

SHORT COMMUNICATION

EXTRACELLULAR ATP SELECTIVELY MODULATES A HIGH-VOLTAGE-ACTIVATED CALCIUM CONDUCTANCE IN SALIVARY GLAND CELLS OF THE LEECH *HAEMENTERIA GHILIANII*

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Accepted 7 April 1993

Extracellular ATP appears to have a widespread role as a neurotransmitter or neuromodulator in mammals (Gordon, 1986; Burnstock, 1990), but little is known about any similar functions in invertebrates. During studies of the effects of cyclic nucleotides on electrically excitable salivary cells of the leech, we found that cyclic GMP produced a rapid (less than 1 min) reduction of spike duration, suggesting an extracellular effect (Wuttke and Berry, 1991). We now show that micromolar concentrations of ATP (and higher doses of other nucleotides) also reduce spike duration, and that this is caused by depression of a specific voltage-dependent Ca^{2+} conductance. Selective modulation of Ca^{2+} current by external ATP has rarely been found, and the effect is also unusual because it changes the kinetics of inactivation rather than those of activation.

Experiments were performed on isolated anterior salivary glands of the Amazon leech *Haementeria ghilianii* (de Filippi). The glands were pinned to Sylgard in a Perspex experimental bath (volume 0.25 ml) and immersed in a continuous flow of physiological saline containing (in mmol l^{-1}): NaCl, 115; KCl, 4; CaCl_2 , 2; MgCl_2 , 1; glucose, 11; Tris maleate, 10 (pH 7.4) at room temperature (18–22°C). The following substances (Sigma) were applied by perfusion at known concentration: ATP (adenosine 5'-triphosphate), AMP (adenosine 5'-monophosphate), cyclic AMP (cyclic 3',5'-adenosine monophosphate), adenosine, GMP (guanosine 5'-monophosphate), cyclic GMP (cyclic 3',5'-guanosine monophosphate) and guanosine. Ba^{2+} (substituting for Ca^{2+}) or tetraethylammonium and 4-aminopyridine (replacing equimolar Na^+) were used to block K^+ channels, and Co^{2+} was used to block Ca^{2+} channels (Hille, 1984; see Marshall and Lent, 1984, and Wuttke and Berry, 1991, for effects on the salivary cells of *Haementeria*). Solutions flowed through the bath at a rate of about $10 \text{ bath volumes min}^{-1}$ and could be changed rapidly without altering recording conditions.

Individual gland cells were impaled with two bevelled KCl-filled microelectrodes (resistance 10–20 M Ω) mounted on high-speed steppers (Digitimer SCAT-02).

Key words: ATP, nucleotides, Ca^{2+} channels, leech, salivary glands, *Haementeria ghilianii*.

Membrane voltage and current were measured with a voltage-clamp amplifier (Axoclamp 2A, Axon Instruments). Signals were monitored on a storage oscilloscope (Tektronix 5111) and pen recorder (Brush 2200S) and stored on tape (Thorn EMI 3000 FM tape recorder). For further details, see Wuttke and Berry (1992). No corrections were made for the small, linear leakage currents. The gland cells are electrically isolated (Wuttke and Berry, 1988) so these experiments are not complicated by electrical coupling between cells.

The salivary gland cells have resting membrane potentials ranging from -40 to -70 mV and produce overshooting Ca^{2+} -dependent action potentials with a duration of 100–400 ms. Sodium ions make no contribution to the action potential (Wuttke and Berry, 1988). Application of $10^{-4} \text{ mol l}^{-1}$ ATP produced little or no change in resting membrane properties: in five cells the membrane depolarized by 1.25 ± 0.6 mV (S.D.) and there was no measurable change in input resistance as measured by injection of constant-current hyperpolarizing pulses (Fig. 1). The duration of action potentials, however, was reversibly reduced by 20–30% (Fig. 2). Much larger effects were produced on artificially prolonged action potentials, such as those recorded in Ca^{2+} -free saline (Fig. 3A; the bathing solution was nominally Ca^{2+} - and Mg^{2+} -free and contained 1 mmol l^{-1} EGTA). In the absence of Ca^{2+} , sodium ions pass through the Ca^{2+} channels (Wuttke and Berry, 1988) and the action potentials become Na^{+} -dependent, reaching 0 mV and sometimes lasting for tens of seconds.

