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Differential modulation of voltage-activated conductances by intracellular and extracellular cyclic nucleotides in leech salivary glands

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1 Two-electrode voltage clamp was used to study the effects of adenosine 3':5'-cyclic monophosphate (cyclic AMP) and guanosine 3':5'-cyclic monophosphate (cyclic GMP) on voltage-dependent ion channels in salivary gland cells of the leech, *Haementeria ghilianii*.

2 Intracellular cyclic AMP specifically blocked delayed rectifier K^+ channels. This was shown by use of 3-isobutyl-1-methylxanthine (IBMX, a phosphodiesterase inhibitor), forskolin (an activator of adenylyl cyclase) and intracellular injection of cyclic AMP and its dibutyryl and 8-bromo analogues. Cyclic AMP appeared to be the second messenger for the putative neuroglandular transmitter, 5-hydroxytryptamine.

3 Intracellular injection of cyclic GMP specifically potentiated high-voltage-activated (HVA) Ca^{2+} current and the effect was mimicked by zaprinast, an inhibitor of cyclic GMP-dependent phosphodiesterase.

4 Extracellularly, cyclic GMP and cyclic AMP specifically decreased the amplitude and increased the rate of inactivation of HVA Ca^{2+} current. These effects of the cyclic nucleotides are identical to those known for extracellular ATP, which activates a presumed purinoceptor. The pyrimidine nucleotide, UTP, was almost equipotent to ATP (threshold dose $<10^{-6}$ M), indicative of a vertebrate-type nucleotide receptor. However, suramin (5×10^{-5} M), a non-specific P_2-receptor antagonist, failed to block the effects of 5×10^{-6} M ATP (higher suramin doses could not be reliably tested because of the depolarization and increase in membrane conductance produced by the drug).

5 Activation of the putative purinoceptor by ATP did not affect inward rectifier Na^+/K^+ current which is known to be potentiated by intracellular cyclic AMP and reduced by intracellular cyclic GMP.

6 The preparation may provide a useful model for study of nucleotide actions, and interactions, in channel modulation. It has technical advantages such as large cells (1200 μm in diameter) which lack intercellular coupling and may be individually dissected for biochemical studies.

Keywords: cyclic AMP; cyclic GMP; ATP; purinoceptor; ion channels; leech salivary glands

Introduction

The electrically excitable salivary gland cells of the giant Amazon leech, *Haementeria ghilianii*, possess a variety of voltage-activated channels which are modulated by cyclic nucleotides. Intracellularly, adenosine 3':5'-cyclic monophosphate (cyclic AMP) modulates delayed rectifier K^+ channels and inwardly rectifying Na^+/K^+ channels, while intracellular guanosine 3':5'-cyclic monophosphate (cyclic GMP) has an opposing action on the inward rectifier (Wuttke & Berry, 1991; 1992). Extracellularly, both nucleotides reduce the duration of action potentials, apparently by blocking Ca^{2+} channels (Wuttke & Berry, 1993). In the present study we analyse in more detail the effects of these substances on the Ca^{2+} and K^+ channels responsible for the generation of gland-cell action potentials.

Cyclic AMP appears to mediate many effects of the putative neuroglandular transmitter, 5-hydroxytryptamine (5-HT). For example, the gland cells produce calcium-dependent action potentials which are increased in duration by 5-HT through a depression of K^+ conductance, and this effect is mimicked by 8-bromo-cyclic AMP. Several other actions of 5-HT are also mimicked by 8-bromo-cyclic AMP, but cyclic AMP, dibutyryl cyclic AMP and the phosphodiesterase inhibitor, theophylline, are without effect (Wuttke & Berry, 1991; 1992). In this study we have examined the possible link between 5-HT and cyclic AMP by use of forskolin (an activator of adenylyl cyclase), 3-isobutyl-1-methylxanthine (IBMX, a more powerful phosphodiesterase inhibitor), and by intracellular injection of cyclic AMP and its 8-bromo and dibutyryl analogues. These experiments support the view that 5-HT acts via cyclic AMP to reduce a voltage-gated K^+ conductance, with no effect on Ca^{2+} currents.

Cyclic GMP, added to the bathing medium, also modulates gland-cell action potentials, though the effect is extracellular (Wuttke & Berry, 1993). To test for intracellular actions, we have used 8-bromo-cyclic GMP (a membrane-permeable analogue), zaprinast (an inhibitor of cyclic GMP-sensitive phosphodiesterase) and intracellular injection of cyclic GMP. The effects of this cyclic nucleotide are shown to be similar to those of cyclic AMP in prolonging the duration of action potentials but the mechanism is different: voltage-activated K^+ channels are unaffected but voltage-activated Ca^{2+} currents are potentiated.

The extracellular effect of cyclic GMP (and cyclic AMP) is a reduction of spike duration which appears to result from stimulation of a P_2-like purinoceptor. ATP (threshold concentration $<10^{-6}$ M) is the most potent of a range of adenine and guanine nucleotides which specifically reduce a high-voltage-activated Ca^{2+} conductance in the salivary cells (Wuttke & Berry, 1993). We show here that the two cyclic nucleotides also reduce this conductance when applied extracellularly.

The unusual diversity of nucleotide actions on single cells, together with special technical advantages of the preparation, may allow electrophysiological and biochemical study of the integration of responses to more than one nucleotide modulator.

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Methods

Preparation

Salivary glands were dissected from specimens of the giant Amazon leech *Haementeria ghilianii* (de Filippi) obtained from our breeding colony and maintained in freshwater aquaria at 25°C. Experiments were performed on the anterior salivary glands which were secured to a layer of Sylgard at the base of a Perspex experimental bath (volume 0.25 ml) by placing pins through the overlying endothelium. Physiological saline or other solutions (see below) flowed through the bath at a rate of about 10 bath volumes min^{-1}. Solutions could be changed rapidly without affecting recording conditions (Wuttke & Berry, 1988).

Electrophysiology

The largest gland cells (800–1200 μm in diameter) were generally chosen for study. Individual cells were impaled under visual control with two bevelled, KCl-filled microelectrodes (10–25 MΩ) mounted on high speed steppers (Digitimer SCAT-02). One electrode was used for recording membrane potential and the other for passing current to alter the membrane potential or elicit action potentials. It was not necessary to remove the overlying sheath or soften it with enzymes, but it was difficult to maintain stable recordings with two electrodes, probably because of the invaginated nature of the cell membrane (Walz *et al.*, 1988); electrodes tended to come out gradually from the cell. The steppers facilitated repositioning or repenetration without loss of membrane potential. The electrodes were connected to an Axoclamp 2-A amplifier (Axon Instruments), and signals were monitored on a storage oscilloscope (Tektronix 5111) and pen recorder (Brush 2200S). An Ag-AgCl pellet in direct contact with the bath fluid was used as a reference electrode.

The Axoclamp amplifier was also used for measuring membrane current, in which case a grounded shield was placed between the two electrodes to minimize capacitative coupling. The gain was normally set around 5000 V/V, and voltage steps were controlled by a Master-8 pulse generator (A.M.P.I.). Leakage currents were subtracted electronically before displaying the data. Electrodes were positioned at an angle of 60° to each other to minimize spatial nonuniformities of membrane potential (Eisenberg & Engel, 1970), and their tips were separated by about one-third of the cell diameter. The degree of space clamp of these giant cells is uncertain but does not appear to introduce serious errors (Wuttke & Berry, 1991); in this respect the cells have the unusual advantage that they lack the intercellular coupling which is a property of most other exocrine gland cells (Wuttke & Berry, 1988). However, the fragility of the cells, and their large size, meant that we had to use relatively high-resistance electrodes that passed a restricted current and limited the amplitude of voltage clamp pulses. The smaller cells probably have similar properties and can be clamped over a larger range, but the giant cells were chosen for comparison with earlier studies and because they form a clearly defined population.

Solutions and drugs

Physiological saline contained (mM): NaCl 125, KCl 4, $CaCl_2$ 1.8, glucose 11, Tris maleate 10, at room temperature (18–22°C). The pH was adjusted to 7.4 with NaOH. In some experiments 50 mM tetraethylammonium (TEA) and 10 mM 4-aminopyridine (with an equimolar reduction in Na^+) were used to block K^+ channels, and 10 mM Co^{2+} was used to block Ca^{2+} influx.

