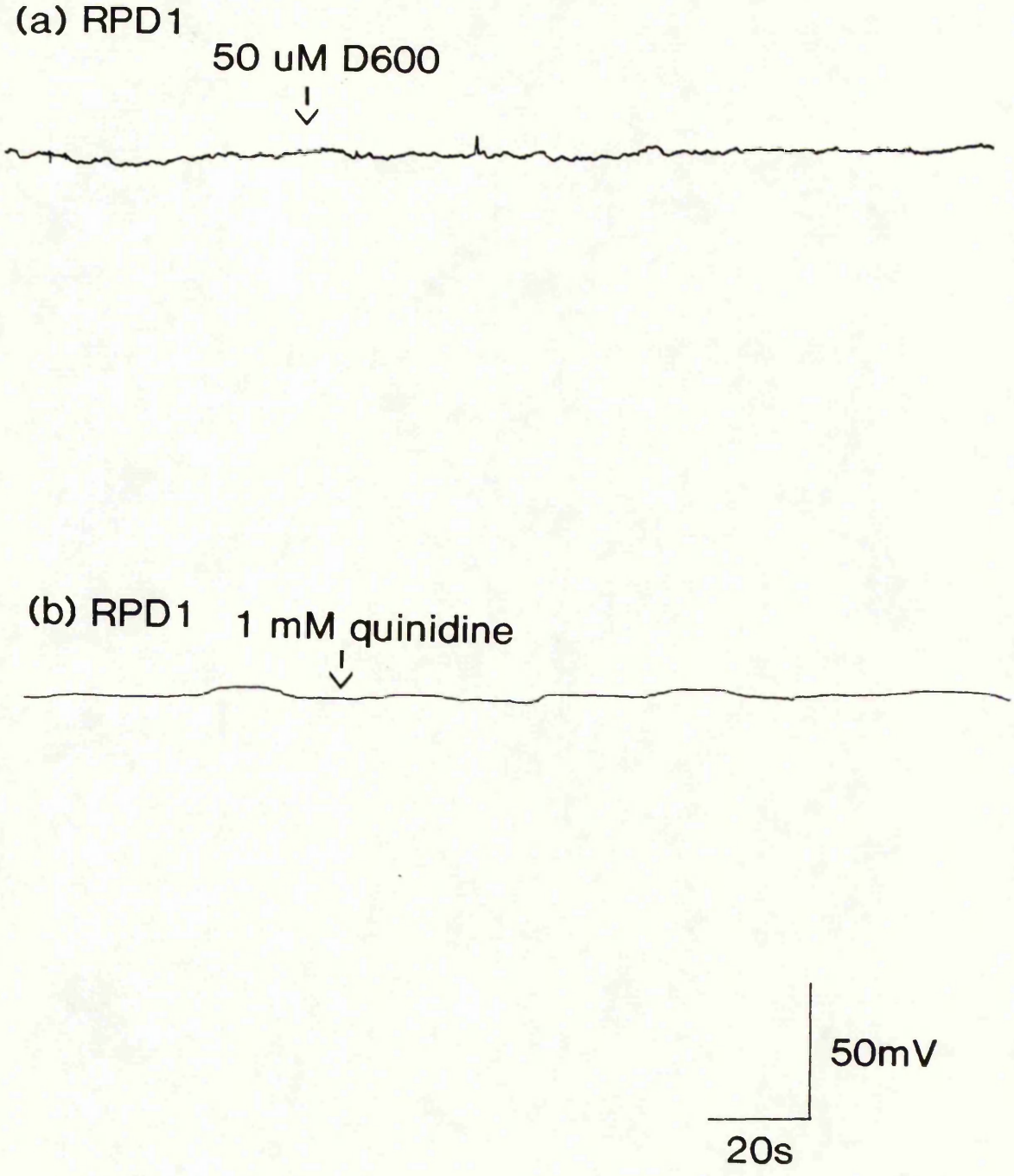


Fig 3.15. Application of 50 μM D600 (a) and 1 mM quinidine (b) did not induce any current response in voltage clamped RPD1 cells (holding potential -60mV). The arrows mark the onset of drug application.

soon after Ca^{2+} injection had ceased. It took between 15 and 30 seconds to decay to control levels (Fig 3.16a, n=5). The outward current induced by Ca^{2+} pressure injection was abolished when 50 μM D600 was added to the bath (Fig 3.16b, n=5) These results indicate that PTZ causes activation of an outward K^+ current, specifically via a Ca^{2+} dependent K^+ channel. The outward current induced by Ca^{2+} also appears to occur through a Ca^{2+} dependent K^+ channel. This suggests that in the RPD1 cell PTZ acts via intracellular Ca^{2+} to activate Ca^{2+}-dependent K^+ channel, resulting in an hyperpolarisation of the cell membrane.

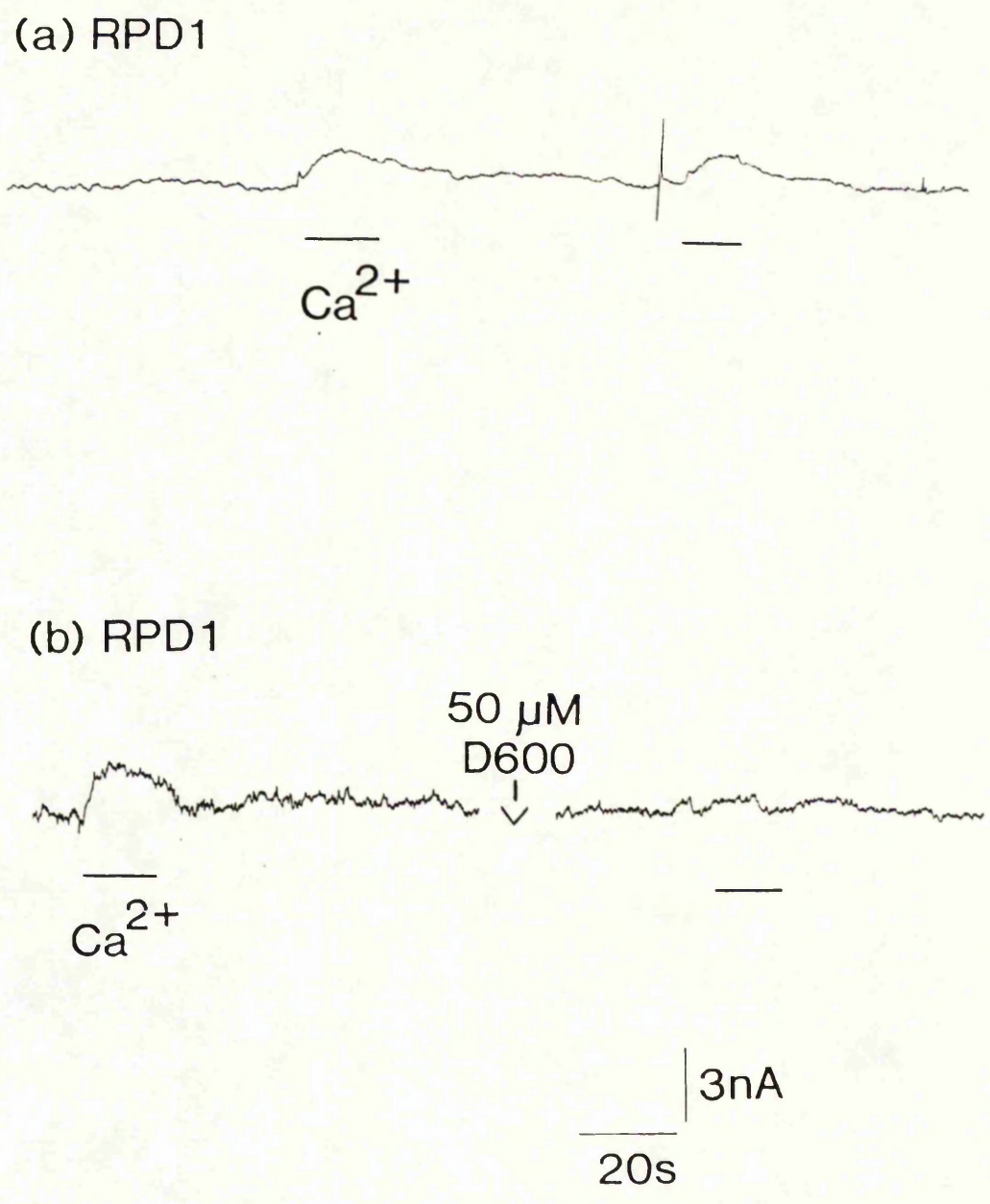


Fig 3.16. (a) Intracellular pressure injection of Ca^{2+} into a voltage clamped RPD1 cell (holding potential -60mV) produced a repeatable slow outward current. The current began to decay soon after the injection period stopped. (b) The outward current produced by Ca^{2+} injection in the RPD1 cell was inhibited by 50 µM D600 suggesting that the outward current produced by Ca^{2+} injection occurred by an efflux of K^+ through Ca^{2+} dependent K^+ channels. The arrow marks application of D600 and the bars indicate periods of Ca^{2+} injection.

Section II

Characteristics of a cyclic AMP-induced inward current in B1 and RPD1 neurones.

Introduction

It has been demonstrated by a number of workers that intracellular iontophoresis of the second messenger cAMP induces an inward current in neurones of gastropod molluscs (Gillette and Green, 1987; McCrohan and Gillette, 1988a). The current induced, however, varies from one species to another, the major differences being the ion(s) which carry the current and the presence or absence of voltage sensitivity of the current. The cAMP induced inward current is carried purely by Na^+ in identified neurones of the following species: Pleurobranchaea californica (Green and Gillette, 1983), Helix pomatia (Aldenhoff et al, 1983), Archidoris montereyensis (Connor and Hockberger, 1984) and Lymnaea stagnalis (McCrohan and Gillette, 1988a). However in neurones of Aplysia californica, a cAMP induced inward current is carried solely by Ca^{2+} (Pellmar, 1981). In neurones of Helix pomatia, cAMP activates Na^+, K^+ and Ca^{2+} conductances (Kononenko et al, 1983), whereas in an identified neurone of Limax maximus, Hockberger and Connor (1984) have described a cAMP induced increase in Na^+ and Ca^{2+} conductance. Clearly there is a diversity between species and even between neurones of the same species with regard to the carrier of the current induced by cAMP.

The presence or absence of voltage sensitivity of the cAMP induced

current also varies. Pellmar (1981) showed that the cAMP induced inward current in neurones of the abdominal ganglia of Aplysia californica is voltage sensitive, amplitude of the current increasing on depolarisation of the cell membrane. Similarly cAMP induced inward current is voltage sensitive in an identified neurone, the ventral white cell, of the marine molluscan carnivore Pleurobranchaea californica (Gillette and Green, 1987); the amplitude of the current increased with depolarisation of the cell membrane. Voltage sensitivity in this cell was abolished when Ca^{2+} was removed from the bathing saline, suggesting that extracellular Ca^{2+} confers voltage sensitivity on the cAMP induced inward current (Gillette and Green, 1987). However in another cell type the amplitude of the cAMP induced inward current decreased on depolarisation of the cell membrane (R. Gillette - personal communication). This suggests that voltage sensitivity varies not only between species but also between cell types of the same species. Thus, characterising the voltage sensitivity of the cAMP induced inward current can be used to distinguish cells and also provide information on the possible function of the cAMP induced inward current.

McCrohan and Gillette (1988b) showed that PTZ causes an increase in the amplitude of the cAMP induced inward Na^{+} current in the B1 cell of Lymnaea. This led them to conclude that PTZ may exert its effect in this neurone via an increase in intracellular cAMP, leading to enhancement of the cAMP-dependent Na^{+} current. If this hypothesis were also applicable to RPD1, then we might expect to see a cAMP-dependent outward current in this cell.

The aim of experiments described in this section was, firstly, to examine and compare cAMP-dependent currents in B1 and RPD1, and secondly, to investigate any relationship between cAMP- and PTZ-induced effects in the two cells.

Results

Properties of cAMP induced current in B1 and RPD1

Intracellular iontophoresis of cAMP induced an inward current in both the B1 (Fig 3.17a) and RPD1 cells of Lymnaea (Fig 3.17b). The amplitude of the current depended on the magnitude of the iontophoretic current pulse. To ensure that similar amounts of cAMP were iontophoresed into each cell type the following protocol was used. Cyclic AMP was iontophoresed into the B1 cell under voltage clamp at a holding potential of -60mV, inducing an inward current. The cAMP electrode and voltage recording electrode were then removed from the B1 cell and inserted into the RPD1 cell. Cyclic AMP iontophoresis was then repeated in the RPD1 cell using the same iontophoretic current pulse. Assuming no blocking of the microelectrode (which was checked by monitoring the electrode tip resistance), this procedure resulted in roughly the same amount of cAMP being iontophoresed into both cell types. In experiments of this kind the amplitude and $T_{\frac{1}{2}}$ (time taken for the current amplitude to decrease to half its maximal value) were not significantly different in the two cell types (Table 3.1a, n=6).

In all experiments where a drug's effect on the cAMP induced current was tested, AMP was iontophoresed to provide a control. The drug was then added to the bath and cAMP iontophoresed again. A comparison was

Fig 3.17. Cyclic AMP dependent inward currents in the B1 and RPD1 cells. Cyclic AMP induced an inward current in the B1 (a) and RPD1 (b) cells. Cells were voltage clamped at a holding potential of -60mV. There was no significant difference in the amplitude or duration of the current in the two cell types. The current began to decay soon after the cAMP iontophoresis had ceased. (c) The cAMP induced inward current in B1 was abolished in zero sodium saline. The recordings are from the same cell, in normal saline and in zero Na^+ saline. (d) In the RPD1 cell, cAMP induced inward current was again abolished in zero sodium saline.

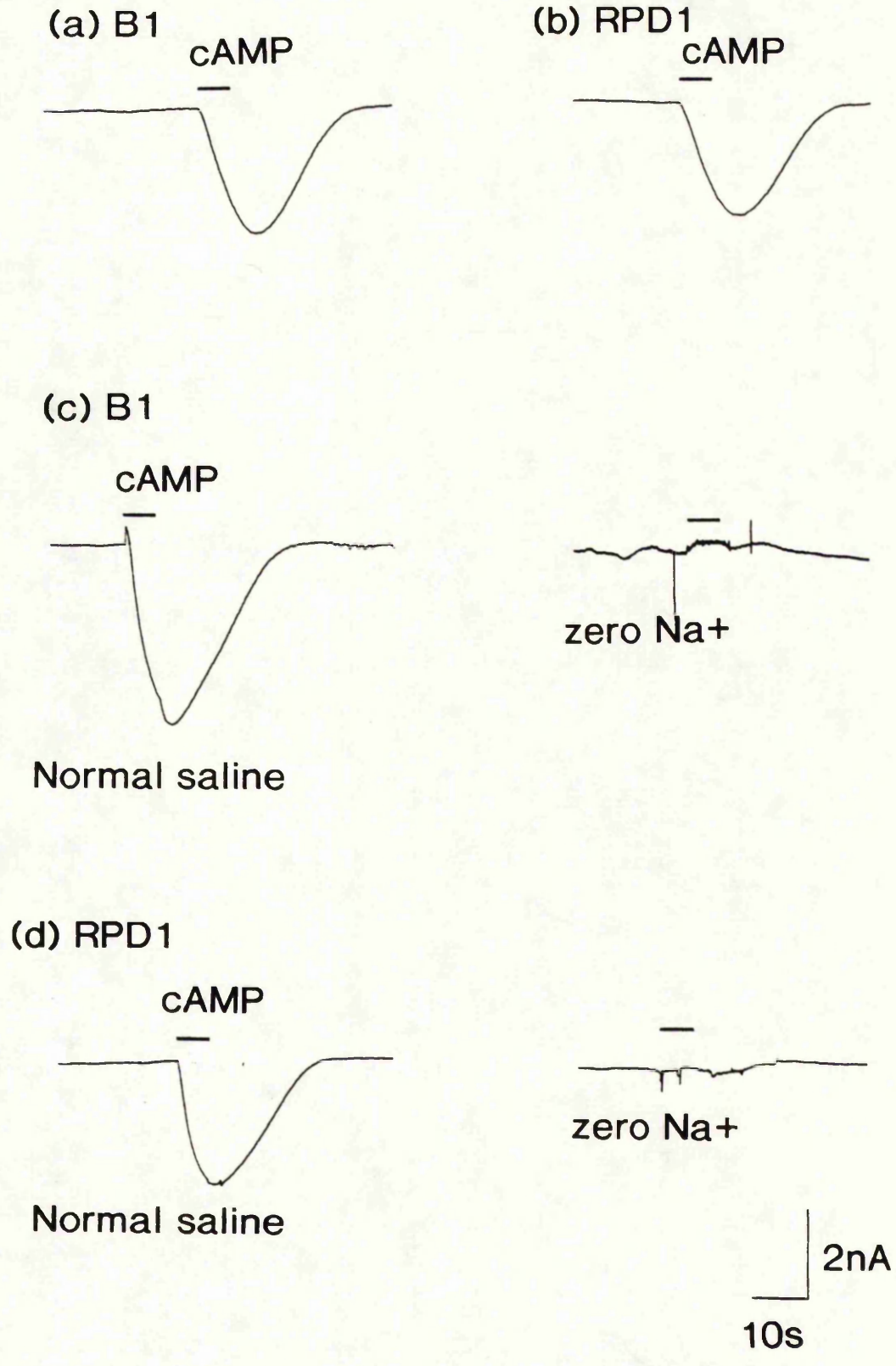

(a) B1
cAMP
(b) RPD1
cAMP
(c) B1
cAMP
zero Na+
Normal saline
(d) RPD1
cAMP
zero Na+
Normal saline
2nA
10s

Table 3.1. Characteristics of cAMP induced inward current in B1 and RPD1 cell types. (a) There was no significant difference between the amplitude or $T_{\frac{1}{2}}$ of the cAMP induced inward current in B1 and RPD1 cells. (b) In a separate experiment tetrodotoxin, an inhibitor of fast Na^+ channels, did not significantly affect the amplitude of the cAMP induced inward current in either B1 or RPD1 cells. Results are presented as the mean $\pm$ SEM.

TABLE 3.1

(a)	B1	RPD1
Amplitude (nA)	2.45 ± 0.48	2.75 ± 0.49
$T_{\frac{1}{2}}$ (secs)	26.9 ± 5.6	22.3 ± 5.6
	(n=6)	(n=6)

(b)	B1 Amplitude (nA)	RPD1 Amplitude (nA)
Control	1.53 ± 0.13	1.19 ± 0.29
100 μM TTX	1.59 ± 0.23	1.38 ± 0.45
	(n=5, n.s.)	(n=5, n.s.)

made between the cAMP induced current in control and test conditions. Statistical analysis involved comparing the two groups (control and test) using a paired t test. Results were considered significant when the value of p was less than 0.05.