The effects of ATP on the membrane currents that underlie action potential shortening were studied by voltage-clamping the cells (Fig. 3B). Stepping from -70 mV to -50 mV elicited a two-phase inward current consisting of an initial fast, transient component (< 500 ms) followed by a longer-lasting component that decayed to zero in less than 30 s. ATP ($10^{-4} \text{ mol l}^{-1}$) had little effect on the amplitude of the inward current but increased

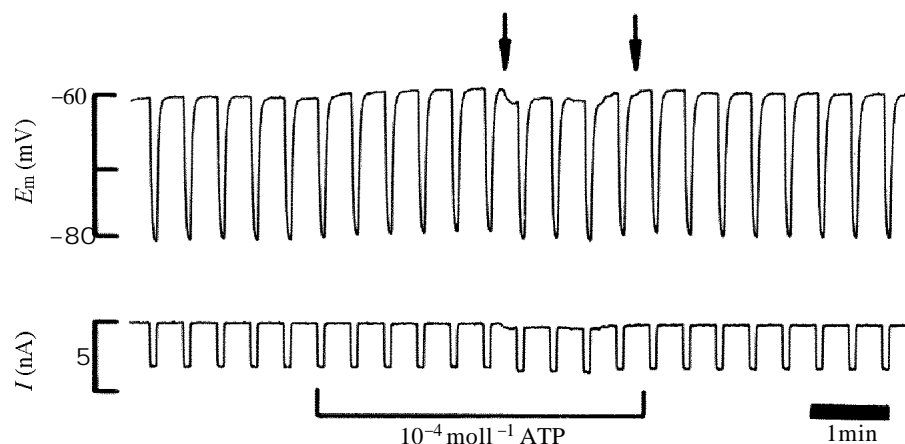


Fig. 1. ATP has little effect on gland-cell resting membrane properties. The upper trace shows the resting membrane potential (E_m), subjected to constant-current hyperpolarizing pulses (lower trace). $10^{-4} \text{ mol l}^{-1}$ ATP caused only a small depolarization and had no measurable effect on input resistance. Between the arrows, the resting membrane potential was reset by applied current.

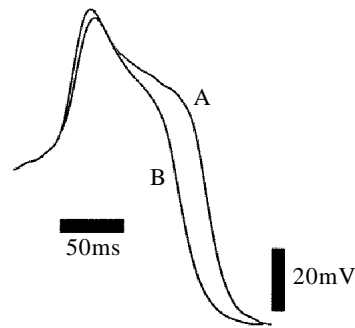


Fig. 2. ATP reduces the duration of gland-cell action potentials. Suprathreshold depolarizing current pulses were applied at constant intervals of about 2min, which ensured that identical action potentials were produced by each stimulus. ATP ($10^{-4} \text{ mol l}^{-1}$) was then introduced into the bath until a maximal effect was produced. It was subsequently washed out to check for reversibility. Recording A shows a spike in normal saline, and the superimposed recording B was taken in the presence of ATP.

its rate of inactivation within 1min (Fig. 3B). The lowest dose of ATP tested ($10^{-6} \text{ mol l}^{-1}$) reduced action potential duration in Ca^{2+} -free saline to $71 \pm 7\%$ of control values ($N=5$) within 1min. An example of its effect on membrane current is shown in Fig. 4.

Depolarising steps applied from more positive levels (approximately -40mV) elicited the longer-lasting component on its own, indicating the presence of at least two types of Ca^{2+} channels: low-voltage-activated (LVA) and high-voltage-activated (HVA). ATP ($10^{-4} \text{ mol l}^{-1}$) in saline containing Ca^{2+} increased the rate of inactivation of the HVA current (reducing the half-decay time to $32 \pm 12\%$ of control, $N=6$) and reduced its amplitude (to $51 \pm 10\%$, $N=6$), but had little effect on its rate of activation (Fig. 5A). Fig. 5B shows that $10^{-4} \text{ mol l}^{-1}$ ATP reduced the duration, but not the amplitude, of the dual (LVA+HVA) inward current in the presence of external Ca^{2+} (2 mmol l^{-1} , cf.