Drugs were applied extracellularly by perfusion or intracellularly by pressure injection from the voltage recording electrode (Narishige IM-200 Microinjector). For injection, drugs were dissolved in 100 mM KCl solution and a positive pressure of 18–30 kPa was applied to the electrodes between applications. In some current clamp experiments, however, a single microelectrode served for recording, passage of current and pressure injection. In this case, depolarizing pulses were not used to elicit action potentials because the large stimulus artifacts could not be reduced sufficiently; the problem was avoided by initiating impulses as anode-break responses to applied hyperpolarizing current (e.g. Figure 2; see Wuttke & Berry, 1988). Preliminary experiments using Fast Green (to stain the cell) or TEA (to prolong the action potential) were carried out to establish appropriate injection procedures. Checks were made that the pressure pulse ejected the substance before and after impalement (it could be seen emerging from the tip). Even this, however, did not guarantee that ejection occurred inside the cell, and in some cases it was clear that nothing was coming out of the electrode. In cases where 'injected' drugs had no effect, it was assumed that the electrode was blocked. All the substances injected produced consistent effects on at least 5 cells.

The following substances were used: adenosine 5'-triphosphate (ATP), adenosine 3':5'-cyclic monophosphate (cyclic AMP), guanosine 3':5'-cyclic monophosphate (cyclic GMP), dibutyryl and 8-bromo derivatives of cyclic AMP, 8-bromocyclic GMP (all sodium salts), 5-hydroxytryptamine creatinine sulphate (5-HT), 3-isobutyl-1-methylxanthine (IBMX), zaprinast (2-*o*-propoxyphenyl-8-azapurin-6-one), forskolin, tetraethylammonium chloride (TEA) and 4-aminopyridine. All chemicals and drugs were obtained from Sigma Chemical Co. apart from zaprinast (M&B22948) and suramin which were kindly supplied by Rhône-Poulenc Rorer and Bayer U.K., respectively.

Results are expressed as mean ± standard deviation, and the significance of effects is evaluated by paired two-tailed *t* tests.

Results

Voltage-activated conductances in the salivary gland cells

The salivary gland cells of *Haementeria* are electrically excitable, producing overshooting, Ca^{2+}-dependent action potentials of 70–110 mV in amplitude and 100–400 ms in duration. The resting membrane potential ranges from −40 to −70 mV (Wuttke & Berry, 1988; 1990). Na^+ ions normally make no measurable contribution to the action potential, but Na^+-dependent action potentials are produced in the absence of external Ca^{2+} (Wuttke & Berry, 1988). Depolarizing voltage clamp steps from the resting membrane potential (−40 to −70 mV) elicit an inward Ca^{2+} current followed by an outward K^+ current. These currents may be studied individually by blocking the Ca^{2+} channels with 10 mM Co^{2+} or the K^+ channels with 50 mM TEA plus 10 mM 4-aminopyridine (Wuttke & Berry, 1991). Depolarizing voltage clamp steps from −70 mV elicit a two-phase inward current consisting of an initial fast, transient component (<500 ms) followed by a longer lasting component that may take up to 30 s to decay to zero (Wuttke & Berry, 1993). Depolarizing steps applied from more positive levels (approximately −40 mV) elicit the longer lasting component on its own, indicating the presence of at least two types of Ca^{2+} channel: low-voltage-activated (LVA) and high-voltage-activated (HVA). Attention was focused on the HVA current, which is specifically modulated by extracellular ATP (Wuttke & Berry, 1993), and voltage steps were generally made from a holding potential close to −40 mV. During the course of an experiment, a voltage or current clamp pulse (or series of pulses) was applied continuously every 1 or 2 min. Consistent responses were obtained for at least 3 h, provided the electrodes remained in position. Control recordings were made for 20–30 min before testing for the effects of drugs.

Depolarizing steps were necessarily restricted in amplitude because it was not usually possible to pass sufficient current through the microelectrode to clamp cells adequately at positive potentials. Use of low resistance electrodes caused reduc-

tion of membrane potential and excitability (this extreme sensitivity may be a feature of leech salivary cells; e.g. Marshall & Lent, 1988).

Intracellular cyclic AMP prolongs gland-cell action potentials by reducing K^+ current

Effects of IBMX Previous attempts to elevate levels of intracellular cyclic AMP by application of the phosphodiesterase inhibitor, theophylline, produced little effect on membrane properties even at concentrations as high as 2 mM (Wuttke & Berry, 1991). However, in the present experiments, IBMX (5×10^{-4} M) increased the duration of action potentials by $209 \pm 35\%$, increased their amplitude by $13 \pm 3\%$ and reduced their undershoot by $44 \pm 5\%$ ($n = 5$) (in each case $P < 0.001$) with no change in resting membrane potential (Figure 1a). Full recovery occurred after washing out the IBMX for about 1 h.

These effects on the action potential are similar to those of 5-HT which specifically reduces a voltage-gated K^+ current (Wuttke & Berry, 1991). Voltage clamp experiments showed that IBMX also reduced the K^+ current, with no effect on Ca^{2+} current (Figure 1b,c). In Figure 1b, a step depolarization of 40 mV from a holding potential of −45 mV elicits an inward Ca^{2+} current followed by an outward K^+ current. The K^+ current was isolated by addition of 10 mM Co^{2+} which eliminated the inward current and showed that IBMX reversibly reduced the amplitude of the outward current ($76 \pm 15\%$, $n = 5$, $P < 0.001$). Tests for effects on the Ca^{2+}