In both cell types the cAMP induced current was abolished when the saline was replaced with zero sodium saline (Fig 3.17c, B1, n=6; Fig 3.17d, RPD1, n=6). This was done by iontophoresing cAMP in a neurone bathed in normal saline to obtain a control value. The experiment was repeated for the same cell bathed in zero sodium saline. In both cell types the cAMP induced inward current was insensitive to application of 10^{-4} M tetrodotoxin (TTX). The amplitude of the cAMP induced inward current was not significantly different from control values in the B1 cell (Table 3.1b, n=5) or in the RPD1 cell (Table 3.1b, n=5). These results agree with the findings of Hara et al (1985) and Connor and Hockberger (1984) who showed that cAMP induced currents were TTX insensitive in identified neurones of Aplysia kurodi and Aplysia californica respectively. These results suggest that cAMP induced inward current in B1 and RPD1 is carried by Na^+ ions but differs from the fast Na^+ conductance associated with the rising phase of the action potential, both in its slow time course and its insensitivity to TTX.

Iontophoresis of the second messenger cGMP and the cAMP breakdown product AMP were carried out to see if either of these compounds could induce an inward current similar to that induced by cAMP. This was achieved by filling one barrel of a double-barrelled microelectrode with 0.2 M cAMP and filling one barrel of a separate

double-barrelled microelectrode with 0.2 M cGMP. Cyclic AMP was iontophoresed into the cell as previously described and an inward current was induced. The cAMP containing microelectrode was then removed from the cell and replaced by the cGMP containing microelectrode, and an iontophoretic current pulse passed between the two barrels to iontophorese cGMP into the cell. Intracellular iontophoresis of cGMP did not induce an inward current in the B1 (Fig 3.18a, n=3) or RPD1 cell (Fig 3.18b, n=3). In some neurones of Archidoris it has been found that cGMP does induce an inward current (Connor and Hockberger, 1984), but it appears that, in B1 and RPD1 at least, this inward current does not occur. In neurones of Helix, Aldenhoff et al (1983) showed that cGMP (as well as ATP, ADP and adenosine) did not induce any current.

The inactive cAMP breakdown product, AMP, was iontophoresed into both cell types. The protocol was the same as that used for cGMP iontophoresis. Iontophoresis of AMP did not induce a current in either the B1 cell (Fig 3.18c, n=3) or in the RPD1 cell (Fig 3.18d, n=3). These results indicate that the mechanism which activates the inward Na^+ current is specifically activated by cAMP.

Voltage insensitivity of cAMP induced current in B1 and RPD1

The cAMP induced inward current was voltage insensitive over the membrane potential range -100 to -20mV in both B1 and RPD1. Cells were voltage clamped at holding potentials between -100 and -20mV, cAMP was iontophoresed and the amplitude of the current measured. There was no significant voltage sensitivity of the amplitude of the cAMP induced inward current in the B1 cell (Fig 3.19, n=5) or the

Fig 3.18. Intracellular iontophoresis of the cyclic nucleotide cyclic GMP (cGMP) did not induce an inward current in either B1 (a) or RPD1 (b). Similarly, iontophoresis of the cAMP breakdown product AMP did not induced a current in B1 (c) or RPD1 (d). The traces in the left hand column indicate control iontophoretic injections of cAMP. The traces in the right hand column show iontophoretic injections of cGMP or AMP. Neurones were voltage clamped at -60mV. The bars indicate 5 second iontophoretic current pulses.

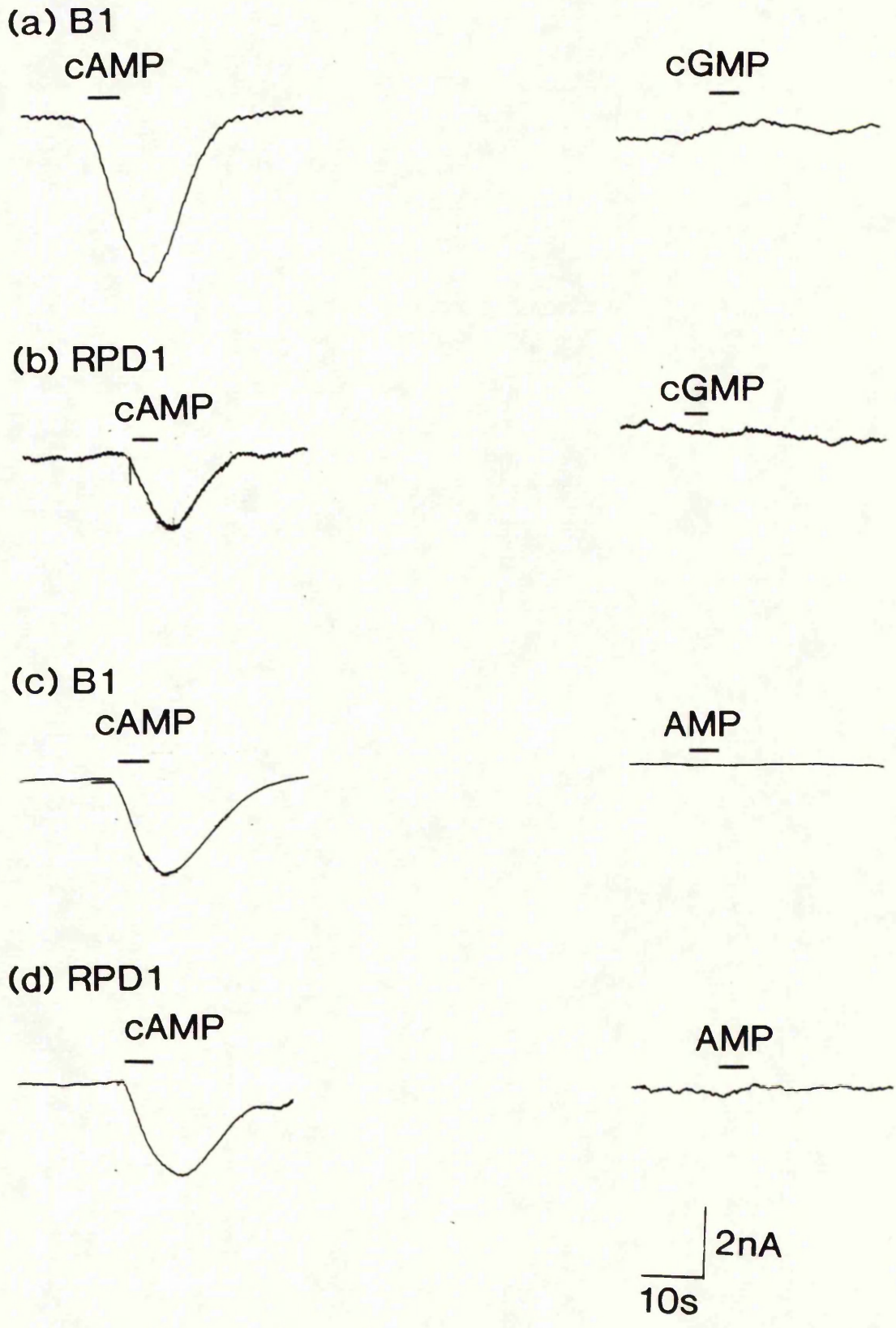
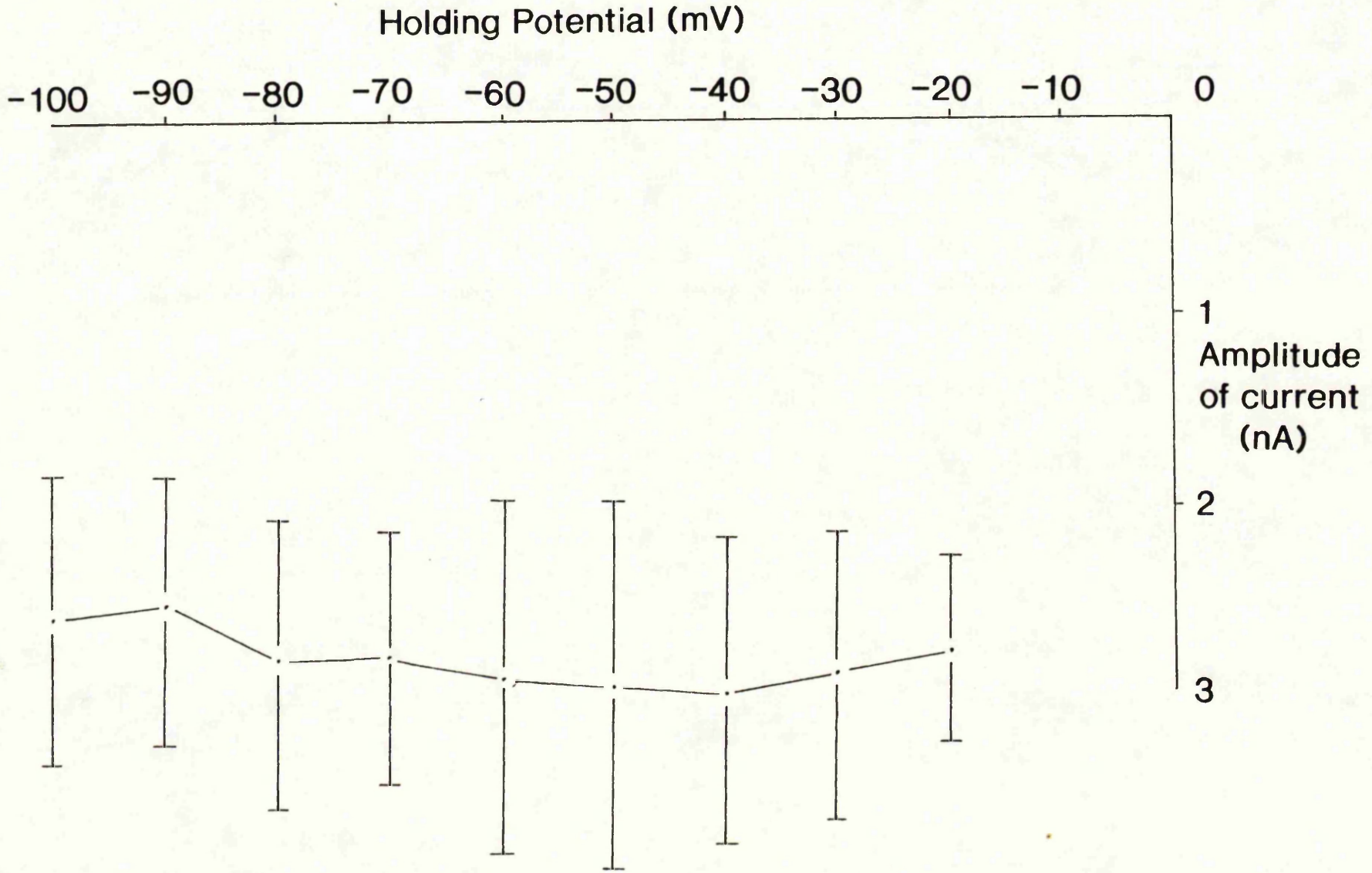

(a) B1
cAMP
cGMP
(b) RPD1
cAMP
cGMP
(c) B1
cAMP
AMP
(d) RPD1
cAMP
AMP
2nA
10s

Fig 3.19. Effect of holding potential on cAMP induced inward current in the B1 cell. Each cell was voltage clamped at holding potentials between -100 and -20mV, cAMP was iontophoresed and the amplitude of the current response measured. The cAMP induced inward current in the B1 cell is voltage insensitive over the membrane potential range -100 to -20mV. The vertical bars represent SEM. (n=5).


Holding Potential (mV)
-100
-90
-80
-70
-60
-50
-40
-30
-20
-10
0
1
Amplitude
of current
(nA)
2
3

RPD1 cell (Fig 3.20, n=5).

Effect of divalent cations on the cAMP induced current in B1 and RPD1

As Pleurobranchaea is a marine mollusc and the Ca^{2+} concentration of its saline is 10 mM, then it is possible that the lower Ca^{2+} concentration in the saline used for Lymnaea (4 mM) is too low to confer voltage sensitivity on the cAMP induced inward current, as it appears to do in the ventral white cell of Pleurobranchaea (Gillette and Green, 1987). Experiments were carried out in which the Ca^{2+} concentration in the saline was increased to 12 mM to see if any voltage sensitivity was induced. The amplitude of the cAMP induced inward current was significantly reduced in both cell types in saline containing 12 mM Ca^{2+}. The reduction in amplitude of the cAMP induced inward current was seen over the membrane potential range -90 to -30mV. In the B1 cell, the amplitude of cAMP induced inward current was significantly ($p < 0.05$) reduced at -90, -60 and -30mV (Fig 3.21, n=5). At a holding potential of -60mV the cAMP induced inward current was reduced by 45% (Table 3.2a, n=5, $p < 0.05$) compared to that in normal saline. In the RPD1 cell the cAMP induced inward current was significantly reduced at all three holding potentials (Fig 3.22, n=5, $p < 0.05$). At a holding potential of -60mV the cAMP induced inward current was reduced by 46% (Table 3.2b, n=5, $p < 0.05$). These results suggest that elevated extracellular Ca^{2+} does not confer voltage sensitivity on the cAMP induced inward current in either cell type. However the fact that Ca^{2+} causes a reduction in its amplitude suggests that it does inhibit the movement of Na^{+} into the cell.

Fig 3.20. Effect of holding potential on cAMP induced inward current in the RPD1 cell. The protocol used was the same as that described for Fig 3.19. The cAMP induced inward current in the RPD1 cell was voltage insensitive over the membrane potential range -100 to -20mV. Bars indicate the SEM. (n=5).

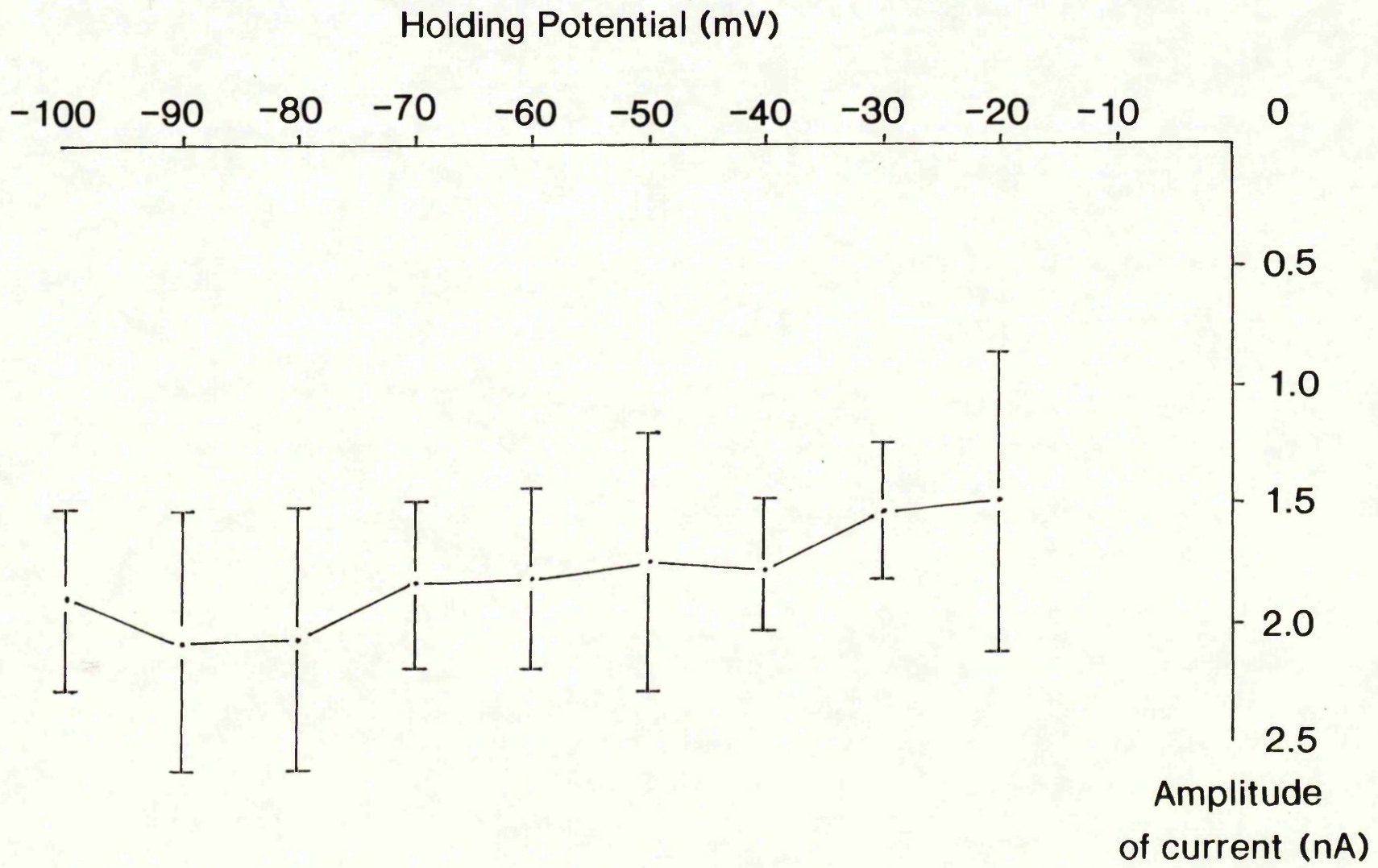

Holding Potential (mV)
-100
-90
-80
-70
-60
-50
-40
-30
-20
-10
0
0.5
1.0
1.5
2.0
2.5
Amplitude
of current (nA)

Fig 3.21. Effect of extracellular high Ca^{2+} (12 mM) on the amplitude of cAMP induced inward current over the membrane potential range -90 to -30mV. Cells were voltage clamped at either -90, -60 or -30mV, cAMP was iontophoresed and the amplitude of the current response measured. This was repeated in high Ca^{2+} saline. High Ca^{2+} caused a significant reduction in the amplitude of the cAMP induced inward current at holding potentials of -90, -60 and -30mV in the B1 cell. Bars indicate SEM. (n=6).