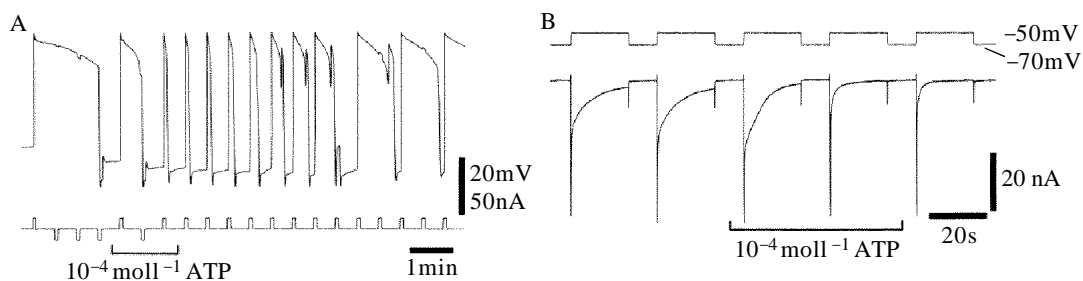


Fig. 3. (A) ATP ($10^{-4} \text{ mol l}^{-1}$) reversibly reduces the duration of gland-cell action potentials that had already been prolonged by perfusion with Ca^{2+} -free saline. Each Na^{+} -dependent action potential (upper trace) was elicited by a depolarizing pulse (lower trace). Initially, hyperpolarizing pulses were also applied to terminate the prolonged spikes (see Wuttke and Berry, 1988). (B) Voltage-clamp steps from a holding potential of -70mV to -50mV (upper trace) elicit two-component inward currents. ATP ($10^{-4} \text{ mol l}^{-1}$) increases the rate of inactivation of the current but has little effect on its peak amplitude. The saline was Ca^{2+} -free.

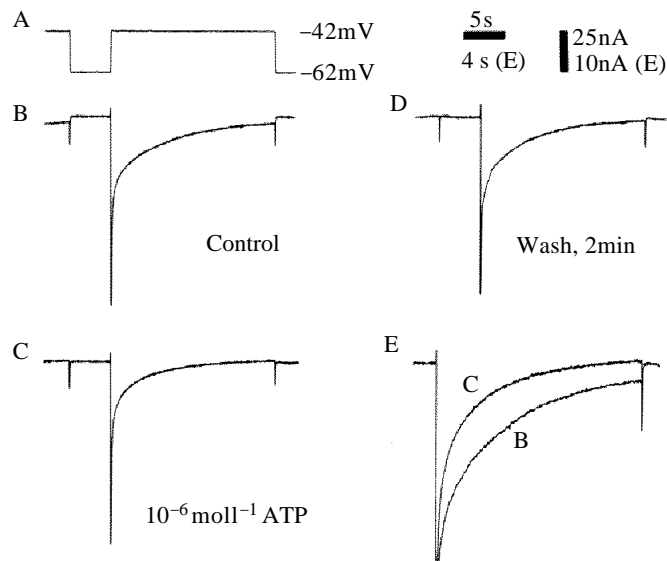


Fig. 4. Effect of $10^{-6} \text{ mol l}^{-1}$ ATP on the two-component inward current in Ca^{2+} -free saline. (A) Pulse protocol, (B) initial (control) current, (C) current after 1 min of exposure to $10^{-6} \text{ mol l}^{-1}$ ATP, (D) recovery after a 2 min wash, (E) records B and C are superimposed and at higher amplification (fast component is clipped) to illustrate more clearly the effect of ATP.

Fig. 3B). The preparation was bathed in saline containing 50 mmol l^{-1} tetraethylammonium and 10 mmol l^{-1} 4-aminopyridine in order to separate the Ca^{2+} currents from K^{+} currents; this also ensured that the effects of ATP on inward current were not caused by an increase in outward K^{+} current. Responses to ATP were unaffected by replacement of Ca^{2+} with 10 mmol l^{-1} Ba^{2+} , which also blocks K^{+} currents ($N=4$; data not shown). Furthermore, when inward current was blocked with 10 mmol l^{-1} Co^{2+} , ATP did not potentiate the voltage-dependent outward current ($N=5$) (Fig. 6; in this cell the current was slightly reduced by ATP).