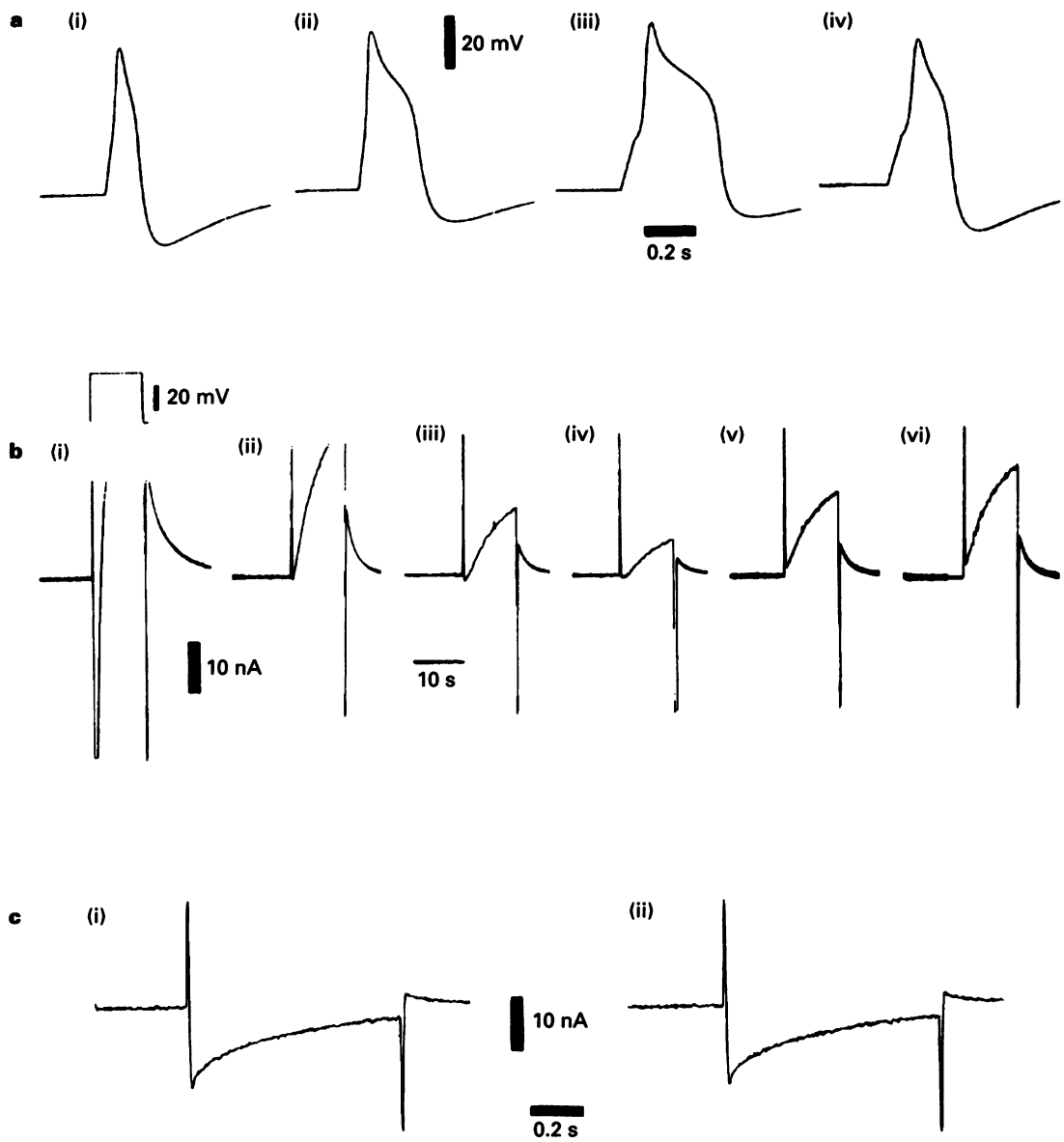

Figure 1 3-Isobutyl-1-methylxanthine (IBMX, 5×10^{-4} M) prolongs gland-cell action potentials by reducing K^+ current. (a) Effect of IBMX on action potentials. (a(i)) Control. (a(ii)) and (a(iii)) Action potentials after exposure to IBMX for 8 and 10 min respectively; (a(iv)) partial recovery after 40 min washout of IBMX. Each action potential was elicited by a depolarizing pulse (25 nA, 200 ms). (b) Effect of IBMX on K^+ current. In each recording the currents were evoked by a 10 s, 40 mV depolarizing pulse from a holding potential of −45 mV (shown for first response only). (b(i)) In normal saline a brief inward current is followed by a maintained outward current with the peak off scale; (b(ii)) 30 min after addition of 10 mM Co^{2+} the inward current is abolished and the outward current substantially reduced. (b(iii) and b(iv)) IBMX reduces the current after 5 and 12 min respectively. Five successive recordings of identical amplitude were made before introducing IBMX. Partial recovery is seen after washout of IBMX with Co^{2+}-containing saline for 12 min (b(v)) and 20 min (b(vi)). (c) IBMX has no effect on inward Ca^{2+} current: (c(i)) control response to a depolarizing pulse (27 mV, 0.8 s) applied from a holding potential of −50 mV in saline containing 50 mM TEA and 10 mM 4-aminopyridine to block K^+ currents. (c(ii)) The current is unaltered after 20 min exposure to IBMX.

current were made by blocking K^+ channels with 50 mM TEA and 10 mM 4-aminopyridine. IBMX had no measurable effect on the amplitude of the Ca^{2+} current (Figure 1c; $n=4$). The response to IBMX was dose-dependent; for example, 2×10^{-4} M IBMX increased spike duration by $24\pm4\%$ ($n=4$, not shown).

The presence of the phosphodiesterase inhibitor should potentiate the effects of any neurotransmitter that mediates its effects by an increase in cyclic nucleotide levels. At 10^{-4} M, IBMX had no measurable effect on gland-cell action potentials but increased the effect of 10^{-6} M 5-HT on spike duration by 13 ± 2 % ($n=4$, not shown). These effects of IBMX, mimicking and potentiating those of 5-HT, lend support to the view that 5-HT operates via cyclic AMP.

Effects of intracellular injection of cyclic AMP analogues Addition of the membrane-permeable dibutyryl cyclic AMP (1 mM) to the bathing medium produced no effect on action potentials ($n=3$) while 8-bromo-cyclic AMP (1 mM) increased their duration by $34\pm7\%$ ($n=3$), confirming the results of Wuttke & Berry (1991). However, these results are potentially ambiguous because of possible extracellular actions in addition to the expected intracellular effects (see below), and we therefore pressure-injected cyclic AMP and its analogues directly into individual salivary cells. The amplitude and duration of the pressure pulse depended on factors such as the extent of microelectrode bevelling and the tendency of the electrode to block within the cell. The experiments reported in this section used 470 kPa pulses applied for 10 s, and electrodes contained a 10 mM concentration of cyclic nucleotide.

Cyclic AMP, dibutyryl cyclic AMP and 8-bromo-cyclic AMP all increased the amplitude and duration of action potentials within 1–1.5 min, reaching a maximum after 3–5 min ($n=5$ in each case; Figure 2). The maximum increase in duration ranged from 45–95%. Recovery was variable (10–30 min), with 8-bromo-cyclic AMP taking no less than 20 min. Experiments similar to those illustrated in Figure 1 showed that intracellularly injected cyclic AMP did not affect the Ca^{2+} current but reduced the K^+ current by $32\pm8\%$ for a voltage step of 30 mV from a holding potential of −40 mV ($n=6$, $P<0.001$; data not shown).

Effects of forskolin The adenylyl cyclase activator, forskolin, mimicked the effects of 5-HT and cyclic AMP in prolonging the duration of action potentials (Figure 3). At 10^{-5} M, spike duration progressively increased by a maximum of $240\pm26\%$ ($n=6$) after 30 min, with complete recovery after washing out the drug for 5 min. Forskolin at 10^{-4} M increased spike duration by $266\pm22\%$ within 5 min and by a maximum of $1720\pm342\%$ after 20 min, with recovery taking 25–35 min ($n=6$). Forskolin (10^{-4} M) also increased spike amplitude by $24\pm3\%$ (in each case, $P<0.001$) and abolished the undershoot ($n=5$; Figure 3).

An additional response to forskolin was a transient increase in excitability, which was seen in every case. This occurred within 2 min of application, before any lengthening of the spike, and then excitability declined below control level after 5–8 min. An increase in excitability was seen with 5-HT and with external application of 8-bromo-cyclic AMP (Wuttke & Berry, 1991) but not with IBMX or intracellularly injected cyclic AMP in the present study. At high doses (> 10^{-3} M) 5-HT also produced a transient increase followed by a decline in excitability ($n=4$, not shown), perhaps indicating that effects of low concentrations of cyclic AMP on excitability may be reversed by higher doses.

The threshold dose of forskolin was about 5×10^{-6} M (6

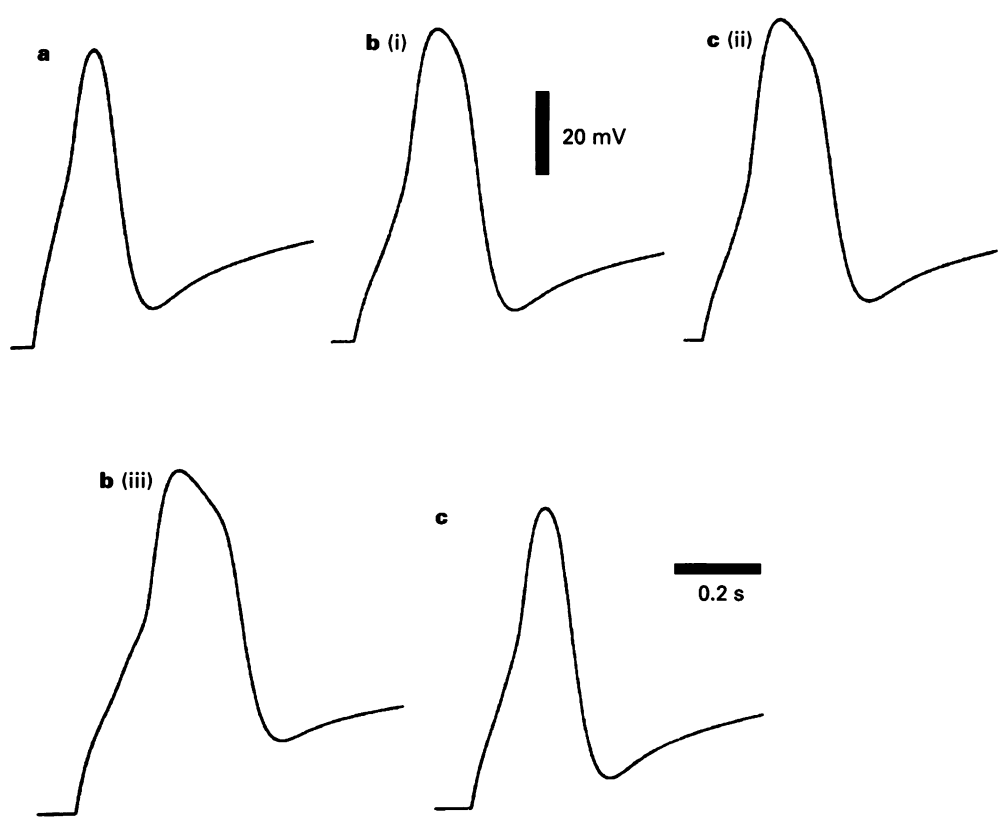

Figure 2 Dibutyryl cyclic AMP, which does not affect gland-cell action potentials when applied externally (10^{-3} M), increases their duration when injected intracellularly. (a) Control action potential. (b(i)–b(iii)) Action potentials recorded at 1, 2 and 4 min respectively after injection of dibutyryl cyclic AMP (10 s, 470 kPa pressure pulse; the electrode contained 10 mM dibutyryl cyclic AMP). (c) Recovery after 20 min. Each action potential was elicited as a rebound response to a hyperpolarizing pulse (10 s, 90 mV) which is off scale. Resting membrane potential remained between −41 mV and −44 mV throughout the experiment.

cells). No measurable effect was seen at 10^{-6} M but this dose increased the effect of 10^{-6} M 5-HT on spike duration by 26±4% and on spike undershoot by 54±8%, and increased the effect on excitability in each case ($n=5$, not shown).