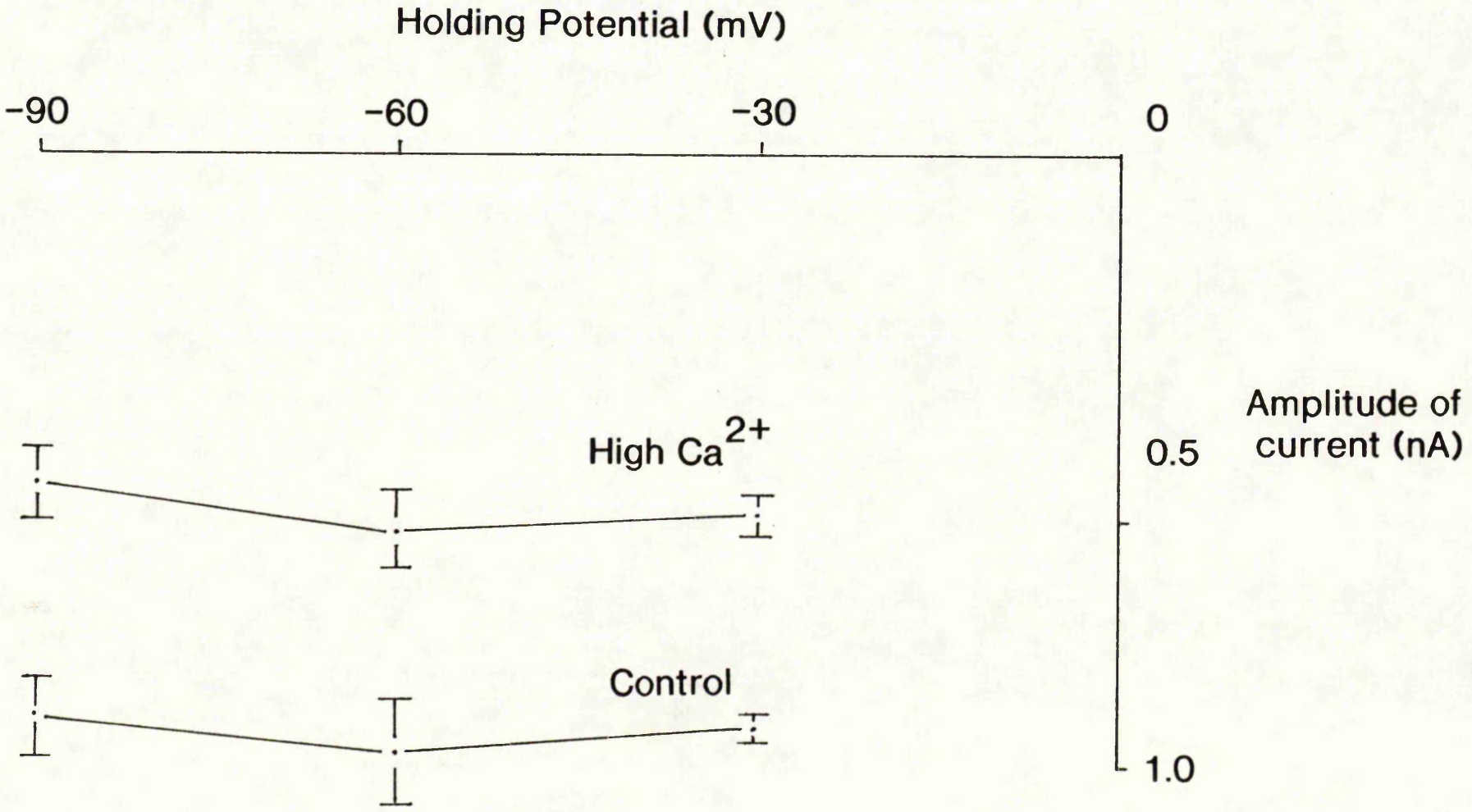
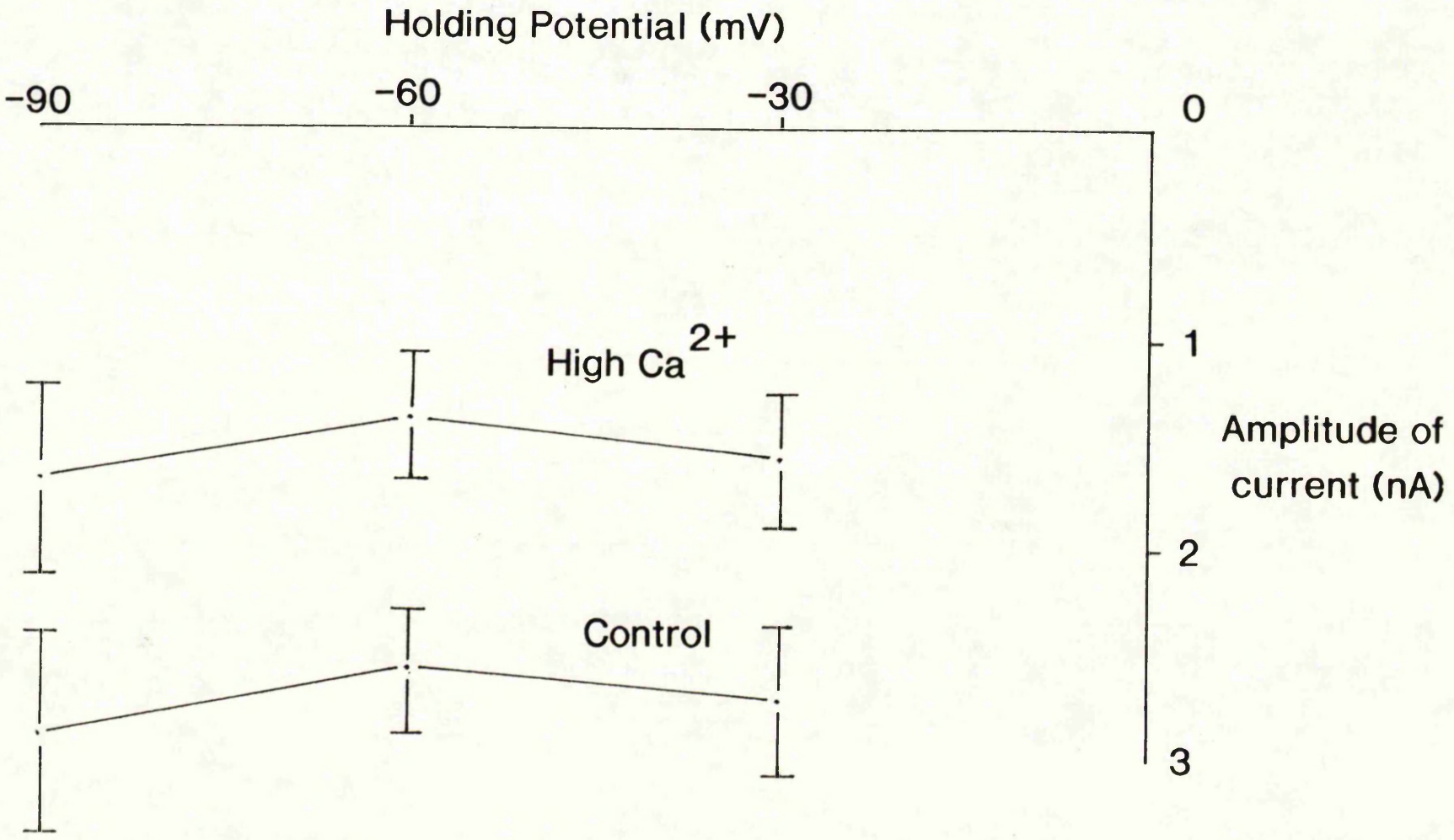

Holding Potential (mV)
-90
-60
-30
0
High Ca2+
Control
0.5
1.0
Amplitude of current (nA)

Fig 3.22. High Ca^{2+} saline caused a significant reduction in the amplitude of cAMP induced inward cuurrent in the RPD1 cell at holding potentials of -90, -60 and -30mV. The protocol used was the same as that described in Fig 3.21. The vertical bars indicate SEM. (n=6)


Holding Potential (mV)
-90
-60
-30
0
1
2
3
High Ca2+
Control
Amplitude of
current (nA)

The effect of another divalent cation, cobalt, was tested to see whether it was also capable of reducing the current amplitude. In the B1 cell addition of 10 mM Co^{2+} to normal saline resulted in a significant reduction of 81% in the cAMP induced inward current amplitude (Table 3.2c, n=5, $p < 0.05$), and similarly in the RPD1 cell 10 mM Co^{2+} caused a significant reduction of 76% in the cAMP induced inward current amplitude (Table 3.2d, n=5, $p < 0.05$). These results suggest that divalent cations possess the ability to reduce the amplitude of the cAMP induced inward current in both cell types. A possible explanation for this is that the divalent cations plug the channel and prevent Na^{+} from crossing the cell membrane (Gillette and Green, 1987).

Effect of removing extracellular Ca^{2+} on the cAMP induced current.

Removal of Ca^{2+} from the saline resulted in a significant increase in the amplitude of the cAMP induced inward current in both cell types. In the B1 cell the amplitude of cAMP induced inward current increased by 52% (Table 3.3a, n=5, $p < 0.05$) and in the RPD1 cell the amplitude of cAMP induced inward current was increased by 36% (Table 3.3b, n=5, $p < 0.05$).

Another means used to reduce extracellular Ca^{2+} was to add 10 mM EGTA, a Ca^{2+} chelator, to normal saline. Addition of 10 mM EGTA to normal saline resulted in a significant increase in the amplitude of cAMP induced current by 78% in the B1 cell (Table 3.3c, n=5, $p < 0.05$) and by 98% in the RPD1 cell (Table 3.3d, n=5, $p < 0.05$). The greater increase in the cAMP induced inward current amplitude seen in cells treated with EGTA compared to cells treated with saline

Table 3.2. Effect of divalent cations on the cAMP induced inward current in the B1 and RPD1 cells. High Ca^{2+} saline caused a significant reduction in the amplitude of the cAMP induced inward in both the B1 (a) and RPD1 cells (b). Likewise addition of 10 mM $CoCl_2$ to normal saline caused a significant reduction in the cAMP induced inward currents of both the B1 (c) and RPD1 cells (d). All experiments were carried out under voltage clamp at a holding potential of -60mV. Results are presented as the mean $\pm$ SEM. The statistical test used was the paired t-test. A value of less than 0.05 for p indicated statistical significance.

TABLE 3.2

(a)	B1 Amplitude (nA)
Control	0.97 ± 0.16
High calcium	0.53 ± 0.07
	($n=5$, $p < 0.05$)
(b)	RPD1 Amplitude (nA)
Control	2.58 ± 0.23
High calcium	1.39 ± 0.30
	($n=5$, $p < 0.05$)
(c)	B1 Amplitude (nA)
Control	2.08 ± 0.30
10mM cobalt	0.39 ± 0.06
	($n=5$, $p < 0.005$)
(d)	RPD1 Amplitude (nA)
Control	3.46 ± 0.81
10mM cobalt	0.82 ± 0.22
	($n=5$, $p < 0.05$)

Table 3.3. Effect of low Ca^{2+} saline on the amplitude of the cAMP induced inward current in the B1 and RPD1 cells. Omission of $CaCl_2$ from the saline resulted in a significant increase in amplitude of the cAMP induced inward current in both the B1 (a) and RPD1 cells (b). Addition of 10 mM EGTA, a Ca^{2+} chelator, to normal saline resulted in a significant increase in the amplitude of the cAMP induced inward current in both the B1 (c) and RPD1 cells (d). Cells were held under voltage clamp at -60mV. Statistical test used was a paired t test. Results are expressed as mean $\pm$ SEM.

TABLE 3.3

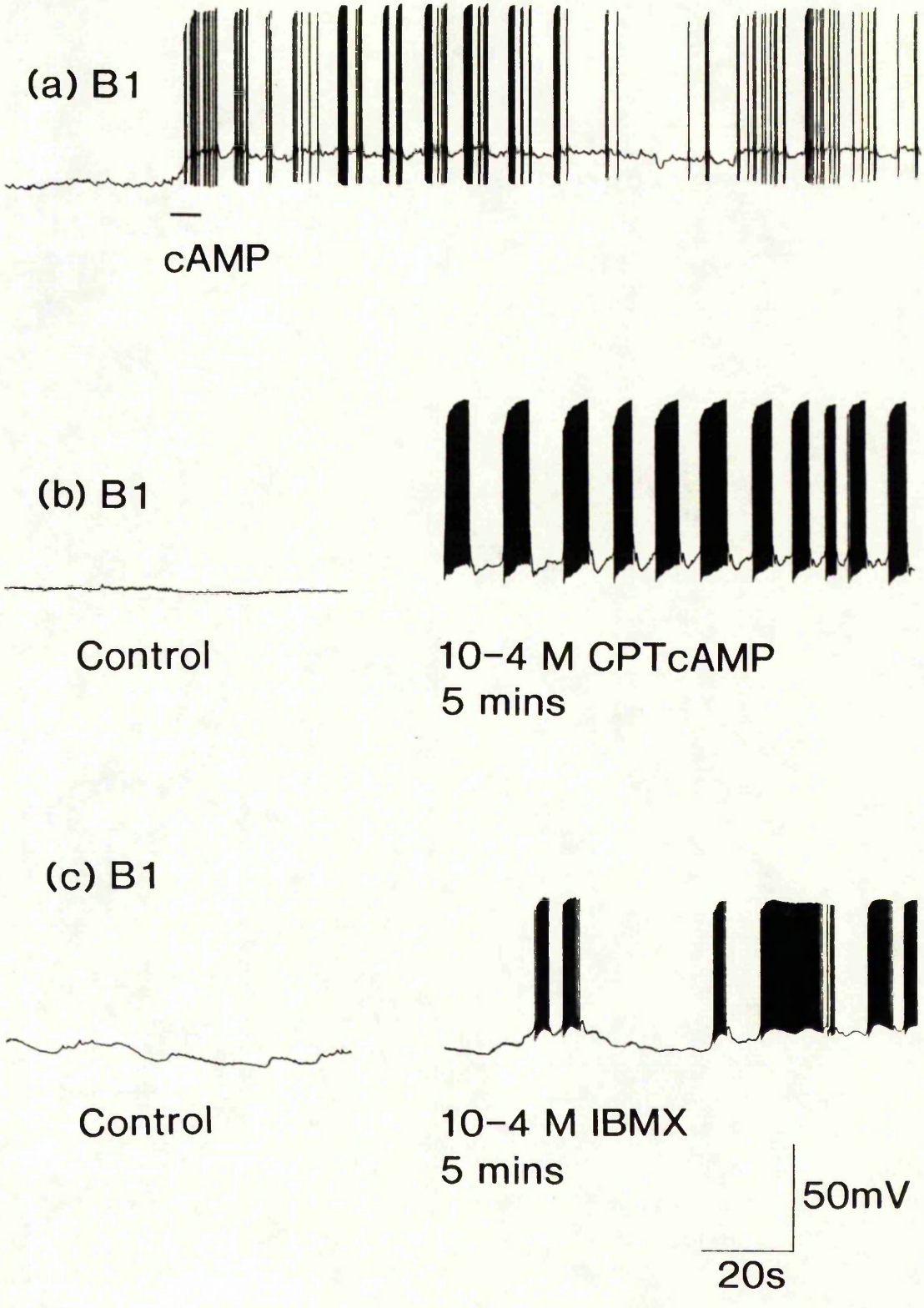
(a)	B1 Amplitude (nA)
Control	1.24 ± 0.14
low calcium	1.88 ± 0.22
	(n=5, $p < 0.01$)
(b)	RPD1 Amplitude (nA)
Control	1.71 ± 0.19
Low calcium	2.32 ± 0.15
	(n=5, $p < 0.005$)
(c)	B1 Amplitude (nA)
Control	2.22 ± 1.05
10mM EGTA	3.95 ± 1.59
	(n=5, $p < 0.05$)
(d)	RPD1 Amplitude (nA)
Control	2.60 ± 0.83
10mM EGTA	5.16 ± 1.35
	(n=5, $p < 0.05$)

in which the Ca^{2+} had been omitted, may be explained by the fact that, in the latter, there is probably a residual Ca^{2+} concentration of about 10 uM owing to leaching and impurities in other chemicals used to make up the saline (Gillette and Green, 1987), but with EGTA the Ca^{2+} concentration is buffered to a lower concentration than this.

Effect on membrane potential of increased intracellular cAMP.

Since iontophoresis of cAMP induced an inward current in B1 and RPD1 it would be expected that increased intracellular cAMP levels in both cell types would cause excitation of the cell and depolarisation of the cell membrane. To investigate this, intracellular cAMP levels were increased in one of two ways; firstly, by directly iontophoresing cAMP into the cell, and secondly, using two pharmacological agents. In the B1 cell, intracellular iontophoresis of cAMP resulted in depolarisation of the cell membrane of between 9 and 17mV, accompanied by bursts of action potentials (Fig 3.23a, n=5). This effect lasted for a considerable period, between 4.5 and 7 minutes, after the cAMP iontophoresis had stopped. This is a very interesting finding as it shows that cAMP's effects last longer in the unclamped cell than they do in the voltage clamped cell. This suggests that the process of voltage clamping cells inhibits the ability of cAMP to induce long lasting effects. Bath application of the membrane permeable cAMP analogue 8-(4-chlorophenylthio)-adenosine 3'5'-cyclic monophosphate (CPTcAMP), resulted in bursting activity in the B1 cell. A concentration of 10^{-4} M CPTcAMP induced a depolarisation of the cell membrane by 13.8 $\pm$ 1.3mV, accompanied by bursts of action potentials (Fig 3.23b, n=11). In a similar fashion,

Fig 3.23. Effect of increased intracellular concentrations of cAMP in the B1 cell. (a) Intracellular iontophoresis of cAMP resulted in depolarisation of the cell membrane accompanied by bursts of action potentials. This effect lasted for up to 7 minutes. (b) Bath application of the membrane permeable cyclic AMP analogue, CPTcAMP, at a concentration of 10^{-4} M, induced depolarisation of the cell membrane accompanied by superimposed bursts of action potentials. (c) Application of 10^{-4} IBMX, a phosphodiesterase inhibitor, produced similar effects. Bar indicates a 5 second iontophoretic current pulse.


