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INHIBITION OF VOLTAGE-DEPENDENT Ca^{2+} INFLUX BY EXTRACELLULAR ATP IN SALIVARY CELLS OF THE LEECH *HAEMENTERIA GHILIANII*

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Summary

The effects of extracellular ATP on intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) and depolarization-induced elevations of $[Ca^{2+}]_i$ were investigated in salivary cells of the leech *Haementeria ghilianii* using the fluorescent Ca^{2+} indicator Fura-2. Simultaneously, the membrane potential was monitored or controlled by voltage-clamp. The cell membrane was depolarized either by transient elevations of the extracellular K^+ concentration ($[K^+]_o$) to 90 mmol l^{-1} or by depolarizing steps under voltage-clamp. The resulting transient elevations of $[Ca^{2+}]_i$ (Ca^{2+} transients) could be repeatedly elicited with little variability in amplitude. Ca^{2+} transients were completely inhibited by 2 mmol l^{-1} Ni^{2+} or in Ca^{2+}-free saline. The transients are, therefore, dependent on Ca^{2+} influx from the external medium through voltage-gated Ca^{2+} channels. The Ca^{2+} influx was rapidly and reversibly inhibited by extracellular application of ATP. The effect was dose-dependent with a threshold concentration below 10^{-7} mol l^{-1}. A 50 % reduction in the amplitude of Ca^{2+} transients was obtained by application of 1–2 μmol l^{-1} ATP or ATP-γ-S (apparent IC_{50}, 1.6 μmol l^{-1} ATP) and Ca^{2+} transients were almost completely inhibited by 30–100 μmol l^{-1} ATP. Resting $[Ca^{2+}]_i$, the resting membrane potential and membrane potential changes induced by 90 mmol l^{-1} $[K^+]_o$ were not affected by ATP. Adenosine (10 μmol l^{-1}) did not affect resting $[Ca^{2+}]_i$, the resting membrane potential or membrane potential changes induced by 90 mmol l^{-1} $[K^+]_o$ and had little effect on Ca^{2+} transients. Suramin, an antagonist of vertebrate P_2 receptors, was without effect on the inhibitory actions of ATP. We conclude that activation of a suramin-insensitive purinoceptor by ATP inhibits Ca^{2+} influx through voltage-gated Ca^{2+} channels in the salivary cells of *Haementeria ghilianii.*

Key words: intracellular Ca^{2+}, ATP, purinoceptor, salivary cell, leech, *Haementeria ghilianii.*

Introduction

Extracellular ATP influences cell functions in a variety of vertebrate preparations (Gordon, 1986). Its biological actions are mediated by purinergic P_2 receptors, and different classes of ATP receptors have been identified by their selectivity for ATP analogues (Burnstock, 1990). At least three different signal transduction pathways are correlated with P_2 receptors (Dubyak, 1991): (1) activation of modulatory G-proteins; (2) activation of ligand-gated cation channels; and (3) formation of non-selective pores permeable to ions and small metabolites.

Little is known about the physiological role of extracellular ATP in invertebrates, but recent evidence suggests that they possess receptors similar to the vertebrate P_2 type (Wuttke and Berry, 1993; Backus *et al.* 1994). For example, ATP modulates the electrical properties of the salivary cells of the giant Amazon leech *Haementeria ghilianii*. These cells are extremely large (up to 1.2 mm in diameter) and do not show electrical or dye-coupling (Sawyer *et al.* 1982; Marshall and Lent, 1984). They produce overshooting action potentials that are Ca^{2+}-dependent with no apparent contribution by Na^+ (Marshall and Lent, 1984; Wuttke and Berry, 1988). The cells possess at least two types of voltage-activated Ca^{2+} channels that have been classified according to their voltage range of activation as low-voltage-activated (LVA) and high-voltage-activated (HVA). The HVA conductance is selectively modulated by micromolar concentrations of external ATP which reduce action potential duration and, in voltage-clamp experiments, decrease peak inward current and increase the rate of current inactivation (Wuttke and Berry, 1993). These results suggested the presence of a receptor similar to the vertebrate P_2 type.

In the present study, we further characterize the receptor by studying the effects of extracellular ATP on $[Ca^{2+}]_i$ of salivary cells of *Haementeria ghilianii*, using the Ca^{2+} indicator Fura-2 in combination with membrane potential measurements or

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voltage-clamp. We demonstrate that micromolar concentrations of external ATP, but not adenosine, reversibly and in a dose-dependent manner inhibit the influx of Ca^{2+} through voltage-activated Ca^{2+} channels in the salivary cells without affecting resting $[Ca^{2+}]_i$ or membrane potential.

Materials and methods

Preparation

Experiments were performed on isolated anterior salivary glands of the giant Amazon leech *Haementeria ghilianii* (de Filippi) obtained from our breeding colony. The glands were pinned to the Sylgard base of a Perspex experimental bath 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; Hepes, 10 (pH 7.4) at room temperature (20–25 °C). Saline with a K^+ concentration of 90 $mmol\,l^{-1}$ was made by equimolar substitution of NaCl with KCl. The following substances (Sigma) were added to the saline at known concentrations: adenosine, adenosine 5′-triphosphate (ATP), ATP-γ-S and $NiCl_2$. Suramin was kindly provided by Bayer AG (Leverkusen, Germany).