Intracellular cyclic GMP prolongs gland-cell action potentials by potentiating Ca^{2+} current

Cyclic GMP is known to reduce the amplitude of inwardly rectifying current in the gland cells, and this effect is mimicked by zaprinast (Wuttke & Berry, 1992). No studies have been made, however, of intracellular effects of cyclic GMP on the other voltage-activated channels in the salivary cells. We attempted to increase the intracellular levels of this cyclic nucleotide by addition of its 8-bromo derivative, addition of zaprinast, and intracellular injection of cyclic GMP.

Effect of 8-bromo-cyclic GMP Extracellular application of 5×10^{-4} M 8-bromo-cyclic GMP reversibly reduced spike amplitude by 25±4% with little change in duration ($n=5$, not shown). At 10^{-3} M the spike amplitude progressively declined until the cell became inexcitable after about 5 min and then took at least 25 min for recovery; spike duration increased by a maximum of 12±2% ($n=5$, not shown). These effects are similar to those of cyclic GMP (Wuttke & Berry, 1991) and almost certainly reflect mainly an extracellular action (see below).

Effect of zaprinast Extracellular application of zaprinast (5×10^{-4} M), which inhibits cyclic GMP-dependent phosphodiesterase in other systems including invertebrates (e.g. Gerschenfeld *et al.*, 1986), increased spike duration by 83±17% ($n=5$). After exposure for 15–25 min, recovery occurred after washing out the drug for 5–8 min (Figure 4a). With longer exposure (30–40 min), however, the effect became irreversible and action potentials continued to lengthen for about an hour after washing. Zaprinast also produced an irreversible reduction of inward rectification in the gland cells (Wuttke & Berry, 1992).

In voltage clamp studies the membrane potential was stepped to 4 different levels from a holding potential of −40 mV. The peak inward current produced by a 30 mV depolarizing step was increased by 12±5% ($n=5$) after 10–15 min exposure to 5×10^{-4} M zaprinast but there was a variable effect on subsequent outward current (little change in 3 cells and a 24–39% increase in 2 others) (not shown). After block of K^+ channels with 50 mM TEA and 10 mM 4-aminopyridine, zaprinast increased the inward current produced by all 4 voltage steps; for example, there was an increase of 43±9% ($n=6$, $P<0.001$) for depolarizing voltage steps of 30 mV from a holding potential of −45 mV (Figures 4 and 6).

Effect of intracellular injection of cyclic GMP Injections were made from microelectrodes containing 10 mM cyclic GMP, using 280 kPa pressure pulses applied for 30–100 ms. After a single pulse, spike duration was increased by 37±5% after 30 s, and by a maximum of 56±8% after 2 min ($n=6$, $P<0.001$). Recovery took 4–10 min (Figure 5a).

Under voltage-clamp, the peak inward current produced by a 30 mV depolarizing pulse from −55 mV was increased by 45±7% and outward current was reduced by 61±16% ($n=6$)

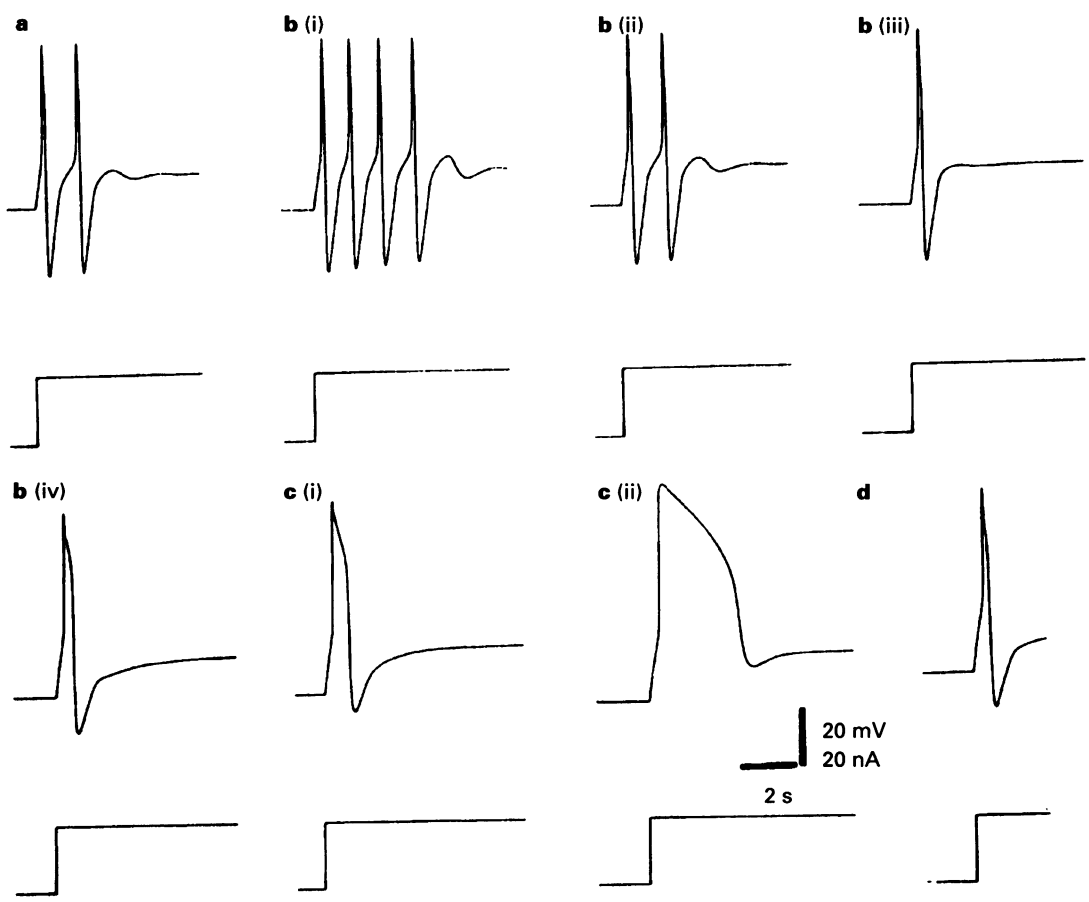

Figure 3 Forskolin mimics the effect of 5-HT on gland-cell action potentials. The recordings are from the same cell and show action potentials (upper) produced in response to a maintained depolarizing pulse (lower). (a) Control. (b(i)–b(iv)) Action potentials recorded at 2, 4, 6 and 40 min respectively after introduction of 10^{-5} M forskolin. Note the transient increase in excitability (b(i)) and progressive lengthening of the spike, with an increase in amplitude and reduction of undershoot. (c(i), c(ii)) Recordings made at 2 and 22 min respectively after introduction of 10^{-4} M forskolin, which immediately replaced the lower dose. (d) Almost complete recovery after washout of the drug for 36 min. The high dose of forskolin produced a depolarization of 7 mV but the resting membrane potential was maintained at its original level of −51 mV by applied current.

(Figure 5b). After blockade of Ca^{2+} current with 10 mM Co^{2+}, intracellular injection of cyclic GMP had little or no effect on K^+ current (0–15% increase; $n=3$, not shown). During exposure to 50 mM TEA and 10 mM 4-aminopyridine, intracellularly injected cyclic GMP increased the peak inward current by 65±13% ($n=6$, $P<0.001$) for a 30 mV depolarizing step from −45 mV. Full recovery took 3–5 min. The effect of intracellular injection on the current-voltage relationship is shown in Figure 6.

Extracellular cyclic AMP and cyclic GMP shorten gland-cell action potentials by reducing Ca^{2+} current

Effects on action potentials TEA (50 mM) plus 4-aminopyridine (10 mM) produce prolonged gland-cell action potentials (4–6.5 s in duration) which are shortened by extracellular cyclic AMP and cyclic GMP (Wuttke & Berry, 1993). Figure 7a illustrates the effect of 10^{-4} M cyclic GMP, which produced a reversible 34±5% shortening ($n=6$). At 10^{-3} M there was a 73±10% reduction in duration and a 28±4% fall in spike amplitude ($n=6$). Cyclic AMP was ineffective at 10^{-4} M but produced a 32±7% shortening of action potentials at 10^{-3} M ($n=6$) (in each case $P<0.001$). The effects were maintained for at least 90 min (maximum time tested).