Qualitatively similar results were obtained with AMP, cyclic AMP, GMP, cyclic GMP and guanosine (data not shown; see Wuttke and Berry, 1991, for the effects of cyclic GMP on spike duration in Ca^{2+} -free saline). Relative potencies were assessed by measuring the effects on the half-amplitude duration of prolonged spikes (4–6.5 s) in saline containing 50 mmol l^{-1} tetraethylammonium and 10 mmol l^{-1} 4-aminopyridine. Control action potentials of constant duration were elicited by a 0.4 s depolarizing pulse applied at 2-min intervals. Nucleotides were passed into the bath at increasing concentrations and spike duration was measured after 10 min of exposure to each dose. There was a 20 min washout between applications, which allowed full recovery from any response. Each nucleotide was tested at all doses on at least four cells. The following order of potency was observed: $\text{ATP} > \text{AMP} > \text{GMP} > \text{cyclic GMP} > \text{cyclic AMP} > \text{guanosine} > \text{adenosine}$. At $10^{-6} \text{ mol l}^{-1}$, only ATP was effective, reducing spike duration by $18 \pm 3.5\%$. At $10^{-5} \text{ mol l}^{-1}$, this reduction increased to

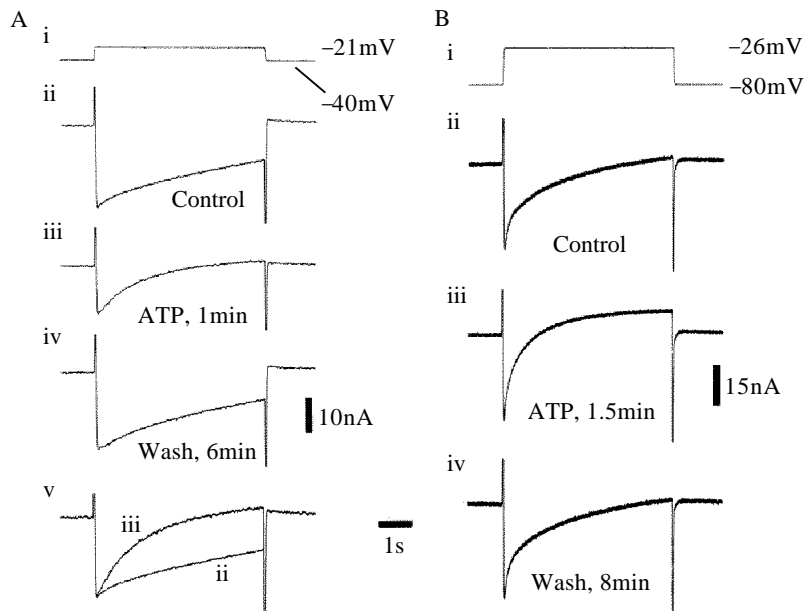


Fig. 5. (A) Voltage steps from -40mV to -21mV (i) elicit only the slowly inactivating component of the inward current (ii) in a salivary gland cell. ATP (10^{-4}mol l^{-1}) reduces the amplitude of the current by 41% in 1min (iii) and increases its rate of inactivation (the inactivation is well described by a single exponential with a time constant of 5.8s; this is reduced to 1.4s by ATP). Full recovery occurs after washing (iv). In v, the peak amplitude of record iii is normalised to that of ii and the traces are superimposed to illustrate more clearly the effect of ATP on the rate of inactivation. (B) Voltage steps from a holding potential of -80mV to -26mV (i) elicit both components of the inward current (ii). ATP (10^{-4}mol l^{-1}) increases the rate of inactivation without altering the maximum amplitude of the current (iii). Washing returns the current to control values (iv). ATP did not affect the holding current. In A and B, the saline contained 50mmol l^{-1} tetraethylammonium and 10mmol l^{-1} 4-aminopyridine.