(a) B1
cAMP
(b) B1
Control
10-4 M CPTcAMP
5 mins
(c) B1
Control
10-4 M IBMX
5 mins
50mV
20s

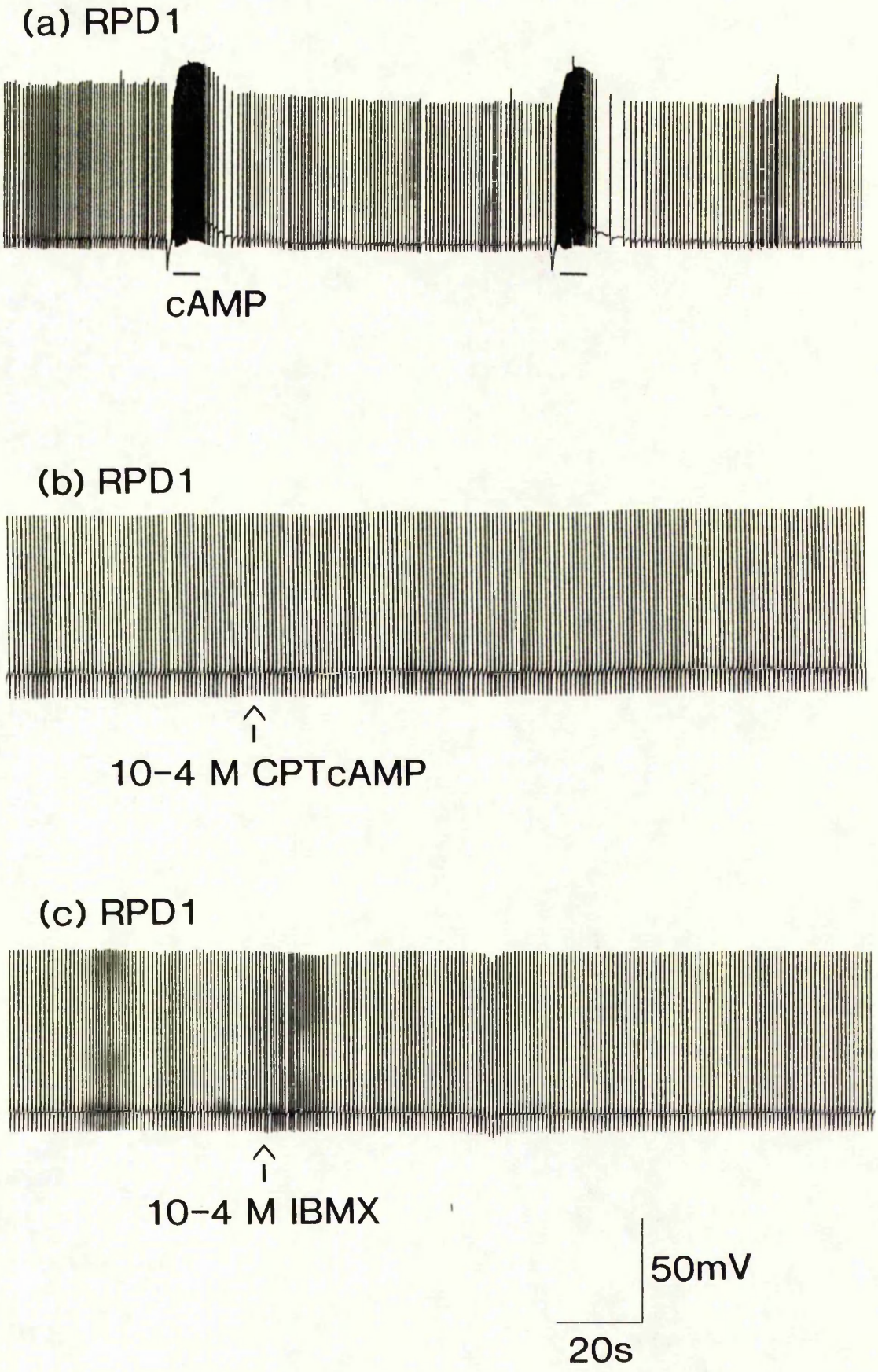
bath application of the cAMP phosphodiesterase inhibitor, IBMX, induced a membrane depolarisation of 15.3 ± 3.7mV with superimposed bursts of action potentials (Fig 3.23c, n=15). These results show that increased intracellular cAMP levels, produced in three different ways in the B1 cell, induced bursting activity similar to that induced by 20 mM PTZ. None of these three manoeuvres was capable of producing PDS in the B1 cell, suggesting that either they do not induce a large enough increase in intracellular cAMP concentrations, or that cAMP, no matter what the concentration, is incapable of inducing PDS in this cell.

In the RPD1 cell, intracellular iontophoresis of cAMP caused a depolarisation of the cell membrane by up to 15mV, and an increase in the firing activity of the cell (Fig 3.24a, n=5). This increase in activity was short lasting and died away almost immediately after the cAMP iontophoresis had ceased. Neither CPTcAMP or IBMX had any marked effect on the activity of the RPD1 cell. Bath application of 10^{-4} M CPTcAMP (Fig 3.24b, n=5) and 10^{-4} M IBMX (Fig 3.24c, n=9) did not result in a significant increase in the firing activity in the RPD1 cell. These effects are difficult to explain since it was been shown in Fig 3.24a that increased intracellular cAMP does cause an increase in firing activity.

Effect of IBMX on the cAMP induced current in B1 and RPD1.

The results described above indicate that, in the RPD1 cell, IBMX's effects are not what one would expect. As IBMX is a phosphodiesterase inhibitor it should result in increased intracellular cAMP levels and correspondingly increase the cAMP induced inward current. The effect

Fig 3.24. (a) Intracellular iontophoresis of cAMP into the RPD1 cell resulted in an increase in activity in the RPD1 cell which lasted for a short period of time before the cell resumed its endogenous pacemaker activity. Bath application of 10^{-4} M CPTcAMP (b) or IBMX (c) did not result in any significant increase in activity in the RPD1 cell. Concentrations of both drugs up to 1 mM were used but did not result in any significant effect. The arrows indicate addition of drugs and the bar indicates a 5 second iontophoretic current pulse.


(a) RPD1
cAMP
(b) RPD1
10-4 M CPTcAMP
(c) RPD1
10-4 M IBMX
50mV
20s

of IBMX on cAMP induced inward current in both cell types was examined. Isobutylmethylxanthine caused an increase in both amplitude and duration of cAMP induced inward current in the B1 cell in a dose dependent manner. The amplitude of the cAMP induced inward current increased in a sigmoid fashion with the maximal amplitude occurring at a concentration of 0.1 mM (Fig 3.25, n=6). In 0.1 mM IBMX, the current amplitude was significantly increased to 169% of its control value (Table 3.4a, n=6). Plotting $T_{\frac{1}{2}}$ of the current against IBMX concentration also revealed a dose dependent increase in $T_{\frac{1}{2}}$ (Fig 3.26, n=6). At a concentration of 0.05 mM IBMX there was no increase in the $T_{\frac{1}{2}}$ which is difficult to explain. As with the current amplitude, the maximal increase in $T_{\frac{1}{2}}$ occurred at the concentration of 0.1 mM IBMX. where the $T_{\frac{1}{2}}$ was increased to 308% of its control value (Table 3.4a, n=6). In the RPD1 cell 0.1 mM IBMX caused a significant increase in the $T_{\frac{1}{2}}$ but did not significantly affect the amplitude of the cAMP induced inward current (Table 3.4b, n=6). The fact that 0.1 mM IBMX did not cause a significant increase in the amplitude of the cAMP induced inward current is difficult to explain, when there was a significant increase of 484% in the $T_{\frac{1}{2}}$ (Table 3.4b, n=6).

Effect of PTZ on the cAMP induced current in B1 and RPD1.

In Section I the possible mode of action of PTZ in B1 and RPD1 was described. The results indicated that PTZ probably acts to increase intracellular Ca^{2+} concentrations in both cell types. This, however, may only be part of its mode of action. McCrohan and Gillette (1988b) suggested that PTZ may act as a phosphodiesterase inhibitor, as it caused a significant increase in amplitude of the

Fig 3.25. Effect of IBMX on amplitude of the cAMP induced inward current in B1. This plot was obtained by adding various concentrations of IBMX (0.005 - 0.5 mM) to saline, iontophoresing cAMP and measuring the amplitude of the current produced. Each point represents the mean of 5 cells; * indicate a significant difference ($p < 0.05$) from the control (100%) value. Statistical test used was a paired t test. The maximal increase in amplitude occurred at a concentration of 0.1 mM IBMX. Bars indicate the SEM.

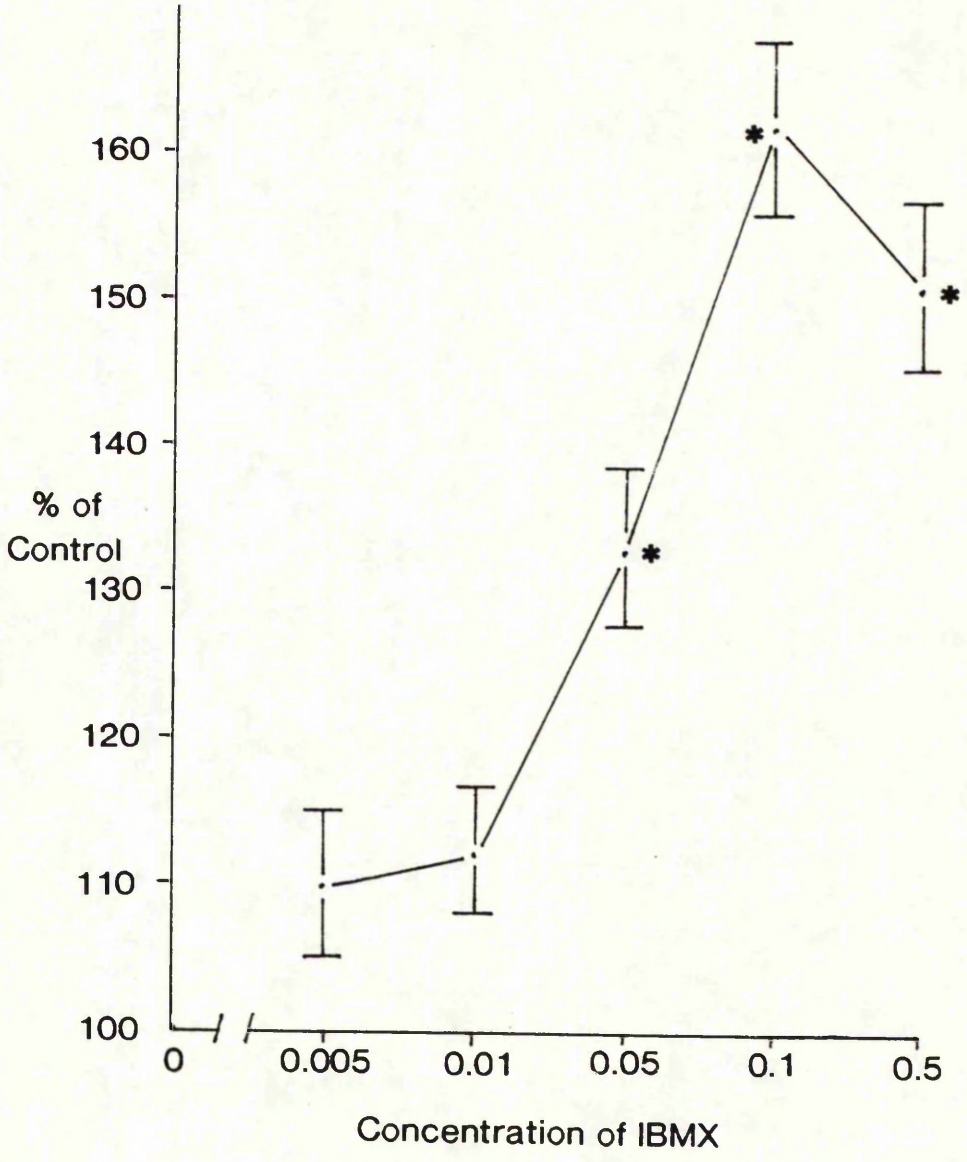

160
150
140
% of
Control
130
120
110
100
0
0.005
0.01
0.05
0.1
0.5
Concentration of IBMX

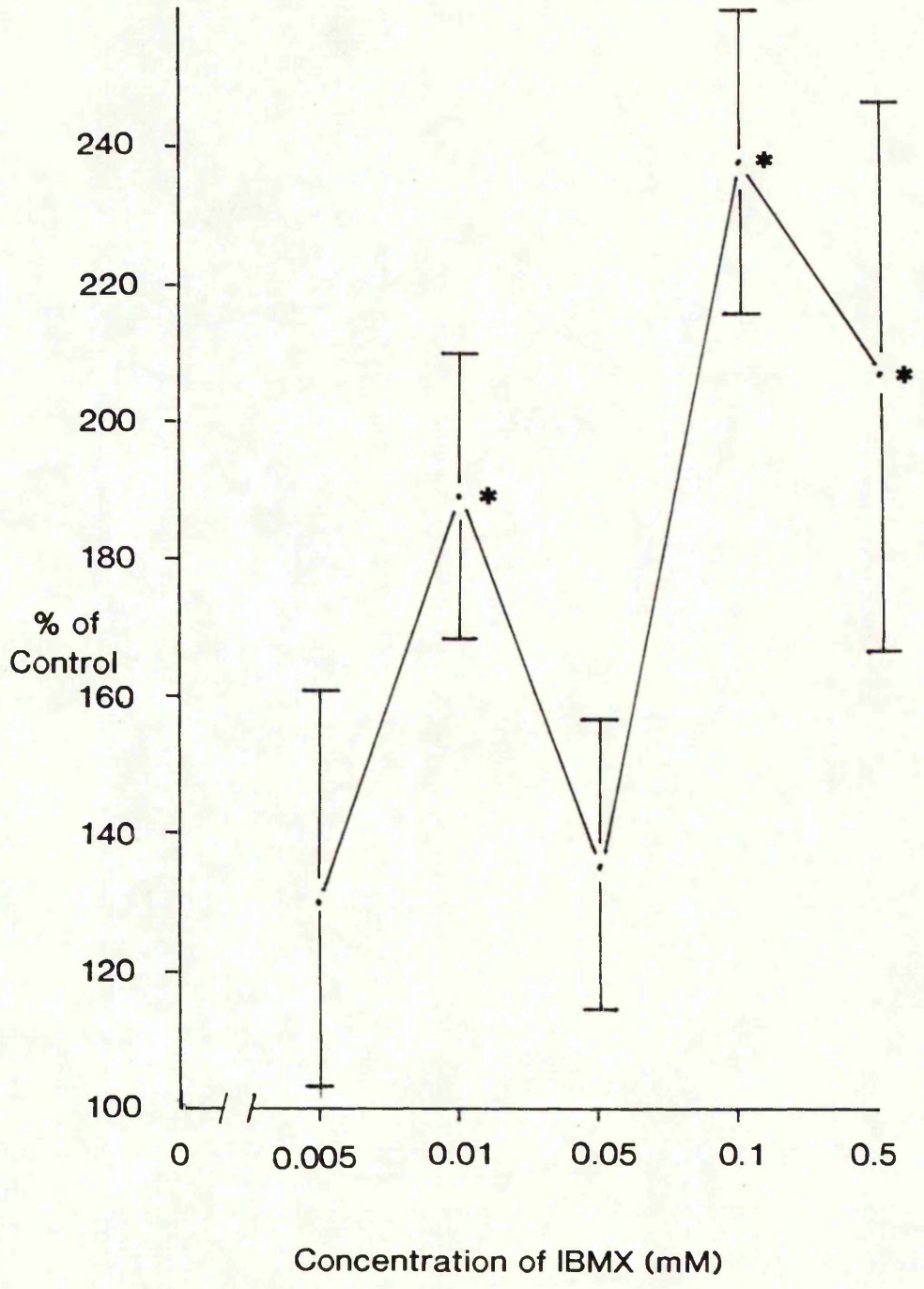
Table 3.4. Effect of 0.1 mM IBMX on the amplitude and $T_{1/2}$ of the cAMP induced inward current in the B1 and RPD1 cells. (a) In the B1 cell 0.1 mM IBMX caused a significant increase in both the amplitude and $T_{1/2}$ of the cAMP induced inward current. (b) In the RPD1 cell 0.1 mM IBMX caused a significant increase in the $T_{1/2}$ but had no significant effect on the amplitude of the cAMP induced inward current. Statistics used was a paired t test. Results are shown as mean $\pm$ SEM.

TABLE 3.4

(a)	B1	
	Amplitude (nA)	$T_{\frac{1}{2}}$ (sec)
Control	1.81 ± 0.29	9.00 ± 3.36
100 μM IBMX	3.07 ± 0.49	27.7 ± 9.44
	(n=6, $p < 0.05$)	(n=6, $p < 0.05$)

(b)	RPD1	
	Amplitude (nA)	$T_{\frac{1}{2}}$ (sec)
Control	2.87 ± 0.29	5.08 ± 1.0
100 μM IBMX	3.19 ± 0.42	24.6 ± 6.4
	(n=6, n.s.)	(n=6, $p < 0.05$)

Fig 3.26. The effect of IBMX concentration on the $T_{\frac{1}{2}}$ of cAMP induced inward curent. The cAMP induced current displayed a dose-dependent response. The protocol was the same as that described in Fig 3.25. The lack of effect using 0.05 mM IBMX is difficult to explain. * indicate the points which are significantly different ($p < 0.05$) from control (100%) values. Statistical test used was the paired t test. Bars indicate SEM.


240
220
200
180
% of
Control
160
140
120
100
0
0.005
0.01
0.05
0.1
0.5
Concentration of IBMX (mM)

cAMP induced current in B1. To investigate whether cAMP is involved in the convulsant effects of PTZ, the effect of PTZ on the cAMP induced current in both cell types was examined. Application of PTZ caused opposite effects on the amplitude of cAMP induced inward current in the two cell types. In the B1 cell 40 mM PTZ caused a significant increase of 59% in the amplitude of the cAMP induced inward current (Table 3.5a, n=6, $p < 0.05$), whereas in the RPD1 cell bath application of 40 mM PTZ caused a significant decrease of 39% in the amplitude of cAMP induced inward current (Table 3.5b, n=9, $p < 0.05$). These results do not support McCrohan and Gillette's (1988b) hypothesis that PTZ acts via an increase in cAMP. at least in the RPD1 cell. An alternative explanation is that elevated intracellular Ca^{2+}, caused by PTZ, acts to increase cAMP-induced current in B1 and to decrease the same current in RPD1. A possible mechanism could be direct enhancement by Ca^{2+} of Na^{+} channel activation in B1, and activation of phosphodiesterase by Ca^{2+} in RPD1. The following experiment was therefore carried out, to see whether elevated Ca^{2+} would mimic PTZ's effects on the cAMP-induced current.

Effect of increased intracellular Ca^{2+} on the cAMP induced current in B1 and RPD1.

Cyclic AMP was iontophoresed into B1 and RPD1 against a superimposed background of Ca^{2+} injection. In B1 Ca^{2+} ions were continuously pressure injected until a steady inward current was seen. This acted as a baseline against which cAMP induced current was measured. Cyclic AMP was then iontophoresed into the cell (Fig 3.27a, n=3). Intracellular injection of Ca^{2+} ions did not have any significant

Table 3.5. Effect of extracellular PTZ on the amplitude of the cAMP induced inward current in the B1 and RPD1 cells. (a) In the B1 cell application of 40 mM PTZ caused a significant increase in the amplitude of cAMP induced inward current, but 40 mM PTZ caused a significant decrease in the amplitude of cAMP induced inward current in the RPD1 cell (b). Statistical test used was the paired t test. Results are shown as mean $\pm$ SEM.

TABLE 3.5

(a)	B1 Amplitude (nA)
Control	1.41 ± 0.22
40mM PTZ	2.24 ± 0.47
	(n=6. $p < 0.05$)

(b)	RPD1 Amplitude (nA)
Control	2.19 ± 0.39
40mM PTZ	1.33 ± 0.30
	(n=9, $p < 0.005$)

Fig 3.27. Effect of increased intracellular Ca^{2+} on cAMP induced inward current. (a) In B1 intracellular pressure injection of Ca^{2+} induced an inward current. The amplitude of cAMP induced inward current was not significantly different from control values when cAMP iontophoresis was superimposed on the Ca^{2+} injection. The dotted line indicates the base line against which cAMP induced current amplitude was measured. (b) Similarly in RPD1 increased concentration of Ca^{2+} had no significant effect on cAMP induced current.