Effects on membrane current The effects of these cyclic nucleotides on membrane current were examined by stepping the membrane potential by 25 mV from a holding potential of −40 mV. After block of K^+ current with TEA and 4-aminopyridine, cyclic GMP (10^{-3} M) reduced the amplitude of Ca^{2+} current by 36±6% ($n=5$, $P<0.001$) and increased its rate of inactivation. The inactivation could be described by a single exponential with a time constant of 5.9±1.8 s, and this was reduced to 1.1±0.3 s by 10^{-3} M cyclic GMP. Cyclic AMP (10^{-3} M) produced qualitatively similar effects.

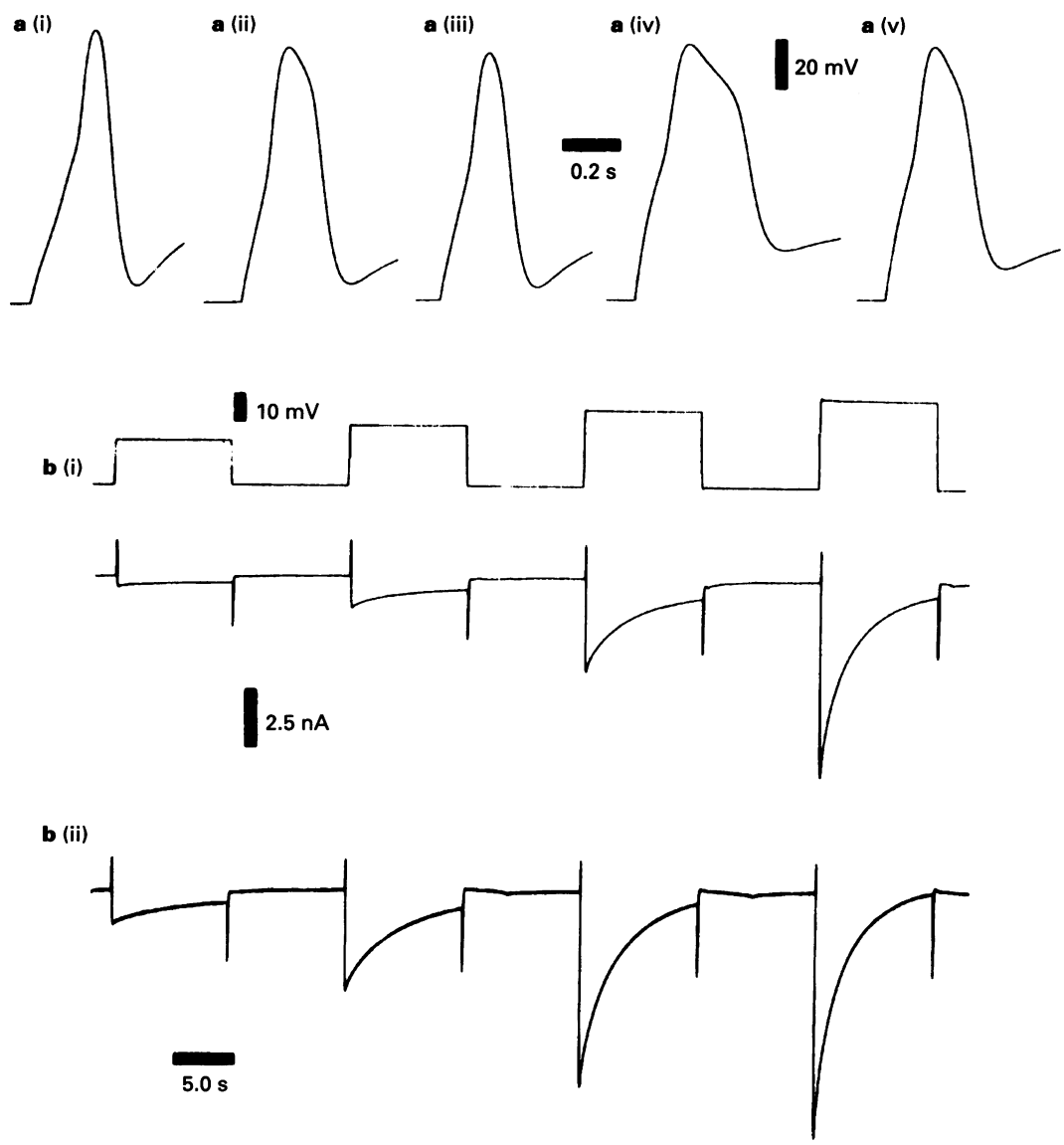

Figure 4 Zaprinast (5×10^{-4} M) prolongs gland-cell action potentials by increasing Ca^{2+} current. (a) Effect of zaprinast on action potentials. (a(i)) Control; (a(ii)) 20 min exposure to zaprinast; (a(iii)) 5 min wash; (a(iv)) 20 min exposure to second application of zaprinast; (a(v)) 5 min wash. No further recovery occurred; the action potential started to increase in duration until it was 27% longer than in (a(iv)) after 1 h of washing (not shown). Each action potential was elicited as a rebound response to a hyperpolarizing pulse (10 s, 70 mV) which is off scale. Resting membrane potential decreased by 9 mV in zaprinast but was held constant at −53 mV by applied current. (b) Effect of zaprinast on Ca^{2+} current. In each recording the membrane potential was stepped to 4 different levels (shown in b(i)) from a holding potential of −40 mV while current was recorded. K^+ current was blocked by addition of 50 mM TEA and 10 mM 4-aminopyridine. (b(i)) Control; (b(ii)) 20 min exposure to zaprinast. Recovery occurred after 12 min washout of the drug (not shown).

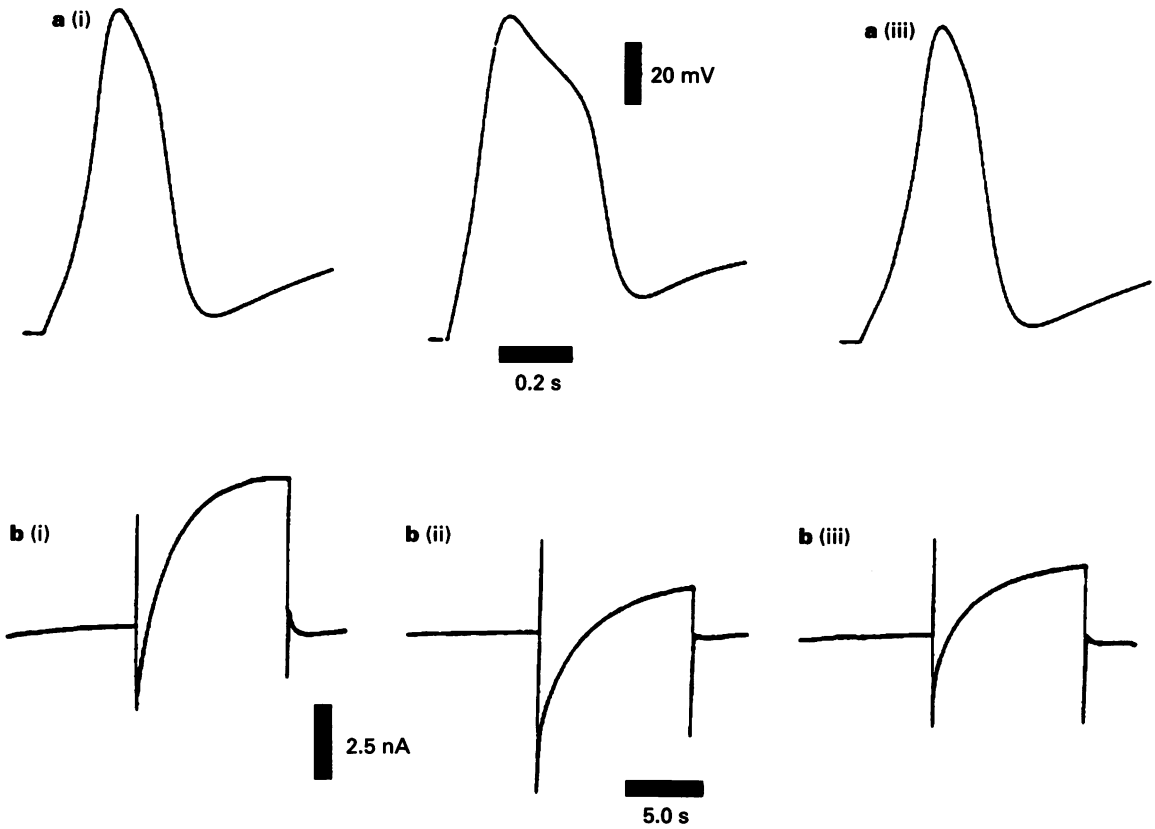

Figure 5 Intracellular injection of cyclic GMP increases action potential duration and net inward current in cells bathed in normal saline. (a) Effect on action potentials; (a(i)) control; (a(ii)) 4 min after injection of cyclic GMP; (a(iii)) recovery after 8 min. Each action potential was elicited as a rebound response to a hyperpolarizing pulse (10 s, 90 mV) which is off scale. Resting membrane potential remained constant at −55 mV. (b) Effect on membrane current. In each recording a depolarizing pulse (10 s, 30 mV) was applied from a holding potential of −55 mV. (b(i)) Control; (b(ii)) 2 min after injection of cyclic GMP; (b(iii)) Partial recovery after 6 min. In (a) and (b), the injection electrode contained 10 mM cyclic GMP which was ejected by a 450 kPa pulse applied for 80 ms.