$47\pm 6\%$; AMP and GMP caused reductions of $26\pm 5.5\%$ and $9\pm 3\%$, respectively, and the other substances had no effect. Cyclic GMP produced a reduction of $31\pm 3.5\%$ at 10^{-4}mol l^{-1} but cyclic AMP, guanosine and adenosine remained ineffective. At 10^{-3}mol l^{-1} , cyclic AMP and guanosine reduced spike duration by $35\pm 4\%$ and $51\pm 11\%$ respectively; adenosine had no effect on four cells but reduced spike duration by 10–13.5% in three others.

Our results show that extracellular ATP reduces action potential duration in leech salivary gland cells by selectively decreasing the amplitude and increasing the rate of inactivation of a high-voltage-activated Ca^{2+} current. The low dose at which the effect occurs ($<10^{-6}\text{mol l}^{-1}$) suggests the presence of a receptor that is similar to the vertebrate P_2 purinoceptor in its relative insensitivity to adenosine. P_2 receptors are not usually coupled to adenylate cyclase, and this enzyme does not appear to be activated by ATP in the salivary cells; for example, 8-bromo-cyclic AMP (presumably acting intracellularly) has the opposing effect of lengthening the normal action potential by blocking outward

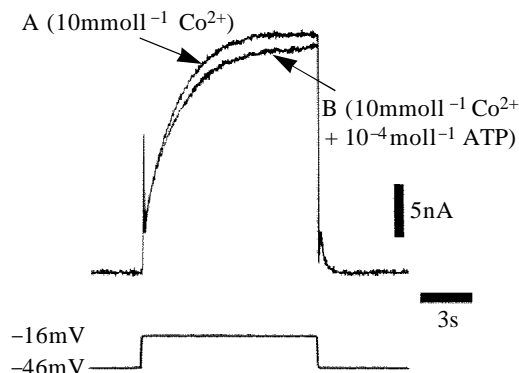


Fig. 6. ATP does not influence net inward current by potentiating outward current. (A) The outward current elicited by a voltage-clamp step to -16mV from a holding potential of -46mV . Inward currents had been blocked by $10\text{mmol l}^{-1}\text{Co}^{2+}$. (B) In the presence of Co^{2+} , the outward current is reduced in amplitude by ATP (10^{-4}mol l^{-1}) and there is no change in its rate of activation.

K^+ currents (Wuttke and Berry, 1991), and we have recently found that 3-isobutyl-1-methylxanthine (a phosphodiesterase inhibitor) also prolongs the action potential (W. A. Wuttke and M. S. Berry, unpublished results). Extracellularly applied cyclic AMP shortens action potentials.

ATP appears to be equipotent in normal saline and in solutions lacking free divalent cations, indicating that both free ATP^{4-} and divalent-cation-complexed ATP can cause the effects outlined above. This also indicates that ectokinase or ecto-ATPase activity is not involved in the mechanism of receptor activation because phosphate transfer reactions require divalent cations.

ATP is unlikely to act indirectly by a Ca^{2+} -induced inactivation of Ca^{2+} current (e.g. Xiong *et al.* 1991) because its effects persist in the absence of external Ca^{2+} . Release of Ca^{2+} from internal stores cannot be excluded, but an effect on membrane conductance might then be expected (e.g. activation of a Ca^{2+} -dependent K^+ conductance; Wuttke and Berry, 1991).

ATP may be a neurotransmitter in the rat salivary gland; it activates Ca^{2+} -permeable channels, and the resultant increase in intracellular Ca^{2+} ultimately stimulates fluid secretion (Soltoff *et al.* 1990). However, the mechanism of secretion by the salivary gland of *Haementeria* is quite different (Wuttke *et al.* 1989), and we have no evidence of any physiological role for ATP. We cannot yet explain why these exocrine cells have the unusual feature of a variety of voltage-gated channels: K^+ (delayed rectifier) (Wuttke and Berry, 1991), Na^+/K^+ (inward rectifier) (Wuttke and Berry, 1992) and Ca^{2+} (HVA), all of which are modulated by putative neurotransmitters.

This work was supported by SERC (grant no. GR/F/17087) and the SmithKline (1982) Foundation.

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