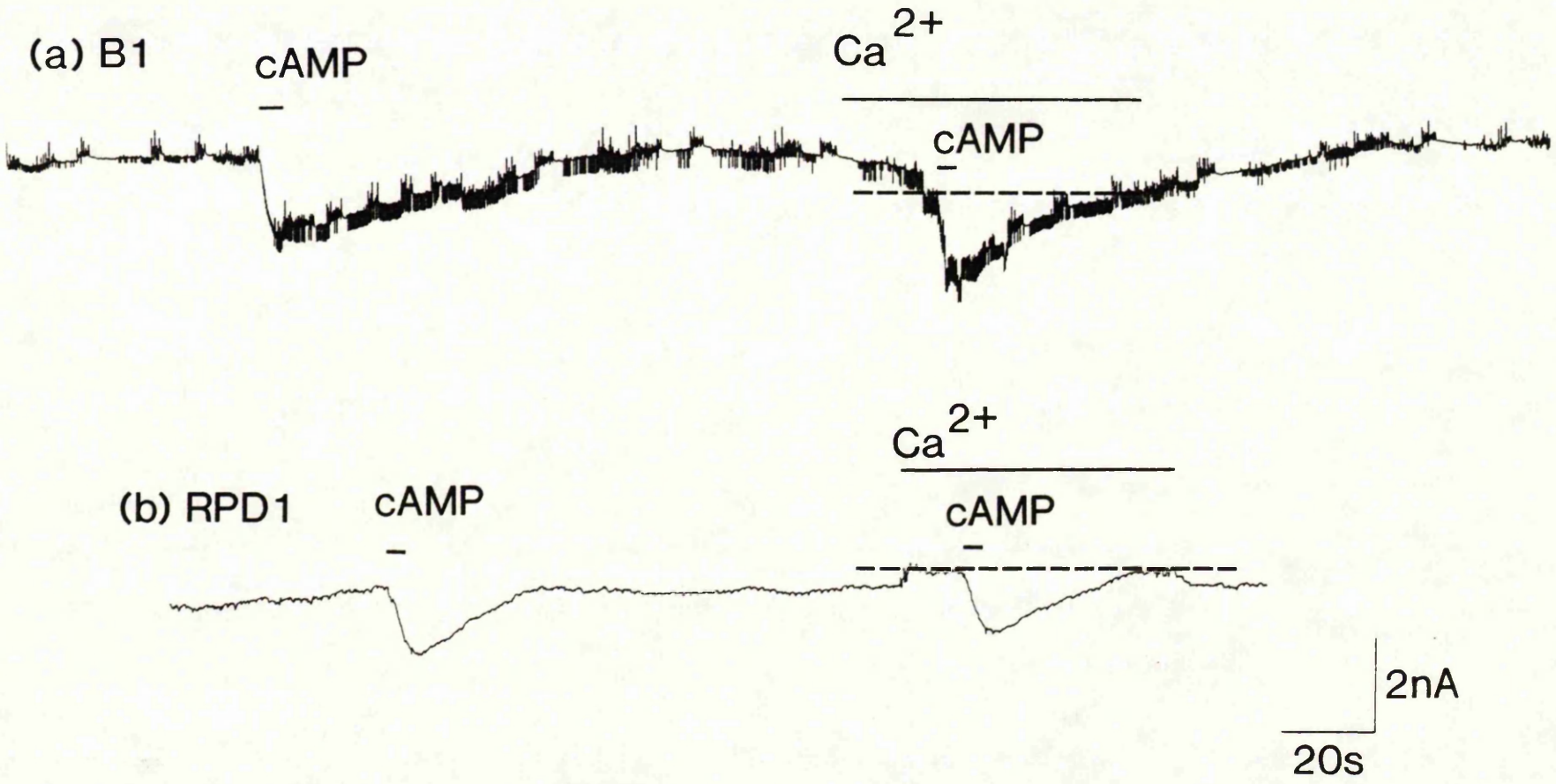

(a) B1
cAMP
Ca2+
cAMP
(b) RPD1
cAMP
Ca2+
cAMP
2nA
20s

Table 3.6. Effect of increased intracellular Ca^{2+} on cAMP induced current in B1 and RPD1. Intracellular pressure injection of Ca^{2+} did not produce any significant effect on the cAMP induced inward current amplitude in the B1 (c) or RPD1 cells (d). Results are shown as mean $\pm$ SEM.

TABLE 3.6

(a)	B1 Amplitude (nA)
Control	2.92 ± 0.57
Calcium inj.	2.85 ± 0.63
	(n=3, n.s.)

(b)	RPD1 Amplitude (nA)
Control	3.54 ± 0.79
Calcium inj.	3.38 ± 0.98
	(n=3 n.s.)

the

effect on amplitude of the cAMP induced current (Table 3.6a, n=3). The same protocol was used for the RPD1 cell except that Ca^{2+} injection resulted in a steady outward current (Fig 3.27b, n=3). Intracellular injection of Ca^{2+} had no significant effect on cAMP induced current amplitude (Table 3.6b, n=3). These results indicate that intracellular Ca^{2+} does not directly affect the cAMP induced inward current in either cell type, and therefore that the effects of PTZ on cAMP-induced current are not mediated via Ca^{2+}.

Section III

Comparison of cAMP and PTZ induced inward currents in B1 neurones.

Introduction.

The results presented in the previous sections have shown similarities between PTZ and cAMP induced currents in the B1 cell of *Lymnaea*. Both are dependent upon extracellular Na^+, are TTX-insensitive and inward in direction. Furthermore, elevated extracellular Ca^{2+} appears to inhibit cAMP induced inward current as well as PTZ-induced depolarisation. In the B1 cell, increased intracellular cAMP levels led to bursting activity, as did bath application of PTZ. This supported McCrohan and Gillette's (1988b) hypothesis that PTZ may act in B1 via increased intracellular levels of cAMP leading to bursting activity. Additional evidence for this is that PTZ caused an increase in the amplitude of the cAMP induced inward current in the B1 cell. These results agree with the findings of Onozuka *et al* (1983), who showed that PTZ activated adenylate cyclase resulting in increased intracellular cAMP concentrations in the identified D neurone of *Euhadra*.

In this section the characteristics of the PTZ- and cAMP-induced currents in B1 are compared in relation to their voltage sensitivity and their response to three pharmacological agents. These three pharmacological agents were carbamazepine, amiloride and TMB-8. Carbamazepine (CBZ) is an anticonvulsant agent in everyday clinical use. Its membrane stabilising properties are thought to be due to its ability to block fast sodium channels associated with the rising

phase of the action potential (Schauf et al, 1974), but there is a growing body of evidence to suggest that it may also bind to adenosine receptors and exert at least part of its effect via this route (Skerrit, 1983a). Amiloride is a diuretic drug which at 100 um inhibits the Na^+:H^+ electroneutral exchanger (Benos, 1982), but which at a lower concentration of 10 uM inhibits Na^+ conductance pathways in epithelial tissue (Sariban-Sohraby and Benos, 1986). More recent findings suggest that amiloride is also capable of inhibiting T-type Ca^{2+} channels in neuronal tissue, with a K_{50} of 30 uM (Tang et al, 1988). Trimethoxybenzoic acid (TMB-8) has been reported to block Ca^{2+} channels (Ikeda et al, 1984). There is some controversy however as to whether these Ca^{2+} channels are in the neuronal membrane or are in the membrane of the intracellular stores. The end result of TMB-8's effects is the same - a reduction in intracellular Ca^{2+} concentrations.

Results

Effect of holding potential on the cAMP and PTZ induced currents.

As illustrated in Fig 3.19 (Section II) the cAMP induced current in B1 is voltage insensitive over the membrane potential range -100 to -20mV. The amplitude of the current was not significantly different over this membrane potential range.

The effect of holding potential on the PTZ induced current was studied by examining the steady state I-V curve in the presence and absence of extracellularly applied PTZ. The steady state I-V curve reveals the sum of the slow currents present over a range of membrane potentials. It does not show fast currents as these inactivate too

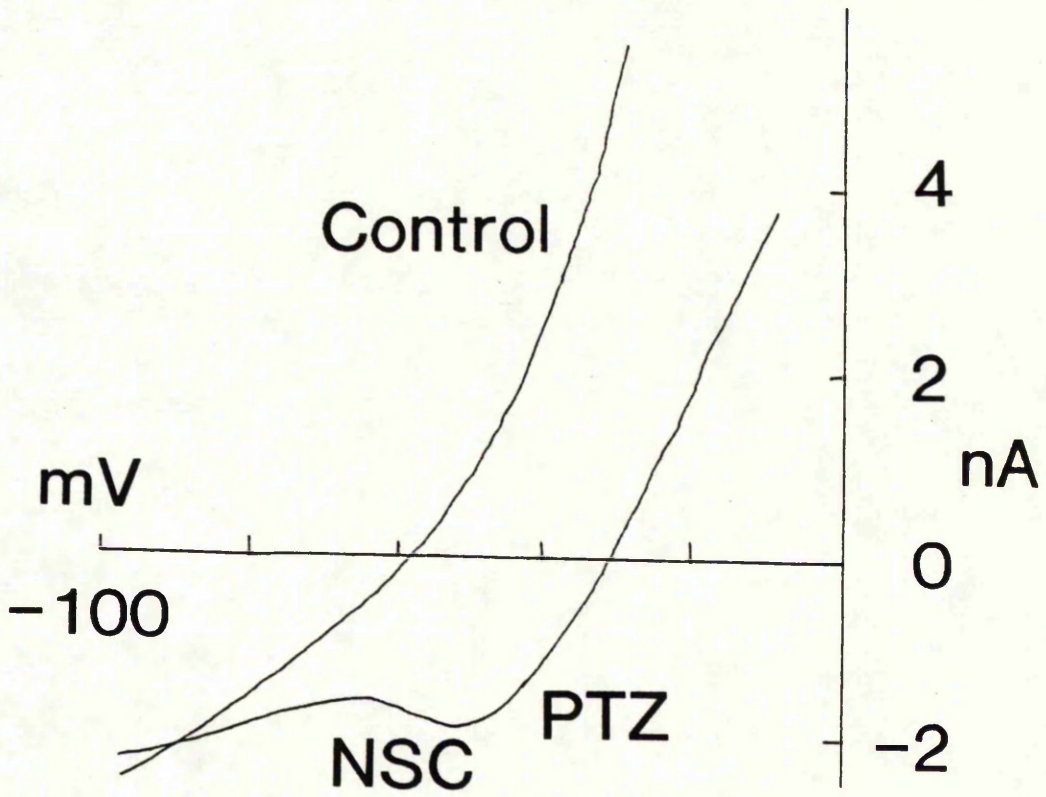
quickly. The point where the curve crosses the x-axis indicates the equilibrium potential (resting potential; Fig 3.28). Application of 40 mM PTZ caused a shift in the curve in the direction of inward current, leading to a shift in equilibrium potential of about 30mV, in the depolarising direction (Fig 3.28, n=7). Pentylenetetrazol also induced a region of negative slope conductance over the membrane potential range -65 to -50mV (Fig 3.28). This signifies a region of inward current development which is voltage sensitive and reaches its peak at -50mV. A similar region of negative slope conductance has also been shown in neurones of Tritonia (Partridge, 1975), Euhadra (Sugaya et al, 1978) and Aplysia (David et al, 1974) and appears to be a property of neurones which are excited by PTZ. The region of negative slope conductance indicates bursting ability in the cell. In the R15 neurone of Aplysia, an endogenously bursting cell, there is a region of endogenous negative slope conductance (Levitan and Levitan, 1988).

Thus there appears to be a fundamental difference in the PTZ and cAMP induced inward currents. Cyclic AMP-induced current in B1 and RPD1 is voltage insensitive (Section II). The negative slope conductance induced by PTZ, signifies that the PTZ-induced current is voltage sensitive. It develops at close to resting membrane potential and increases in amplitude with depolarisation. This would tend to cause excitation in the cell and explains the depolarisation and bursting activity induced by PTZ in B1.

Effect of carbamazepine on the PTZ and cAMP induced currents.

Carbamazepine inhibited PTZ-induced effects in B1. Application of 20

Fig 3.28. In the B1 cell, 40 mM PTZ caused a shift in the steady state I-V curve. This experiment was carried out by voltage clamping the cell at -60mV and imposing a slow ramp of $\pm$ 50mV on the membrane potential via a function generator connected to the clamp input of the voltage clamp. The curve shifted in the direction of inward current and a region of negative slope conductance (NSC) appeared over the membrane potential range -65 to -50mV.


Control
4
2
nA
mV
0
−100
PTZ
NSC
−2

mM PTZ induced a membrane depolarisation of 13.3 ± 2.7mV, accompanied by bursts of action potentials. Subsequent addition of 1 mM CBZ resulted in a delayed reversal of these PTZ-induced effects, and repolarised the membrane by 11.4 ± 2.1mV (Fig 3.29a, n=5) after a period of 170 ± 33 secs. Following pretreatment with 1 mM CBZ, application of 20 mM PTZ still induced bursting activity and a depolarisation of 14.9 ± 4.2mV in the B1 cell. However, this was short lived and the membrane potential repolarised by 13.7 ± 4.1mV within 198 ± 27 secs (Fig 3.29b, n=5). Carbamazepine inhibited the PTZ induced inward current in B1. Application of 40 mM PTZ induced an inward current of 1.89 ± 0.42 nA (n=5). Subsequent addition of 1 mM CBZ caused a reduction of this current by 1.54 ± 0.34 nA (Fig 3.29c), after a period of 235 ± 52 secs. Prior application of 1 mM CBZ in the voltage clamped B1 cell did not prevent 40 mM PTZ from inducing an inward current of 2.45 ± 0.45 nA, but this inward current was transient and decayed by 2.01 ± 0.37 nA, after 210 ± 37 secs (Fig 3.29d, n=5). As CBZ does not prevent the PTZ induced current, but inhibits it after it has been produced, this suggests that CBZ can only block the PTZ activated channel after it has been opened by PTZ. Carbamazepine reversed PTZ's effects on the the steady state I-V curve. Figure 3.30 shows that, as previously described, PTZ caused a shift in the steady state I-V curve in the direction of inward current and induced a region of negative slope conductance. Subsequent application of 1 mM CBZ caused a reversal of these PTZ-induced effects and returned the steady state I-V curve to close to its control level (n=5).

Carbamazepine had no effect on the amplitude or $T_{\frac{1}{2}}$ of the cAMP

Fig 3.29. Effect of carbamazepine (CBZ) on the PTZ-induced effects in the B1 cell. (a) Application of 20 mM PTZ induced depolarisation of the cell membrane accompanied by bursts of action potentials. Subsequent addition of 1 mM CBZ abolished these PTZ-induced effects. (b) Prior application of 1 mM CBZ did not prevent 20 mM PTZ from inducing bursting activity but this was short lived and died out with a few minutes. (c) Application of 40 mM PTZ induced an inward current in a voltage clamped cell, held at -60mV. Subsequent application of 1 mM CBZ inhibited the current after a delay of about 3 minutes. (d) Prior application of 1 mM CBZ did not stop 40 mM PTZ from inducing an inward current in the B1 neurone but this current was short lived a decayed after several minutes.

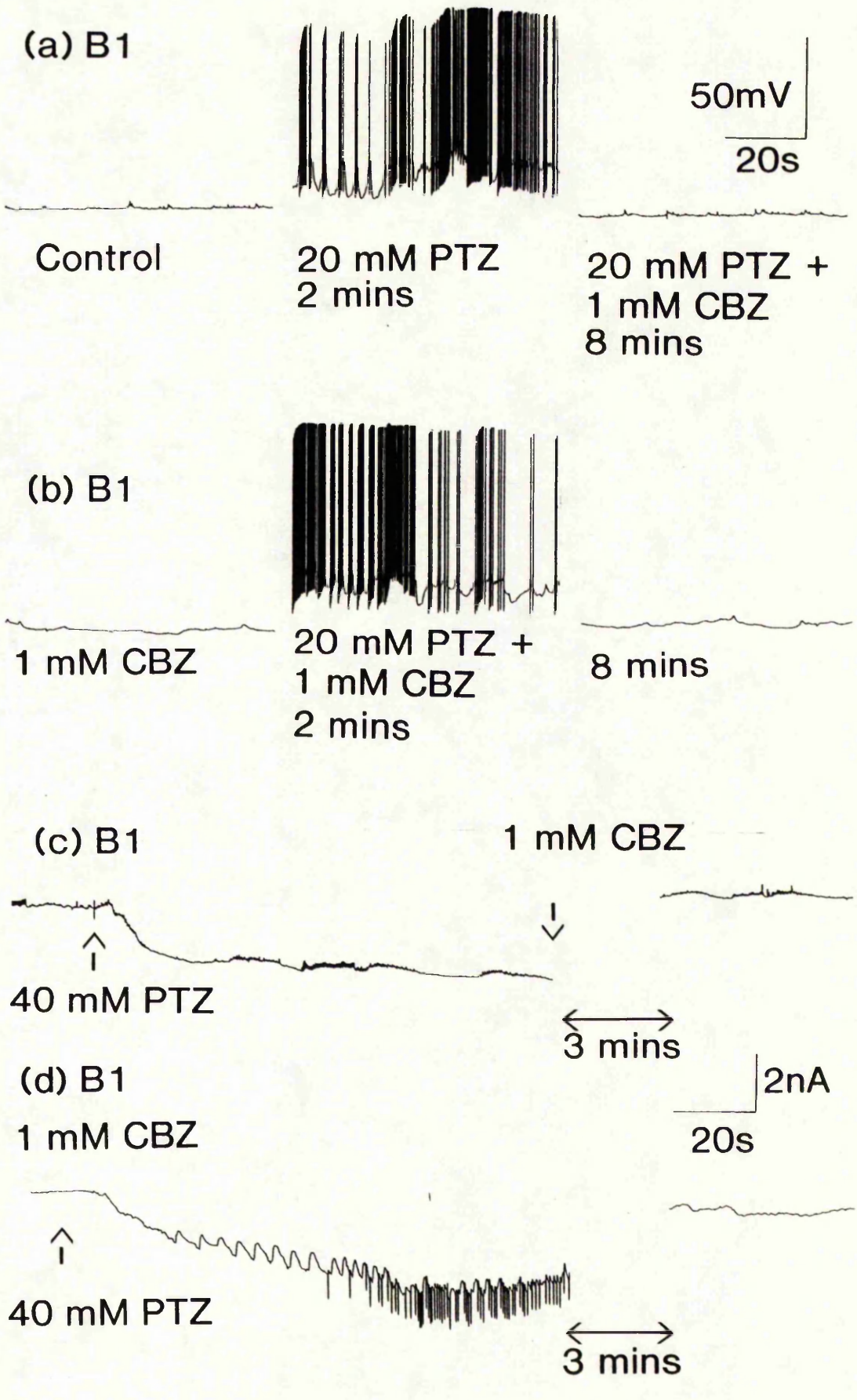
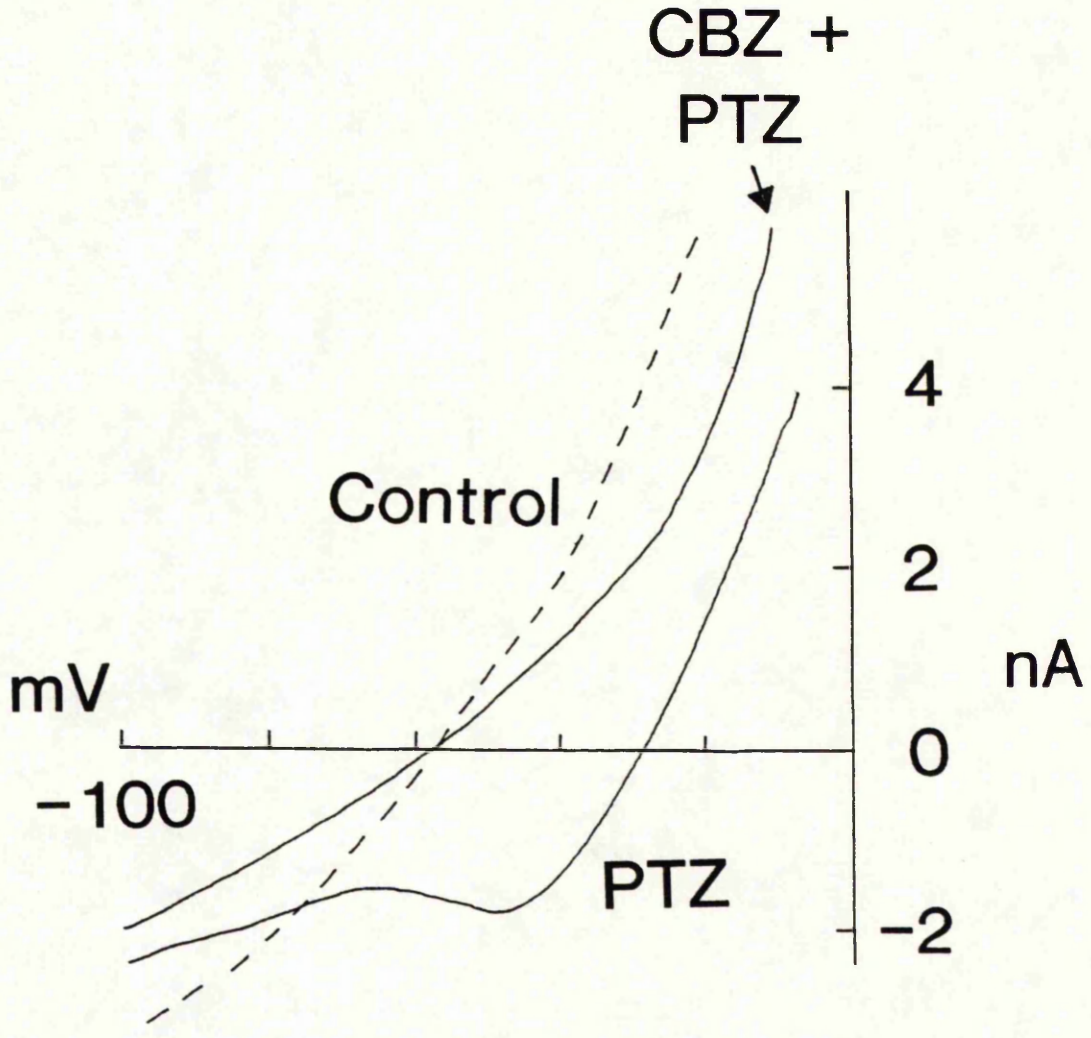