The effects on spike duration and Ca^{2+} current are identical to those of ATP (Wuttke & Berry, 1993). ATP has no effect on LVA Ca^{2+} current, and this was also found for cyclic AMP and cyclic GMP. The LVA current was elicited by step depolarizations from a holding potential of about −80 mV; Figure 7b(i) shows an example of this current recorded in normal saline. TEA and 4-aminopyridine greatly reduced its amplitude (Figure b(ii)). Typically, the peak amplitude of Ca^{2+} currents containing both components was not affected by cyclic GMP or cyclic AMP, but the rate of inactivation was increased (Figure 7b(iii)). It was possible that enhanced K^+ current contributed to the changes in net inward current produced by the cyclic nucleotides in normal saline. This was examined by blocking Ca^{2+} current with 10 mM Co^{2+}. In six cells, 10^{-3} M cyclic GMP did not affect the peak amplitude of K^+ current or increase its rate of development (Figure 7c).

It was also of interest to test extracellularly applied 8-bromo-cyclic AMP, which prolongs gland-cell action potentials recorded in normal saline. This is almost certainly an intracellular effect because it was identical to the response to intracellular injection (block of K^+ channels). When K^+ channels were blocked with TEA and 4-aminopyridine, extracellular 8-bromo-cyclic AMP mimicked extracellular cyclic AMP and cyclic GMP in shortening action potentials by a block of HVA Ca^{2+} current. At 10^{-3} M, 8-bromo-cyclic AMP shortened prolonged action potentials by more than 50% (3 cells) whereas it prolonged normal action potentials by 34%. This indicates the potential problems of interpretation when using membrane-permeable analogues. P_1-purinoceptors may also be activated by 8-bromo-cyclic AMP (Dolphin *et al.*, 1986).

Do the cyclic nucleotides activate a purinoceptor? The effects of the extracellular cyclic nucleotides on gland-cell electrophysiology are identical to those of ATP (Wuttke & Berry, 1993). These substances are not readily membrane permeable and they work very quickly (usually within 1 min), suggesting

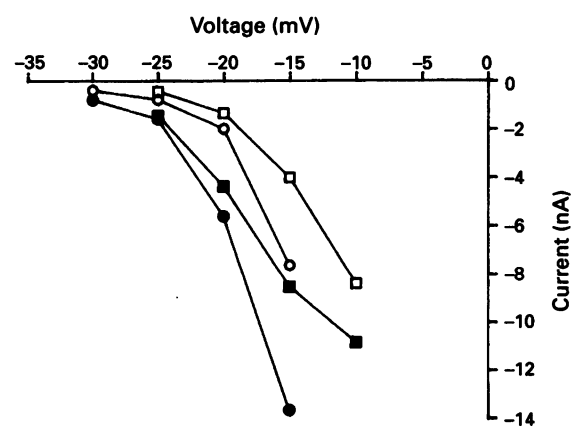

Figure 6 Effect of zaprinast (data from Figure 4) and intracellular injection of cyclic GMP (representative example) on peak Ca^{2+} current-voltage relationship. (○) Control; (●) 2 min after cyclic GMP injection; (□) control; (■) 20 min exposure to 5×10^{-4} M zaprinast. The holding potentials were −40 mV and −45 mV respectively. K^+ current was blocked by 50 mM TEA and 10 mM 4-aminopyridine.

that they operate by a surface membrane-limited mechanism. Wuttke & Berry (1993) proposed that ATP activates a P_2-type purinoceptor because it is effective at submicromolar concentrations whereas adenosine is inactive at millimolar doses; also, ATP does not activate adenylyl cyclase, as generally expected of P_2-receptors (Burnstock, 1990). We therefore tested suramin, a P_2-receptor antagonist which is relatively non-specific for different receptor subtypes (Dubyak & El-Moatassim,

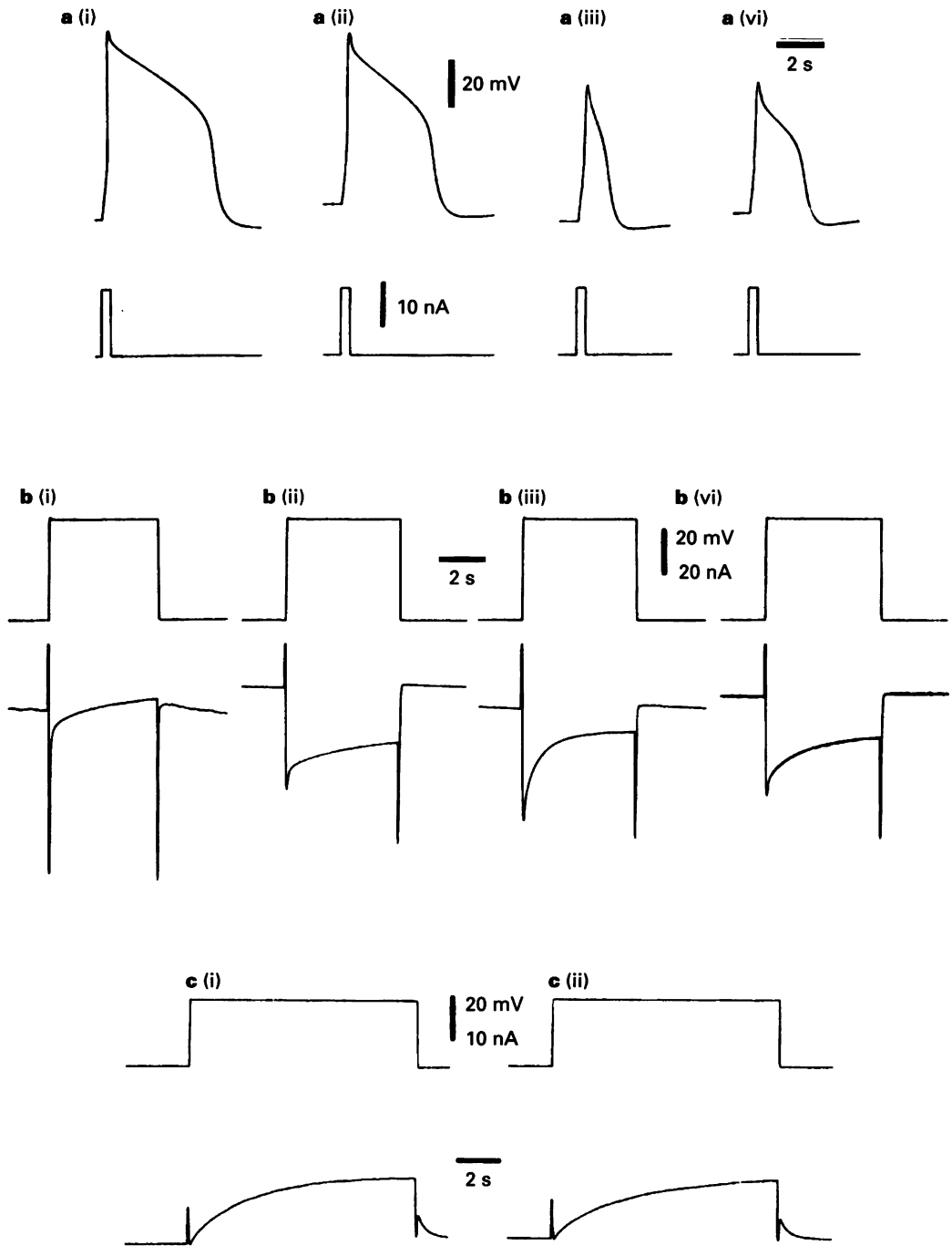

Figure 7 Extracellular cyclic GMP reduces the amplitude and duration of artificially lengthened action potentials, and increases the rate of inactivation of Ca^{2+} current. (a) Effect on action potentials (upper) elicited by depolarizing pulses (lower) in saline containing 50 mM TEA and 10 mM 4-aminopyridine. (a(i)) Control; (a(ii)) 12 min exposure to 10^{-4} M cyclic GMP; (a(iii)) 6 min exposure to 10^{-3} M cyclic GMP; (a(iv)) partial recovery after 12 min wash (full recovery took 35 min). The high concentration of cyclic GMP produced a depolarization of 7 mV, but the resting membrane potential at the start of the experiment (−44 mV) was maintained by applied current. (b) Effect of cyclic GMP on Ca^{2+} current (lower) elicited by depolarizing pulses (45 mV, upper) from a holding potential of −70 mV. (b(i)) Normal saline. Note the large, transient LVA current. (b(ii)) After 25 min exposure to 50 mM TEA and 10 mM 4-aminopyridine, the transient component is almost abolished and the slow component (HVA current) is potentiated; (b(iii)) 4 min after addition of cyclic GMP (10^{-3} M), increased rate of inactivation of the Ca^{2+} current occurs. (b(iv)) Partial recovery after washing for 10 min. (c) Extracellular cyclic GMP does not affect K^+ current: (c(i)) control K^+ current after blocking Ca^{2+} current with 10 mM Co^{2+}; (c(ii)) 18 min after addition of cyclic GMP (10^{-3} M).

1993). After equilibration of the preparation in saline containing 50 mM TEA and 10 mM 4-aminopyridine, 5×10^{-6} M ATP was run into the bath until a maximum shortening of action potentials occurred ($27 \pm 6\%$, $n = 5$). The ATP was then washed out until the original spike duration was obtained and suramin was run in, followed after 40 min by suramin and 5×10^{-6} M ATP. At 5×10^{-5} M, suramin produced a depolarization of 14–24 mV (which was offset by applied current) but had no blocking effect ($n = 5$). The decrease in membrane potential and associated increase in membrane conductance, precluded reliable testing of higher doses.

To determine whether the presumed receptor was sensitive to pyrimidine nucleotides, the effect of UTP was examined on prolonged action potentials. At 10^{-6} M, UTP decreased spike duration by $9 \pm 2\%$; this was increased to $23 \pm 5\%$ at 10^{-5} M and $51 \pm 9\%$ at 10^{-4} M ($n = 3$), with complete recovery after washing out for about 20 min. This compared with a reduction of $19 \pm 5\%$ ($n = 3$) produced by 10^{-6} M ATP. In the absence of blocking drugs the possibility of distinct purine and pyrimidine receptors (or even cyclic AMP receptors; e.g. Sorbera & Morad, 1991) could not be determined. Cross desensitization between ATP and UTP could not be tested because the latter also showed no decline in response after prolonged application (90 min).

Activation of the putative purinoceptor does not appear to affect K^+ or LVA Ca^{2+} currents, but there remained the possibility of modulation of the inward rectifier channel. The inward rectifier was activated by a series of hyperpolarizing command pulses, applied every 2 min from a holding potential of −70 mV; pulse amplitude was 5–30 mV, producing currents of 0–22 nA. ATP (10^{-5} M) had no measurable effect on the current (6 cells) indicating that it specifically modulates HVA Ca^{2+} current.

Discussion

The salivary gland cells of *Haementeria* provide a potentially useful model for study of the modulation of ion channels by purine nucleotides. Step depolarizations under voltage clamp are shown here to produce delayed rectifier K^+ current which is blocked by intracellular cyclic AMP, and HVA Ca^{2+} current which is potentiated by intracellular cyclic GMP and reduced by extracellular cyclic GMP (and other nucleotides). Hyperpolarizing clamp pulses produce inward rectifier Na^+/K^+ current which is potentiated by intracellular cyclic AMP and reduced by intracellular cyclic GMP (Wuttke & Berry, 1992). The lack of electrical coupling between cells is an unusual feature which facilitates electrophysiological experiments on intact glands because there is no modification of electrical activity by neighbouring cells. The large size of the cells (up to 1200 μm) enables them to be dissected individually for biochemical analysis (for example, secretory products of single cells have been determined and shown to vary between different cell types; Wuttke *et al.*, 1989). These properties of the salivary cells are not shared by those of other leeches. The medicinal leech, *Hirudo medicinalis*, has relatively small salivary cells which are not collected together into a specific gland and can be held with a single microelectrode for no more than about 3 min (Marshall & Lent, 1988).

Cyclic AMP appears to serve as a second messenger for the putative neuroglandular transmitter, 5-HT. Intracellular injection of cyclic AMP analogues or presumed elevation of cyclic AMP levels with IBMX or forskolin all mimicked the effects of 5-HT. Furthermore, forskolin and IBMX potentiated the effects of 5-HT.

Unlike the situation with cyclic AMP, where previous work had indicated an effect on K^+ channels (Wuttke & Berry, 1991), the actions of intracellular cyclic GMP were not predicted. Wuttke & Berry (1991, 1993) had shown that extracellular cyclic GMP reduced the duration of action potentials which had been prolonged by TEA or EGTA, and abolished action potentials recorded in normal saline. Our attempts to introduce cyclic GMP into cells by use of its 8-bromo analogue suggested that its intracellular effect might be similar. Intracellular injection of cyclic GMP or addition of zaprinast, however, showed that this cyclic nucleotide potentiated HVA Ca^{2+} current. Although a different mechanism from that of cyclic AMP, the effect on the action potential is the same, increasing its duration.

It is not known whether cyclic GMP acts as a second messenger in the salivary cells. It cannot be a messenger for 5-HT because it has opposing or different actions, and it does not produce similar effects to any of the common neurotransmitters that were screened by Wuttke & Berry (1991). Although salivary glands in invertebrates appear to differ from those of vertebrates in having single-transmitter innervation (see Petersen, 1980) the salivary cells of *Haementeria* may have two transmitters because of the presence of small clear vesicles and larger dense-cored vesicles seen in synaptic terminals supplying the gland cells (Walz *et al.*, 1988). A second transmitter may perhaps operate *via* cyclic GMP.

The potential value of the preparation as a model system is determined by its relevance for mammalian studies. For example, are the channels comparable to those of mammals? The channels described here have not been fully characterized, but there are no reasons to suppose that they have unusual properties. For example, the Ca^{2+} channels show typical high and low activation thresholds with different inactivation rates; they are blocked by Co^{2+}; they pass no measurable Na^+ ions unless external Ca^{2+} is removed; Ba^{2+} will substitute for Ca^{2+}. The K^+ channels are blocked by the usual drugs: TEA and 4-aminopyridine (Wuttke & Berry, 1988; 1991; 1993). The inward rectifier channel shows typical characteristics of such channels in mammals: slow activation; an increase in current with increasing external $[K^+]$ without a shift in the activation curve; a block by external Cs^+ and a resistance to external Ba^{2+} (Wuttke & Berry, 1992). Although channels in invertebrates cannot always be easily grouped with those of vertebrates, they appear to be based on similar protein subunits controlling voltage-dependent activation, ion conductance and inactivation. Differences between channels seem to be derived from subtle variations on a common structural theme (Swandulla *et al.*, 1991; Catterall & Striessnig, 1992).

Similarly with the second messengers, no major distinctions are made between the actions of cyclic nucleotides in vertebrates and invertebrates; the same substances are used in the same ways in response to activation by the same neurotransmitters. In our preparation, the drugs which modify cyclic nucleotides in mammals have their expected effects, for example IBMX, forskolin and zaprinast. Goy (1991) mentions that the prominence of the cyclic GMP pathway at many levels of phylogeny provides an array of model systems.

The situation with the extracellular nucleotides is less clear. As far as we are aware, there is little or no information on invertebrate purinoceptors to compare them with those of vertebrates. Receptors for neurotransmitters or neuromodulators often show differences between vertebrates and invertebrates in their pharmacological classification, but these are usually differences in detail, and the same agonists and antagonists are generally effective. However, comparisons may have to await clarification of receptor classification in vertebrates, where the situation is increasingly complex. The discoveries of 'novel' purinoceptors, 'nucleotide' receptors and pyrimidinoceptors may necessitate reassessment of current receptor classification (Von Kugelgen *et al.*, 1987; Seifert & Schultz, 1989; Davidson *et al.*, 1990; Saiag *et al.*, 1990; Henning *et al.*, 1993; Uneyama *et al.*, 1994). A further source of confusion is the possible change in rank order of potency of agonists (an important criterion in receptor classification) with the concentration of divalent cations (Trezise *et al.*, 1994), perhaps because of the preference of cell-surface ectonucleotidase enzymes for ATP complexed with Mg^{2+}. In our preparation ATP is equipotent in normal saline and in solutions lacking free divalent cations (Wuttke & Berry, 1993).