(a) B1
50mV
20s
Control
20 mM PTZ
2 mins
20 mM PTZ +
1 mM CBZ
8 mins
(b) B1
1 mM CBZ
20 mM PTZ +
1 mM CBZ
2 mins
8 mins
(c) B1
1 mM CBZ
40 mM PTZ
3 mins
(d) B1
1 mM CBZ
2nA
20s
40 mM PTZ
3 mins

Fig 3.30. Effect of carbamazepine on the 40 mM PTZ induced effects on the steady state I-V curve. Carbamazepine (1 mM) caused a reversal of PTZ induced effects on the steady state I-V curve. The shift in direction of inward current and the region of negative slope conductance were reversed and the steady state I-V curve returned to close to its control level.


CBZ +
PTZ
Control
PTZ
mV
−100
4
2
0
−2
nA

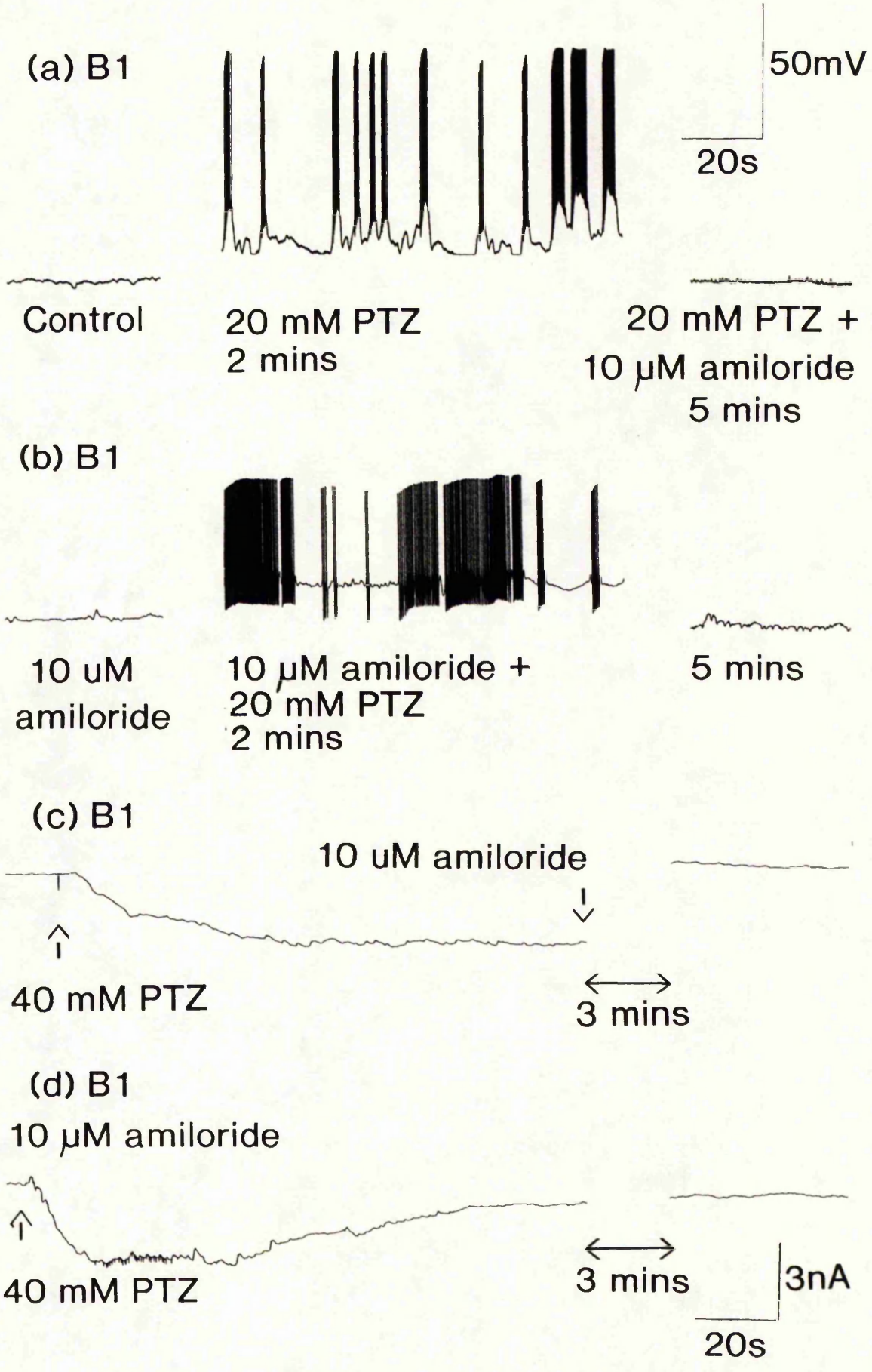
induced inward current in the B1 cell (Fig 3.33a, Table 3.7a, n=5).

These results suggest that CBZ blocks the slow inward current induced by PTZ in the B1 cell. This would inhibit the depolarising current which probably underlies the production of bursting activity induced by PTZ in the B1 cell, and hence explain CBZ's anticonvulsant effects in this preparation. Since CBZ did not affect the cAMP induced inward current, this suggests that the depolarisation produced by PTZ is not due to cAMP induced inward current. Thus it seems that the PTZ- and cAMP-induced inward currents are distinct and are manifested via separate conductance pathways.

Effect of amiloride on PTZ and cAMP induced effects.

Amiloride inhibited PTZ induced effects in the B1 cell. In the B1 cell treated with 20 mM PTZ the membrane depolarised by 14.6 $\pm$ 2.1mV (n=5) and bursting activity was induced. Subsequent addition of 10 uM amiloride caused a delayed reversal of this effect and repolarised the cell membrane by 13.3 $\pm$ 1.8mV (Fig 3.31a). The time taken for amiloride to exert its full effect was 145 + 40 secs (Fig 3.31a). Pretreatment of cells with 10 uM amiloride did not prevent 20 mM PTZ from inducing bursting activity in the B1 cell, leading to depolarisation of the cell membrane by 16.0 $\pm$ 3.2mV (n=5). However this effect of PTZ was short lived and the cell membrane repolarised by 15.8 $\pm$ 4.7mV after 190 $\pm$ 17 secs (Fig 3.31b). Amiloride also inhibited the inward current induced by PTZ in the B1 cell. Application of 40 mM PTZ to a voltage clamped B1 cell induced an inward current of 2.77 $\pm$ 0.38 nA. Addition of 10 uM amiloride caused a reduction of this current to 0.83 $\pm$ 0.28 nA over a period of about

Fig 3.31. Effect of amiloride on PTZ induced effects in the B1 cell. (a) Application of 20 mM PTZ induced bursting activity in the B1 cell. Subsequent application of 10 μM amiloride caused an inhibition of bursting activity and repolarisation of the cell membrane. This effect took about 2 minutes to occur. (b) Prior application of 10 μM amiloride did not prevent 20 mM PTZ from inducing bursting activity in the B1 cell but this activity only lasted about 2 minutes. (c) Application of 40 mM PTZ induced an inward current in the B1 cell, held at -60mV. This current was inhibited by 10 μM amiloride after a period of about 2 minutes. (d) Prior application of 10 μM amiloride did not prevent PTZ from inducing an inward current in the B1 cell but this inward current decayed after about 2 minutes.


(a) B1
50mV
20s
Control
20 mM PTZ
2 mins
20 mM PTZ +
10 μM amiloride
5 mins
(b) B1
10 uM
amiloride
10 μM amiloride +
20 mM PTZ
2 mins
5 mins
(c) B1
10 uM amiloride
40 mM PTZ
3 mins
(d) B1
10 μM amiloride
40 mM PTZ
3 mins
3nA
20s

170 ± 29 secs (Fig 3.31c, n=7). Pretreatment with 10 uM amiloride did not prevent PTZ from inducing an inward current of 5.53 ± 0.29 nA in the B1 cell, but this current however was short lived and decreased by 3.67 ± 0.49 nA after 268 ± 34 secs (Fig 3.31d, n=5). The cAMP induced inward current was not significantly affected by bath application of 10 uM amiloride (Fig 3.33b, Table 3.7b, n=5). The fact that amiloride's blocking effect on PTZ-induced responses was not immediate suggests that its effect is not directly on the PTZ activated channel.

Effect of TMB-8 on PTZ and cAMP induced effects.

In the B1 cell 0.2 mM TMB-8 inhibited PTZ induced bursting (Fig 3.32, n=5). This is the same effect as described in Section I (Fig 3.9a). Application of 20 mM PTZ to the B1 cell resulted in bursting activity. Subsequent addition of 0.2 mM TMB-8 caused an inhibition of this firing activity and repolarised the cell membrane. The cAMP induced inward current was not significantly affected by 0.2 mM TMB-8 (Fig 3.33c, Table 3.7c, n=7).

These results support the hypothesis that increased intracellular Ca^{2+} concentrations play a part in PTZ's mode of action. The lack of effect of TMB-8 on the cAMP induced inward current suggests, as do the effects of CBZ and amiloride, that the PTZ and cAMP induced inward currents are distinct.

Fig 3.32. Effect of trimethoxybenzoic acid (TMB-8) on PTZ induced bursting activity in the B1 cell. Application of 20 mM PTZ induced bursting activity in the B1 cell. Subsequent application of 0.2 mM TMB-8 inhibited this activity and repolarised the cell membrane. This effect took about 10 minutes to occur.

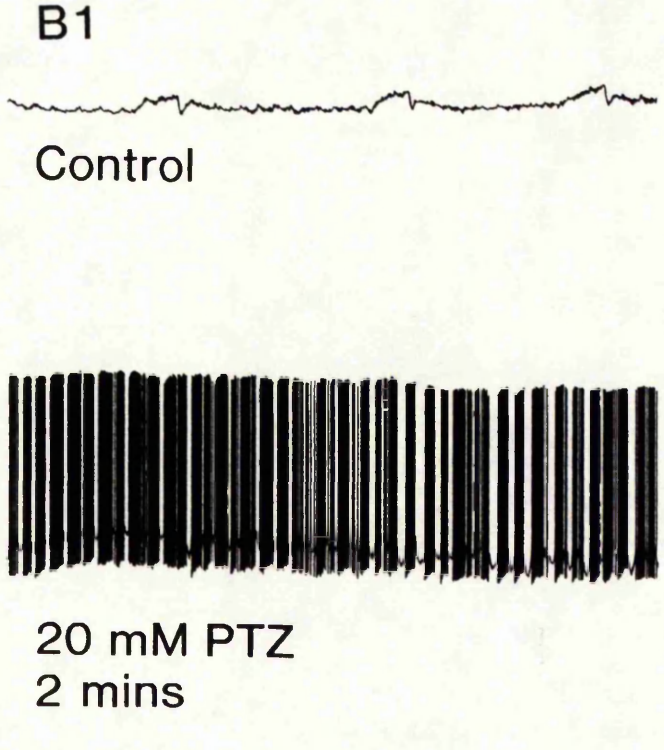

B1
Control
20 mM PTZ
2 mins
20 mM PTZ + 0.2 mM TMB-8
10 mins
50mV
20s

Fig 3.33. Effect of CBZ, amiloride and TMB-8 on cAMP induced current. The cAMP induced current was not significantly affected by either 1 mM CBZ (a), 10 μM amiloride (b) or 0.2 mM TMB-8 (c). Bars indicate 5 second iontophoretic current pulses. See also Table 3.7.

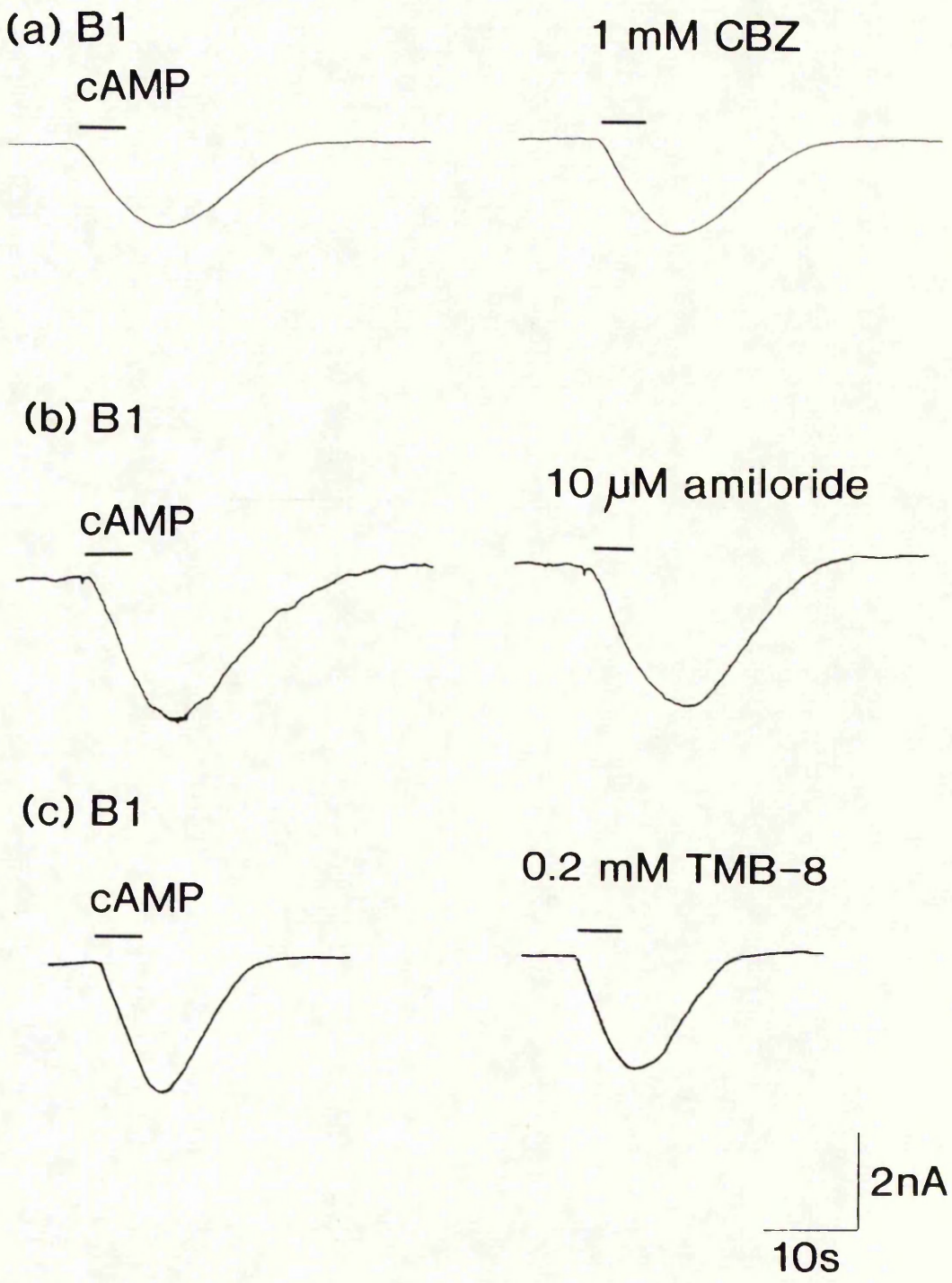

(a) B1
cAMP
1 mM CBZ
(b) B1
cAMP
10 μM amiloride
(c) B1
cAMP
0.2 mM TMB-8
2nA
10s

Table 3.7. Effect of CBZ, amiloride and TMB-8 on the cAMP induced inward currents in the B1 cell. The cAMP induced inward current was not significantly affected by 1 mM CBZ (a), 10 μM amiloride (b) or 0.2 mM TMB-8 (c). Statistics used were a paired t test. Results are shown as mean ± SEM.

TABLE 3.7

(a)	Amplitude (nA)	$T_{\frac{1}{2}}$ (s)
Control	3.23 ± 0.25	23.0 ± 6.0
1 mM CBZ	3.26 ± 0.33	21.0 ± 6.6
	(n=5, n.s.)	