Although the lack of effect of suramin in the present ex-

periments may indicate a fundamental difference between vertebrate and invertebrate purine receptors, further work is needed because the dose of suramin (5×10^{-5} M) was necessarily lower than is often used with mammals (up to 1 mM; e.g. Bailey & Hourani, 1994). Also, suramin-insensitive ATP effects are known in mammals (Von Kugelgen *et al.*, 1990; Bailey & Hourani, 1994) and low doses of the drug may take several hours to produce effects (Leff *et al.*, 1990).

P_2-receptor-mediated modulation (usually inhibition) of voltage-activated Ca^{2+} channels is found in mammals (e.g. Diverse-Pierluissi *et al.*, 1991; Gandia *et al.*, 1993; Qu *et al.*, 1993). The response in *Haementeria* is unusual in showing an increased rate of inactivation with no change in the kinetics of activation, and may indicate that the nucleotides are acting as open Ca^{2+} channel blockers. However, ATP increases Ca^{2+} current inactivation *via* P_{2Y} receptors in ferret ventricular myocytes at potentials depolarized to 0 mV (Qu *et al.*, 1993).

In summary, the salivary glands of *Haementeria* show an unusual variety of nucleotide and cyclic nucleotide actions on ion channels (Ca^{2+}, K^+ and Na^+/K^+), and provide special technical advantages for pharmacological and biochemical analysis of the modulatory and transduction processes in single cells.

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References

BAILEY, S.J. & HOURANI, S.M.O. (1994). Differential effects of suramin on P_2-purinoceptors mediating contraction of the guinea-pig vas deferens and urinary bladder. *Br. J. Pharmacol.*, **112**, 219–225.

BURNSTOCK, G. (1990). Purinergic mechanisms. *Ann. N.Y. Acad. Sci.*, **603**, 1–18.

CATTERALL, W.A. & STRIESSNIG, J. (1992). Receptor sites for Ca^{2+} channel antagonists. *Trends Pharmacol. Sci.*, **13**, 256–262.

DAVIDSON, J.S., WAKEFIELD, I.K., SOHNIUS, U., ANTON VAN DER MERWE, P. & MILLAR, R.P. (1990). A novel extracellular nucleotide receptor coupled to phosphoinositidase-C in pituitary cells. *Endocrinology*, **126**, 80–87.

DIVERSE-PIERLUISSI, M., DUNLAP, K. & WESTHEAD, E.W. (1991). Multiple actions of extracellular ATP on calcium currents in cultured bovine chromaffin cells. *Proc. Natl. Acad. Sci. U.S.A.*, **88**, 1261–1265.

DOLPHIN, A.C., FORDA, S.R. & SCOTT, R.H. (1986). Calcium-dependent currents in cultured rat dorsal root ganglion neurons are inhibited by adenosine analogue. *J. Physiol.*, **373**, 47–61.

DUBYAK, G.R. & EL-MOATASSIM, C. (1993). Signal transduction via P_2-purinergic receptors for extracellular ATP and other nucleotides. *Am. J. Physiol.*, **265**, C577–C606.

EISENBERG, R.S. & ENGEL, E. (1970). The spatial variation of membrane potential near a small source of current in a spherical cell. *J. Gen. Physiol.*, **55**, 736–757.

GANDIA, L., GARCIA, A.G. & MORAD, M. (1993). ATP modulation of calcium channels in chromaffin cells. *J. Physiol.*, **470**, 55–72.

GERSCHENFELD, H.M., HAMMOND, C. & PAUPARDIN-TRITSCH, D. (1986). Modulation of the calcium current of molluscan neurons by neurotransmitters. *J. Exp. Biol.*, **124**, 73–91.

GOY, M.F. (1991). cGMP: the wayward child of the cyclic nucleotide family. *Trends Neurosci.*, **14**, 293–299.

HENNING, R.H., DUIN, M., DEN HERTOG, A. & NELEMANS, A. (1993). Characterization of P_2-purinoceptor mediated cyclic AMP formation in mouse C2C12 myotubes. *Br. J. Pharmacol.*, **110**, 133–138.

LEFF, P., WOOD, B.E. & O'CONNOR, S.E. (1990). Suramin is a slowly equilibrating but competitive antagonist at P_{2x}-receptors in the rabbit isolated ear artery. *Br. J. Pharmacol.*, **101**, 645–649.

MARSHALL, C.G. & LENT, C.M. (1988). Excitability and secretory activity in the salivary gland cells of jawed leeches (Hirudinea: Gnathobdellida). *J. Exp. Biol.*, **137**, 89–105.

PETERSEN, O.H. (1980). *The Electrophysiology of Gland Cells.* Monographs of the Physiological Society, no. 36. London: Academic Press.

QU, Y., CAMPBELL, D.L. & STRAUSS, H.C. (1993). Modulation of L-type Ca^{2+} current by extracellular ATP in ferret isolated right ventricular myocytes. *J. Physiol.*, **471**, 295–317.

SAIAG, B., MILON, D., ALLAIN, H., RAULT, B. & VAN DEN DRIESSCHE, J. (1990). Constriction of smooth muscle of rat tail and femoral arteries and dog saphenous vein is induced by uridine triphosphate via 'pyrimidinoceptors', and by adenosine triphosphate via P_{2x} purinoceptors. *Blood Vessels*, **27**, 352–364.

SEIFERT, R. & SCHULTZ, G. (1989). Involvement of pyrimidinoceptors in the regulation of cell functions by uridine and uracil nucleotides. *Trends Pharmacol. Sci.*, **10**, 365–369.

SORBERA, L.A. & MORAD, M. (1991). Modulation of cardiac sodium channels by cAMP receptors on the myocyte surface. *Science*, **253**, 1286–1289.

SWANDULLA, D., CARBONE, E. & LUX, H.D. (1991). Do calcium channel classifications account for neuronal calcium channel diversity? *Trends Neurosci.*, **14**, 46–51.

TREZISE, D.J., BELL, N.J., KENNEDY, I. & HUMPHREY, P.P.A. (1994). Effects of divalent cations on the potency of ATP and related agonists in the rat isolated vagus nerve: implications for P_2 purinoceptor classification. *Br. J. Pharmacol.*, **113**, 463–470.

UNEYAMA, H., UNEYAMA, C., EBIHARA, S. & AKAIKE, N. (1994). Suramin and reactive blue 2 are antagonists for a newly identified purinoceptor on rat megakaryocyte. *Br. J. Pharmacol.*, **111**, 245–249.

VON KUGELGEN, I., BULTMAN, R. & STARKE, K. (1990). Interaction of adenine nucleotides, UTP and suramin in mouse vas deferens: suramin-sensitive and suramin-insensitive components in the contractile effect of ATP. *Naunyn-Schmied. Arch. Pharmacol.*, **342**, 198–205.

VON KUGELGEN, I., HAUSSINGER, D. & STARK, K. (1987). Evidence for a vasoconstriction mediating receptor for UTP, distinct from the P_2-purinoceptor, in rabbit ear artery. *Naunyn-Schmied. Arch. Pharmacol.*, **336**, 556–560.

WALZ, B., SCHAFFNER, K.H. & SAWYER, R.T. (1988). Ultrastructure of the anterior salivary gland cells of the giant leech *Haementeria ghilianii* (Annelida, Hirudinea). *J. Morphol.*, **196**, 321–332.

WUTTKE, W.A. & BERRY, M.S. (1988). Calcium-dependent action potentials in giant salivary gland cells of the leech *Haementeria ghilianii*: calcium removal induces dependence on sodium. *J. Exp. Biol.*, **138**, 431–453.

WUTTKE, W.A. & BERRY, M.S. (1989). Extraglandular innervation of the salivary cells of the leech *Haementeria ghilianii*: neuronal stimulation elicits gland-cell action potentials and secretion. *J. Exp. Biol.*, **143**, 389–410.

WUTTKE, W.A., SAWYER, R.T. & BERRY, M.S. (1990). Rapid co-transport of sodium and chloride ions in giant salivary gland cells of the leech *Haementeria ghilianii*. *J. Physiol.*, **427**, 51–69.

WUTTKE, W.A., & BERRY, M.S. (1991). Initiation and modulation of action potentials in salivary gland cells of *Haementeria ghilianii* by putative transmitters and cyclic nucleotides. *J. Exp. Biol.*, **157**, 101–122.

WUTTKE, W.A. & BERRY, M.S. (1992). Modulation of inwardly rectifying Na^+-K^+ channels by serotonin and cyclic nucleotides in salivary gland cells of the leech, *Haementeria ghilianii*. *J. Membr. Biol.*, **127**, 57–68.

WUTTKE, W.A. & BERRY, M.S. (1993). Extracellular ATP selectively modulates a high-voltage-activated calcium conductance in salivary gland cells of the leech *Haementeria ghilianii*. *J. Exp. Biol.*, **181**, 313–319.

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