(b)	Amplitude (nA)	$T_{\frac{1}{2}}$ (s)
Control	4.00 ± 0.13	34.0 ± 3.0
10 μM amiloride	3.80 ± 0.24	33.0 ± 6.0
	(n=5, n.s.)	

(c)	Amplitude (nA)	$T_{\frac{1}{2}}$ (s)
Control	2.57 ± 0.47	22.3 ± 5.6
0.2 mM TMB-8	2.81 ± 0.51	26.9 ± 10.3
	(n=5, n.s.)	

CHAPTER 4

DISCUSSION

The opposing effects of PTZ in the two cell types, B1 and RPD1 in Lymnaea offered an excellent opportunity to study the cellular mechanisms underlying PTZ's effects. The particular information sought in this study was to classify specific membrane conductances activated by PTZ in each cell. This would indicate whether PTZ acted via a common mechanism in both cells. The fact that, in most cell types examined, PTZ caused an increase in excitability, suggests that there is a common distribution of receptors and channels which produce this increased activity among the population of cells. Studying the opposing effects of PTZ in B1 and RPD1 might give an insight into the mechanism underlying different cells' susceptibility to seizure, and furthermore provide information about certain intrinsic characteristics possessed by cells which have the capacity to exhibit seizure activity.

Concentration of PTZ used.

The concentration of PTZ used to induce epileptiform activity in molluscan preparations varies from 20 mM (Pacheco et al, 1981) to 120 mM (Williamson and Crill, 1976). The concentration range used in this study was 20 to 40 mM. It has been shown by Lucke et al (1989) that the concentration of PTZ required to induce epileptic attacks in rats and mice varies from 2 mM (80 mg/kg injected i.p.) to 8 mM (125 mg/kg injected i.p.). The concentrations used in this study were higher than this but were still within a reasonable concentration

range.

The effect of 40 mM PTZ on the B1 cell was to induce a PDS. This type of behaviour is never seen in 'normal', untreated neurones.

The bursting activity induced by 20 mM PTZ did not indicate epileptiform activity as this type of behaviour is endogenous to certain cells; e.g. the R15 cell of Aplysia (Levitan and Levitan, 1988).

Site of action of PTZ.

Pentylenetetrazol is an uncharged molecule and is able to cross the cell membrane (Pacheco et al, 1981). However, results presented by Onozuka et al (1983), Pacheco et al (1981) and Hartung and Hermann (1987) indicate that PTZ's site of action is extracellular. Using the D neurone of Euhadra peliomphala, Onozuka et al (1983) gave evidence that PTZ binds to a receptor on the extracellular side of the cell membrane, which is associated with the adenylate cyclase enzyme system. The binding of PTZ to the receptor is thought to result in increased adenylate cyclase activity leading to raised intracellular cAMP levels (Onozuka et al, 1983). The cAMP is thought to cause release of intracellularly stored Ca^{2+} which results in bursting activity in the cell. Pacheco et al (1981) injected PTZ intracellularly into neurones in the visceral and parietal ganglia of Lymnaea stagnalis, to produce an intracellular concentration equivalent to the concentration required extracellularly to induce

bursting activity. They found that intracellularly injected PTZ resulted in a brief increase in activity which ceased as soon as the PTZ injection had stopped. They concluded from this that intracellular injection of PTZ had no significant effect on the neurones. Hartung and Hermann (1987) showed that extracellular application of PTZ induced bursting activity in identified neurones of Aplysia. They found that PTZ inhibited $I_{K,V}$, $I_{K,Ca}$, I_{Na} and I_K. However intracellular injection of PTZ did not affect $I_{K,V}$ or $I_{K,Ca}$ and so it was concluded that PTZ was not acting intracellularly.

The results presented in this thesis show that intracellular injection of PTZ into the B1 and RPD1 neurones, resulting in a calculated intracellular concentration of about 40 mM, caused a transient increase in activity in both B1 and RPD1 cells. This activity cannot be put down to an increase in the cell volume activating stretch receptors as control injections of a similar volume of 0.1 M KCl produced no significant effect. There are several other possible explanations, however. Firstly, PTZ may act intracellularly but is broken down so quickly that the effect is very short lived. Secondly, injected PTZ may move out of the cell and bind with extracellular receptor sites; owing to dilution in the bath, the concentration of PTZ at the external side of the membrane would rapidly decrease leading to short lived effects. However, the finding that injection of PTZ resulted in a transient increase in activity in RPD1 is in direct contrast to the inhibitory effects induced by extracellular application of PTZ, arguing against this second possibility.

The fact that extracellularly applied PTZ induced long lasting effects in both cell types does not necessarily mean that it does act extracellularly. It could cross the cell membrane and act intracellularly. Thus the site of action of PTZ in B1 and RPD1 is unclear; PTZ may act intracellularly, extracellularly or both.

The effects of PTZ are not due to osmolarity changes.

As the concentration of PTZ used in this study was comparatively high, it could be argued that the effects of PTZ were due to osmolarity changes. This has been tested by Williamson and Crill (1976), Pacheco et al (1981) and Fowler and Partridge (1984). Williamson and Crill (1976) and Fowler and Partridge (1984) substituted equimolar sucrose for PTZ and found that this had no significant effect on the cell membrane. Sucrose however, unlike PTZ, is not membrane permeable. Pacheco et al (1981) substitiuted equimolar urea, which is membrane permeable, for PTZ and found that this too had no significant effect on the cell membrane potential. These results provide good evidence that PTZ's effects in molluscan neurones are not due to changes in osmolarity.

The role of extracellular Ca^{2+} in PTZ's effects.

This has been studied by a number of workers, notably Pacheco et al (1981) and Papp et al (1990). Pacheco et al (1981) omitted Ca^{2+} from saline and found that this did not significantly affect the ability of PTZ to induce bursting activity in Lymnaea neurones. However it has been demonstrated by Gillette and Green (1987) that saline from which Ca^{2+} is omitted still contains between 6 and 10 uM Ca^{2+}, owing to leaching from the glassware and impurities in

other chemicals. This would probably still contain enough Ca^{2+} to provide a considerable concentration gradient across the cell membrane (intracellular concentration of Ca^{2+} in molluscan neurones is about 100 nM; Connor and Hockberger, 1985). Thus the conclusion that Ca^{2+} is not needed for PTZ's effects is not necessarily valid. Papp et al (1990) investigated the effect of organic and inorganic Ca^{2+} channel antagonists on PTZ induced bursting in Helix neurones. They found that the divalent cations nickel, cobalt and manganese, at a concentration of 15 mM, inhibited the slow inward current induced by PTZ. They also found that the inorganic Ca^{2+} channel antagonists diltiazem and verapamil inhibited the PTZ-induced slow inward current at concentrations of between 1 and 2 mM. This finding has also been reported by Altrup et al (1991). These results indicate that extracellular Ca^{2+} is important in PTZ-induced effects in Helix neurones, possibly as a carrier or modifier of this current.

Results presented in this thesis agree with the finding that extracellular Ca^{2+} is necessary for PTZ-induced effects. The finding that saline containing Ca^{2+} buffered to 1 uM did not prevent PTZ from inducing bursting activity in the B1 cell does not mean that extracellular Ca^{2+} has no role to play in PTZ-induced effects. Indeed, lowering the concentration of Ca^{2+} in the saline led to PTZ inducing a depolarisation in the B1 cell which was greater than that seen in control saline. This agrees with the findings of Papp et al (1990) who showed that divalent cations inhibit the inward current induced by PTZ. The reason for this is not clear but it is possible that divalent cations "plug" the PTZ activated conductance pathway, or alternatively that divalent cations have surface

potential effects. Extracellular Ca^{2+} may also be used to 'recharge' intracellular Ca^{2+} stores. This is discussed below.

The role of intracellular Ca^{2+} in PTZ's effects.

Intracellular Ca^{2+} is thought to play a pivotal role in PTZ-induced epileptiform activity in molluscan neurones (Sugaya and Onozuka, 1978; Doerner et al, 1982). Onozuka et al (1983) proposed that PTZ activates adenylate cyclase leading to increased levels of cAMP which cause release of stored Ca^{2+} from intracellular lysosome-like granules. The Ca^{2+} is thought to migrate to the internal side of the cell membrane where it acts to produce conformational changes in the cell membrane resulting in bursting activity. Doerner et al, (1982) also suggested that intracellular release of Ca^{2+} is involved in at least part of PTZ's mode of action since carbonyl cyanide m-chlorophenyl hydrozone, a substance which releases mitochondrial Ca^{2+}, mimics the effects of PTZ. Hartung and Herman (1987) however, showed that PTZ is capable of inducing epileptiform activity in Aplysia neurones where the intracellular Ca^{2+} has been buffered by EGTA, suggesting that increased Ca^{2+} may not play a role in PTZ-induced effects in all cell types.

The results in this thesis provide strong evidence to indicate that PTZ does act via an increase in intracellular Ca^{2+} in B1 and RPD1, probably by causing a release of Ca^{2+} from intracellular stores. Intracellular injection of Ca^{2+} mimicked the effects of PTZ in both neurones. In the B1 cell intracellular injection of Ca^{2+} produced similar effects to those induced by bath application of PTZ, namely it induced bursting activity and a sodium dependent, TTX insensitive

inward current. In the RPD1 cell there was again a similarity between the effects produced by extracellular application of PTZ and intracellular injection of Ca^{2+}. Both induced an outward current which was inhibited by D600 which, at the concentration used, inhibits Ca^{2+} dependent K^{+} channel (Gola and Ducreux, 1985). Injection of EGTA, the Ca^{2+} chelator, reversed the effects of PTZ in both cells, again suggesting that PTZ acts via elevation of intracellular Ca^{2+}.

The intracellular Ca^{2+} appears, at least in part, to come from intracellular stores. The finding that TMB-8 reversed the effects of PTZ in both cell types supports this view. There is some controversy however, as to TMB-8's precise mode of action. Ikeda et al (1984) showed that TMB-8 inhibits release of Ca^{2+} from intracellular stores in rat pancreatic acini. However Kojima et al (1986) suggested that TMB-8 inhibits Ca^{2+} influx across the external membrane of adrenal glomerulosa cells. The end result of TMB-8's actions in both cases is the same; it will inhibit an increase in intracellular Ca^{2+}. The time taken for TMB-8 to inhibit the PTZ-induced effects (about 10 minutes) may be due to at least two factors: firstly, the time taken for TMB-8 to diffuse across the cell membrane and block Ca^{2+} release, and secondly, the time taken for the intracellular Ca^{2+} concentration to decrease to a level where it is incapable of inducing excitation. Additional evidence for the hypothesis that PTZ causes release of intracellularly stored Ca^{2+} is found in the effects of extracellularly applied Co^{2+}. If PTZ simply caused a Ca^{2+} influx the addition of Co^{2+}, a Ca^{2+} antagonist (Byerly et al, 1982), would prevent PTZ from having any effect. The results

produced by Co^{2+} in the B1 cell indicate that PTZ has two effects involving Ca^{2+}. The fact that Co^{2+} did not inhibit PTZ's effects completely suggests that increased intracellular Ca^{2+} originates from intracellular rather than extracellular sources. However, the bursting activity induced by PTZ was not maintained in the presence of Co^{2+}. Bursting declined and ceased over a period of about 2 minutes, although it took over 10 minutes for the membrane potential to return to its control level.

Amiloride also inhibited the inward current and bursting activity induced by PTZ in B1. Prior application of amiloride, like Co^{2+}, did not prevent PTZ from inducing short lived effects. It has been reported by Tang et al (1988) that amiloride (30 μM) inhibits T-type Ca^{2+} channels in neuronal tissue. Therefore a possible mode of action of amiloride is that it inhibits Ca^{2+} influx into B1. Since it has been proposed that PTZ causes the release of Ca^{2+} from intracellular stores, any PTZ induced effect relying on this release would die away as the stores became depleted. However, in the absence of Ca^{2+} channel blocker, PTZ's effects were maintained for as long as PTZ was present in the bath, suggesting that the Ca^{2+} stores were being replenished; this could occur via an influx of Ca^{2+} across the neuronal membrane. Prior treatment of the B1 cell with amiloride or Co^{2+} did not prevent PTZ from inducing a short period of bursting activity. This could be due to the release of Ca^{2+} from intracellular stores. However if Ca^{2+} influx is blocked the stores might be unable to refill and PTZ-induced effects would die away. The fact that prior application of amiloride or Co^{2+} did not prevent PTZ from inducing a short period of bursting activity supports the

view that the increase in Ca^{2+} thought to be induced by PTZ is intracellular in origin. Amiloride has also been reported to directly inhibit Na^{+} conductance pathways in epithelial tissue (Sariban-Sohraby and Benos, 1986), but it appears unlikely that amiloride directly inhibits the PTZ-activated conductance pathway as its effect is not immediate.

Carbamazepine inhibited bursting activity and the inward current induced by PTZ in B1. The fact that prior application of CBZ did not prevent PTZ from inducing short lived effects suggests that CBZ does not block the inactive PTZ activated conductance pathway but that it can only block it after it has been opened by PTZ. As CBZ's effects are similar in nature and time course to amiloride's, it seems reasonable to hypothesize that CBZ may be acting in a similar way to amiloride i.e. inhibiting Ca^{2+} influx. Indeed phenytoin, another clinically used anticonvulsant, has been reported to inhibit Ca^{2+} channels (Ferrendelli and Kinscherf, 1977a).

The effects of Co^{2+} in the RPD1 cell were different from those in B1. Cobalt ions did not inhibit PTZ-induced hyperpolarisation even over a period of 60 minutes. This suggests a difference in Co^{2+}'s effects in B1 and RPD1. It is possible that the Ca^{2+} stores in RPD1 contain a greater amount of Ca^{2+} than in the B1 cell, and hence the blocking effects of Co^{2+} did not have such an immediate effect. This seems unlikely as the PTZ-induced hyperpolarisation was maintained for more than an hour, and the Ca^{2+} stores would surely be exhausted in that time (Miller, 1988). Another possibility could be that in RPD1, Ca^{2+} is recycled intracellularly, but in B1 new

extracellular Ca^{2+} is required to replenish depleted Ca^{2+} stores. A less likely possibility is that the Ca^{2+} channels in RPD1 are, unlike those in B1, insensitive to Co^{2+}, but such heterogeneity seems unlikely.

The type of intracellular Ca^{2+} store involved is not known. In Euhadra Sugaya and Onozuka (1978) describe the Ca^{2+} stores as lysosome-like due to their appearance, but no such studies have been carried out in Lymnaea.

It appears therefore that Ca^{2+} ions are important in the generation of PTZ-induced epileptiform activity in Lymnaea neurones. In mammalian tissue this also appears to be the case. In rat cerebral cortex it has been shown that there is a decrease in extracellular Ca^{2+} during PTZ-induced epileptiform activity (Heinemann et al, 1977). This is thought to be due to an influx of Ca^{2+} into cortical neurones. Calcium channel antagonists have also been shown to inhibit bursting activity in rat CA3 hippocampal neurones (Prince, 1978). More recently DeLorenzo (1988) has shown that Ca^{2+} channel antagonists are capable of inhibiting epileptiform activity in mammalian preparations. It is not clear whether the Ca^{2+} influx is maintained during the seizure in mammalian preparations, as it seems to be in B1 and RPD1. It appears that the initial Ca^{2+} influx has two functions: firstly to depolarise the cell, leading to activation of voltage sensitive Ca^{2+} channels resulting in further Ca^{2+} influx, and secondly, to activate Ca^{2+} dependent processes which maintain the epileptiform activity (Traub and Llinas, 1979).

Specific conductances activated by PTZ.

It appears that in each cell type PTZ activated only one conductance pathway; Na^+ current in B1 and K^+ current in RPD1. This contrasts with the findings of Hartung and Hermann (1987), who showed that PTZ affects at least four conductance pathways in an identified Aplysia neurone. It is likely that the PTZ-induced currents in B1 and RPD1 are Ca^{2+} activated since injection of Ca^{2+} ions induced similar currents in the two cells. In B1 blockade of PTZ-induced Na^+ conductance by substitution of extracellular Na^+ did not reveal any residual outward K^+ current. This suggests that Ca^{2+}-dependent K^+ channel is absent in B1 and explains why there was no repolarising phase during PDS. Doerner et al (1981) showed that PTZ-induced PDS in neurones in the visceral and parietal ganglia of Lymnaea is interrupted by periods of repolarisation where the cell membrane repolarised to control levels. This could be due to activation of a Ca^{2+}-dependent K^+ channel. In RPD1, PTZ did not appear to activate Ca^{2+}-dependent Na^+ current as blockade of the PTZ-induced outward current did not reveal any underlying inward current. This would explain PTZ's inability to induce depolarisation and bursting in this cell type.

Both Ca^{2+}-activated Na^+/cation currents and Ca^{2+} activated K^+ current have been documented previously (Ewald and Levitan, 1987). Inward Ca^{2+} activated cation current has been shown to be important during bursting activity. In the R15 neurone of Aplysia, there is a build up of Ca^{2+} inside the cell during a burst of action potentials (Gorman and Thomas, 1980). This Ca^{2+} activates a Ca^{2+}-dependent inward cation current, which results in further excitation

of the cell. (The burst is interrupted by a period of hyperpolarisation caused by activation of a Ca^{2+} dependent K^+ channel). A similar situation may occur in B1, pentylenetetrazol results in increased intracellular Ca^{2+} which activates a Ca^{2+}-dependent Na^+ conductance and leads to an inward current and increased excitation in the cell. The Ca^{2+}-dependent K^+ current is functionally opposite to the Na^+ current in that it results in hyperpolarisation of the cell membrane. In RPD1 the increased intracellular Ca^{2+} produced by PTZ leads to activation of this current, resulting in a long lasting hyperpolarisation of the cell membrane. It appears that B1 and RPD1 each possess only one of these currents; Na^+ and K^+ currents respectively. In D neurones of Euhadra, it appears that both conductances are present (Sugaya et al, 1988); application of PTZ leads to an initial period of bursting activity, due to activation of the Ca^{2+}-dependent Na^+ channel. This gives way however to a period of hyperpolarisation thought to be due to activation of a Ca^{2+}-dependent K^+ channel.

Possible functional roles for the PTZ-activated currents.

The B1 cell is normally quiescent and its function is thought to be to activate the salivary glands during feeding, when it generates bursts of action potentials (Benjamin et al, 1979). In order to do this it must depolarise and an obvious mechanism for this depolarisation is via an influx of Na^+. Whether this involves PTZ/Ca^{2+}-activated Na^+ influx, cAMP-induced Na^+ influx, or both is not known.

The RPD1 cell normally fires in a pacemaker fashion. Presumably such

activity is necessary for its normal function (which is unknown) and this might be impaired if it became either too depolarised or hyperpolarised. It would appear that this cell never or rarely fires bursts of action potentials, and its activity remains fairly constant. Any tendency for the cell to become depolarised, and hence activate voltage dependent cation channels would tend to be countered by the increased intracellular Ca^{2+} activating the Ca^{2+}-dependent K^{+} channel, resulting in hyperpolarisation of the cell membrane.

Cyclic AMP induced a TTX-insensitive inward Na^{+} current in B1 and RPD1.

The mode of action of PTZ in B1 was previously suggested to be via increased intracellular cAMP (McCrohan and Gillette, 1988b), which activated the cAMP induced inward current leading to depolarisation. Since PTZ had been shown to increase the amplitude of the cAMP induced current this appeared plausible (McCrohan and Gillette, 1988b). Indeed PTZ has been shown to increase intracellular cAMP concentrations in rat (Ferrendelli and Kinscherf, 1977a; Onozuka et al, 1989) and molluscan CNS (Onozuka et al, 1983). Since elevated levels of cAMP are intimately linked with epileptiform activity (Ferrendelli, 1984) a role for cAMP in PTZ induced activity in B1 seemed possible. It was also interesting to see whether PTZ's opposing effects on B1 and RPD1 were somehow mediated by cAMP.

Intracellular iontophoresis of cAMP into both B1 and RPD1 induced an inward current. Similar responses have been reported in neurones of Aplysia californica (Pellmar, 1981), Archidoris odheneri (Connor and Hockberger, 1984), Helix pomatia (Aldenhoff et al, 1983), Limax

maximus (Hockberger and Connor, 1984), Lymnaea stagnalis (McCrohan and Gillette, 1988a), Pleurobranchaea californica (Gillette and Green, 1987) and Triopha catalinea (Connor and Hockberger, 1984). Thus this cAMP induced current appears to be a phenomenon common to a number of gastropod molluscs. The resting level of cAMP in molluscan neurones has been calculated to be between 10 and 20 uM (Hockberger and Yamane, 1984). Connor and Hockberger (1984) showed, in neurones of Archidoris, that intracellular iontophoresis of 30-35 uM cAMP was enough to induce depolarisation and firing of action potentials. The amount of cAMP iontophoresed in the present study was not known.

Ionic carrier of cAMP induced current in B1 and RPD1.

The ionic carrier of the cAMP induced current varies between the species. The current appears to be carried solely by Na^+ in B1 and RPD1 neurones of Lymnaea; no residual current (e.g. carried by Ca^{2+}) was seen in zero Na^+ saline. Likewise, in the ventral white cell (VWC) of Pleurobranchaea (Gillette and Green, 1987) and in neurones of Helix (Aldenhoff et al, 1984) the cAMP induced current is carried by Na^+. In neurones of other species, the cAMP induced current is not carried solely by Na^+. For example in Limax maximus, it is carried by both Na^+ and Ca^{2+} (Hockberger and Connor, 1984). In Aplysia, cAMP induced current is not carried by Na^+ at all; rather it is carried by Ca^{2+} (Pellmar, 1981).

The cAMP induced current was TTX insensitive in both B1 and RPD1. This TTX insensitivity has also been demonstrated for neurones of Aplysia kurodi (Hara et al, 1985) and Aplysia californica (Connor and Hockberger, 1984). Thus it appears that in B1 and RPD1 the cAMP

induced current is distinct from the fast Na^+ current which is associated with the rising phase of the action potential.

The cAMP induced current is voltage insensitive in B1 and RPD1.

In this study the cAMP induced current was found to be voltage insensitive over the membrane potential range -100 to -20mV. Similar voltage insensitivity has been shown for the cAMP induced current in the RP1GC of Archidoris (Connor and Hockberger, 1984). This contrasts with cAMP-induced current in the ventral white cell of Pleurobranchaea (Gillette and Green, 1987) and in neurones of Aplysia (Pellmar, 1981; Connor and Hockberger, 1984), which is voltage sensitive, depolarisation resulting in increased amplitude of the current. The presence or absence of voltage sensitivity of the cAMP induced current may have a functional significance. The ventral white cell of Pleurobranchaea is a higher order neurone which initiates feeding behaviour in the animal (Gillette and Gillette, 1983). Its function depends on its ability to generate sustained bursts of action potentials. A possible means by which this is achieved is by the fact that when the cell depolarises the amplitude of the cAMP induced current increases, leading to further depolarisation and increased activity. This induces a region of negative slope conductance on the steady state I-V curve, which is characteristic of bursting neurones (Levitan and Levitan, 1988).

Connor and Hockberger (1984) proposed that apparent voltage insensitivity of cAMP-induced current is due to a decreased driving force for Na^+ ions on depolarisation being equally balanced by a weak voltage sensitivity of the Na^+ current. This would explain why

the cAMP induced current in B1 and RPD1 showed complete independence of voltage for different holding potentials, rather than an ohmic relationship between current and voltage. Thus the voltage sensitivity seen in other neurones may simply be because voltage sensitivity overcomes the decreasing driving force for Na^+ ions. This explanation infers that the cAMP induced current in B1 and RPD1 is, in fact, voltage sensitive, if only weakly.

The function of cAMP induced current in RPD1 is not known, but its function in B1 may be to modulate the input from the buccal central pattern generator. This is a neural network which generates rhythmic feeding motor output (Elliott and Benjamin, 1985). The B1 is a motoneurone which innervates the salivary glands (Benjamin et al, 1979). It receives an input from the central pattern genetrator causing it to fire bursts of action potentials in phase with rhythmic radula movements (Benjamin, 1983). B1 also receives a direct excitatory synaptic input from the cerebral giant cells (CGC: McCrohan and Benjamin, 1980a). These are a pair of giant serotonin containing cells which modulate feeding activity (McCrohan and Benjamin, 1980b). It has been shown that local application of serotonin to B1 induces a depolarisation (Tuersley and McCrohan, 1989) and this is fairly strong evidence that the EPSP's seen in B1 following stimulation of the CGC's are mediated by serotonin. Thus stimulation of the CGC's may result in an excitatory synaptic input to B1 due to release of serotonin from their presynaptic terminals. It has been shown that activation of serotonin receptors on many neurones stimulates adenylate cyclase to produce cAMP (Cedar and Schwartz, 1972). Ocorr and Byrne (1985) have shown that serotonin

produces a dose dependent increase in cAMP in sensory neurones of Aplysia. Therefore activation of the CGC's may result in increased cAMP in B1 which modulates the output to salivary glands via activation of cAMP induced inward current.

The finding that Ca^{2+} did not confer voltage sensitivity on the cAMP induced current in B1 and RPD1 demonstrates a fundamental difference in the cAMP induced current in B1 and RPD1 of Lymnaea and the ventral white cell of Pleurobranchaea. In the latter, Gillette and Green (1987) found that extracellular Ca^{2+} conferred voltage sensitivity on the cAMP induced current. The mechanism of this, however, is not known.

Divalent cations inhibited cAMP induced current in B1 and RPD1.

Divalent cations such as Ca^{2+} and Co^{2+} inhibited the cAMP induced current in B1 and RPD1. Similar results have been found by Gillette and Green (1987) and Aldenhoff et al (1983). Gillette and Green (1987) found that a series of divalent cations inhibited the current, but that Ca^{2+} was the most effective. They concluded from this that there was a fairly specific receptor site for Ca^{2+} binding. The results presented in this thesis, however, showed that, in spite of concentration differences, Co^{2+} inhibited the cAMP induced current in B1 and RPD1 to a greater extent than Ca^{2+}, so that Gillette and Green's theory of a Ca^{2+} selective receptor site does not apply here. A possible way in which the divalent cations could inhibit the current is to 'plug' the cAMP activated conductance pathway. The inhibition may not be as simple as that, however, and may a involve a more complicated mechanism, such as changes in surface potential. In

neurones of Limax, the current is insensitive to the divalent cations Cd^{2+} and Mn^{2+} (Hockberger and Connor, 1984). This suggests that the cAMP activated conductance pathway in Limax is different from those described in this thesis and by Gillette and Green (1987). Indeed the cAMP induced current in neurones described here and by Gillette and Green (1987) is carried by Na^{+}, whereas the cAMP induced current is carried by Na^{+} and Ca^{2+} in neurones of Limax (Hockberger and Connor, 1984).

Increased intracellular cAMP excites B1 and RPD1.

Intracellular iontophoresis of cAMP induced prolonged bursting activity in B1. Similar results have been shown in neurones of Archidoris (Connor and Hockberger, 1984), Helix (Aldenhoff et al, 1983; Kononenko et al, 1983), Pleurobranchaea (Gillette and Green, 1987). This was reinforced by the finding that agents which result in increased intracellular cAMP (membrane permeable cAMP analogues; e.g. CPTcAMP, and phosphodiesterase inhibitors; e.g. IBMX) induced bursting activity in B1.

In RPD1, intracellular iontophoresis of cAMP induced only a short lived increase in activity. This increase lasted for only as long as the period of iontophoretic injection. This seems an odd result. Since the cAMP induced currents were very similar in B1 and RPD1 it would be expected that injected cAMP would have similar effects on membrane potential. Moreover, this effect cannot be explained by RPD1 having a greater activity of phosphodiesterase, as if this were the case the $T_{\frac{1}{2}}$ of RPD1 would be less than that for B1.

It is surprising that CPTcAMP had no effect on RPD1. The most likely explanation for this is that CPTcAMP was unable to cross the cell membrane. It cannot be explained by CPTcAMP diffusing across the membrane very slowly, as CPTcAMP is not broken down by phosphodiesterase (McCrohan and Gillette, 1988a) no matter how slowly it diffused across the membrane, eventually a high enough concentration would accumulate in the cell to cause excitation.

In B1 and RPD1, the effects of IBMX on the cAMP induced current were what would be expected of a compound which inhibited phosphodiesterase. There was an increase in the amplitude of the current in B1, presumably due to the iontophoresed cAMP not being broken down quickly by phosphodiesterase. The increase in $T_{\frac{1}{2}}$ in both cells would be due to a decrease in the rate of breakdown of cAMP. The effect was dose dependent and appeared to saturate at about 0.1 mM IBMX.

The lack of effect of IBMX on firing activity in RPD1, however, was unexpected. IBMX is membrane permeable in this cell, as it affected the cAMP induced current. The absence of any significant effect may be put down to RPD1 having a very low resting concentration of cAMP. Thus IBMX would be unable to increase cAMP levels and no increased excitation would be seen.

PTZ has opposing effects on cAMP induced current in B1 and RPD1.

McCrohan and Gillette (1988b) reported that bath application of PTZ caused an increase in the amplitude of the cAMP induced inward current in the B1 cell. They drew the conclusion that PTZ was acting

to increase intracellular cAMP levels, by acting as a phosphodiesterase inhibitor. This seemed a logical conclusion as both PTZ and cAMP induced bursting activity in the B1 cell, probably due to activation of a TTX-insensitive, inward Na^+ current. In RPD1, PTZ resulted in a significant decrease in the amplitude of cAMP induced current. This PTZ induced effect cannot be explained by a decrease in intracellular cAMP as the same amount of cAMP was iontophoresed under control and test conditions. A possible explanation is that PTZ acts to increase phosphodiesterase activity; Gillette and Green (1987) have shown that increased intracellular Ca^{2+} may act to stimulate phosphodiesterase. However this theory can be discounted; although fairly strong evidence has been provided to show that PTZ results in increased Ca^{2+} in RPD1, intracellular injection of Ca^{2+} did not affect the amplitude of the cAMP induced current in RPD1. Another possible way that PTZ may act in RPD1 is either to affect the cAMP activated conductance pathway directly, or to affect cAMP induced protein phosphorylation. What is clear is that changes in the magnitude of cAMP-induced effects cannot account for PTZ's actions, particularly in RPD1.

The cAMP induced current is not altered by intracellular Ca^{2+}.

In B1 it seems unlikely that PTZ acts in the same way as it is thought to act in the D neurone of Euhadra (Onozuka et al, 1983); namely that PTZ increases intracellular cAMP which releases intracellularly stored Ca^{2+} resulting in bursting activity. The fact that increased intracellular Ca^{2+} (injected) did not affect the cAMP induced current suggests that Onozuka's theory is not applicable in the B1 cell. Kononenko et al (1986), have shown that

increased intracellular Ca^{2+} results in an increase in amplitude of the cAMP induced current in Helix neurones. They concluded that the cAMP activated channel had two phosphorylation centres, one Ca^{2+} / calmodulin dependent, and the other cAMP dependent. Phosphorylation of the Ca^{2+} / calmodulin dependent centre converts the channel to a "pre active" non conducting state, with subsequent addition of cAMP resulting in phosphorylation of the second centre and converting the channel into a conducting state. Hence, the greater the intracellular Ca^{2+} concentration the greater the number of channels available for activation.

In contrast the results in this thesis suggest that increased intracellular Ca^{2+} concentrations do not play a role in activation or inactivation of the cAMP induced inward current in either B1 or RPD1.

Distinction between PTZ- and cAMP-induced currents.

The difference in voltage sensitivity of the PTZ and cAMP induced currents in B1 indicates a fundamental difference between them, and suggests that they are distinct. Pentylenetetrazol induced a region of negative slope conductance in the steady state I-V curve, over the membrane potential range -65 to -40mV. This region of negative slope conductance indicates a region of inward current development. In a cell where there is a region of negative slope conductance close to threshold, there is a continuous flow of inward current (assuming the region of negative slope conductance is below the zero current axis). Release from voltage clamp of this cell would drive the membrane potential to action potential threshold and result in high frequency

action potential firing. Thus the cell appears unstable and highly excitable. Such an I-V curve is seen in the endogenously bursting cell, R15, of Aplysia (Benson and Adams, 1987). The ability of PTZ to induce a region of negative slope resistance has also been shown in neurones of Aplysia (David et al, 1974), Euhadra (Sugaya et al, 1978) and Tritonia (Partridge, 1975). In each of the above examples PTZ induces bursting activity which can be explained by the development of an inward current occurring around threshold.

The distinction of the two currents was also demonstrated in the effects of amiloride, CBZ and TMB-8. The actions of Co^{2+}, TMB-8 and amiloride suggested that PTZ acts to release Ca^{2+} from intracellular stores, which are subsequently refilled by an influx of Ca^{2+} across the neuronal membrane. Carbamazepine may act in the same way as amiloride. Since none of these drugs inhibited the cAMP induced current in B1 this provides evidence, along with the difference in voltage sensitivity, that the PTZ and cAMP induced inward currents are distinct.

Comparison of epileptogenic activity in B1 and mammalian preparations.

Generation of seizure activity in both mammalian and molluscan preparations is brought about by depolarisation of the neuronal membrane leading to PDS. In mammalian studies it has generally been shown that an inward current, sometimes accompanied by an outward current, leads to PDS. In cat cortical neurones the PDS is accompanied by a decrease in extracellular Ca^{2+} and an increase in extracellular K^{+} in the medium in intimate proximity with the

neurones (Heinemann et al, 1977). This was interpreted as a Ca^{2+} influx and a K^{+} efflux occurring simultaneously, and supporting seizure activity. In CA3 neurones of guinea pig Bingmann and Speckmann (1986) showed that decreased extracellular Ca^{2+} caused a reduction in frequency and duration of experimentally induced PDS. Experiments with Ca^{2+} channel antagonists have provided further evidence that Ca^{2+} influx is vital in the generation of seizures. In DBA/2 mice, a strain genetically prone to sound-induced seizures, injection of Ca^{2+} channel antagonists inhibited electroshock induced seizures in mice (DeSarro et al, 1988). Similarly Ca^{2+} channel antagonists inhibited electroshock induced seizures in mice (Wong and Rahwan, 1989). Thus it appears a general feature of mammalian epilepsy that generation of epileptic seizures is brought on by an influx of Ca^{2+} ions.

In B1 it appears that the current underlying PTZ-induced PDS is solely carried by Na^{+}. The influx of Na^{+} is brought about by increased intracellular Ca^{2+}. Similarly, in mammalian neurones, it is thought that the influx of Ca^{2+} does not induce the seizure; rather it is the intracellular effect of this Ca^{2+} that stimulates epileptiform activity (Traub and Llinas, 1979). It has been shown that there is a delay between the influx of Ca^{2+} and the onset of seizure activity (Traub and Llinas, 1979). This has been explained as the time taken for Ca^{2+} to have its intracellular effects. The suggested intracellular actions of Ca^{2+} include enhanced neurotransmitter release and phosphorylation of protein kinases (Katz and Miledi, 1967; Cheung 1980). It is possible that similar events occur in B1.

In mammalian cells cAMP has been shown to be intimately associated with epileptic seizures. In rat brain, increased intracellular cAMP was shown to precede seizure discharges (Purpura and Shofer, 1972). However in rat cortical cells, PTZ-induced seizure activity was accompanied by an increase in cAMP levels which preceded onset of seizure activity (Onozuka et al, 1989). In mouse brain, it has been shown that PTZ induced seizure activity is accompanied by localised increases in cAMP (Ferrendelli and Kinscherf, 1977b). This increase in cAMP occurred after development of the seizure, and as such appears to be the result of, rather than the cause of seizure activity. The results of the present study suggest that, contrary to the previous hypothesis (McCrohan and Gillette, 1988b), cAMP may play only a minor role in initiating PDS in the B1 neurone. Indeed, cAMP and its analogues were incapable of inducing PDS in this cell, though they did increase excitation. The major effect of PTZ appears to be via increased intracellular Ca^{2+}. However, enhancement of cAMP-induced Na^{+} current by PTZ would contribute to depolarisation. In RPD1, cAMP-induced effects would, if anything, oppose the inhibitory actions of PTZ.

Summary and Conclusions.

It appears that PTZ's mode of action in B1 and RPD1 is similar, i.e. release of intracellularly stored Ca^{2+}, presumably replenished by an influx of Ca^{2+} across the cell membrane. The opposing responses seen in the two cells appear to be due to different ionic conductances activated by the increased levels of Ca^{2+} - I_{Na} in B1 and Ca^{2+}-dependent I_K in RPD1. Thus cells with different intrinsic membrane properties may show differential susceptibility to

seizure. Such underlying mechanisms can be dissected in this way (i.e. at the level of ion currents) in identified molluscan neurones. However it seems likely that differential susceptibility to seizure also occurs in mammalian cells. It appears that Ca^{2+} ions, rather than cAMP are the major intracellular mediator of PTZ's effects. However, enhanced cAMP-induced current may supplement Ca^{2+} mediated effects in B1. In RPD1 however, a role for cAMP in mediating PTZ's action is unlikely.

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FULL PAPERS

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Differential responses of two identified neurones of the pond snail Lymnaea stagnalis to the convulsant drug pentylenetetrazol. (In prep)

A M BROWN & C R McCROHAN

Properties of a cyclic AMP-induced inward current in two identified neurones of the snail Lymnaea stagnalis. (In prep).

A M BROWN & C R McCROHAN

Separation of two inward Na^+ currents in an identified molluscan neurone using voltage clamp and pharmacological agents. (In prep).

BOOK CHAPTERS

A M BROWN, C R McCROHAN & P PAMPLIN

Properties of Na^+ currents induced by pentylenetetrazol and cyclic AMP in an identified neurone of Lymnaea stagnalis.

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