Electrophysiology

Microelectrodes for the injection of Fura-2 and for applying current were bevelled, mounted on high-speed steppers (SPI, Oppenheim, Germany) and connected to an Axoclamp 2A amplifier (Axon Instruments, USA). Membrane potential and applied current were displayed on an oscilloscope and recorded by a computer and a chart recorder.

Measurement of intracellular [Ca^{2+}] with Fura-2

Fura-2 was used to determine changes in the intracellular free Ca^{2+} concentration of single salivary cells. Large cells with a diameter of up to 1200 μm were usually chosen (see Sawyer *et al.* 1982; Walz *et al.* 1988, for details of the anatomy and ultrastructure of the preparation). The experimental bath was mounted on the stage of an upright microscope (Axioskop, Zeiss) and microelectrodes were positioned using low magnification (10× objective). Individual cells were impaled under visual control with a microelectrode containing 10 $mmol\,l^{-1}$ Fura-2 dissolved in 100 $mmol\,l^{-1}$ KCl. For voltage-clamp experiments, cells were impaled with a second microelectrode containing 3 $mol\,l^{-1}$ KCl. A steady hyperpolarizing current of 10–20 nA was passed for 10–30 min through the Fura-2-containing electrode to inject the dye into the cell. The intracellular Fura-2 was excited alternately with monochromatic light at 350 nm and 380 nm (band width 4–8 nm) from a Deltascan dual-wavelength spectrofluorimeter (PTI, Wedel, Germany) using a 75 W xenon arc lamp. The light was directed onto the preparation through a water immersion 40× objective (Achroplan 40*/w, Zeiss). Fluorescence intensity was measured at 510 nm using a photon-counting photomultiplier tube. Measurements were limited by a diaphragm to a rectangular field of view from a central region of the cell soma. The microelectrodes were outside this field to exclude fluorescence light from the electrode containing the Fura-2. Shutters, monochromators and data acquisition were controlled by computer software and by interfaces from PTI. The fluorescence light signals were not calibrated, and results are expressed as the ratio of the fluorescence light upon excitation at 350 and 380 nm (F_{350}/F_{380}).

Results are presented as mean values ± S.D.

Results

On termination of the injection of Fura-2 into a cell, the fluorescence measured at 350 nm was stable or increased slightly during the first 2–5 min. In the majority of cells, however, it decreased exponentially with a half-time of 6–12 min before stabilizing at a steady-state level (54±19 % of the initial maximum fluorescence, N=17). This suggests diffusion of Fura-2 out of the field of view within the large cell. To avoid recording Ca^{2+} measurements during the initial period of relatively fast fluorescence decrease, experiments were begun when the fluorescence had stabilized about 10 min after dye injection.

In contrast to the absolute fluorescence, the ratio of the fluorescent light (F_{350}/F_{380}) emitted from a resting cell was stable from the beginning of the fluorescence measurements throughout an experiment (i.e. >2 h) with a typical value of about 0.4 (0.42±0.05, N=27; see Figs 2–5). No fluorescence could be detected in neighbouring cells, confirming earlier studies that have demonstrated a lack of both dye- and electrical coupling between the salivary cells (Sawyer *et al.* 1982; Marshall and Lent, 1984). The resting membrane potential, measured a few minutes after dye injection, was between −35 and −60 mV (−43±10 mV, N=9), and no spontaneous changes in membrane potential or $[Ca^{2+}]_i$ were observed.

Depolarization-induced transient elevations of $[Ca^{2+}]_i$

It has been shown previously that an increase in $[K^+]_o$ to 90 $mmol\,l^{-1}$ elicits a transient elevation of $[Ca^{2+}]_i$ (Ca^{2+} transient) in salivary cells of *Haementeria ghilianii* (Wuttke *et al.* 1994). In the present study, either this method or controlled current injection under voltage-clamp was used to depolarize the cell membrane. Both methods produced large Ca^{2+} transients that could be repeatedly elicited with little variation in peak amplitude (see Figs 1, 2). In all experiments, the depolarization was maintained beyond the rise time of the Ca^{2+} signal to ensure a maximal increase in $[Ca^{2+}]_i$.

Simultaneous measurements of $[Ca^{2+}]_i$ and membrane potential showed that the depolarization induced by 90 $mmol\,l^{-1}$ $[K^+]_o$ initiated action potentials that caused most of the Ca^{2+} transient. A typical example of such an experiment is illustrated in Fig. 1A. The increase in $[K^+]_o$ depolarized the cell membrane from −60 to −5 mV, and four action potentials were elicited. The depolarization leading to the first action potential caused an initial rise in $[Ca^{2+}]_i$, followed by four discrete increases caused by the four action potentials (arrows in Fig. 1A). $[Ca^{2+}]_i$ peaked shortly after the last action potential and then declined towards the resting level while the membrane was still depolarized. In similar experiments, an increase in

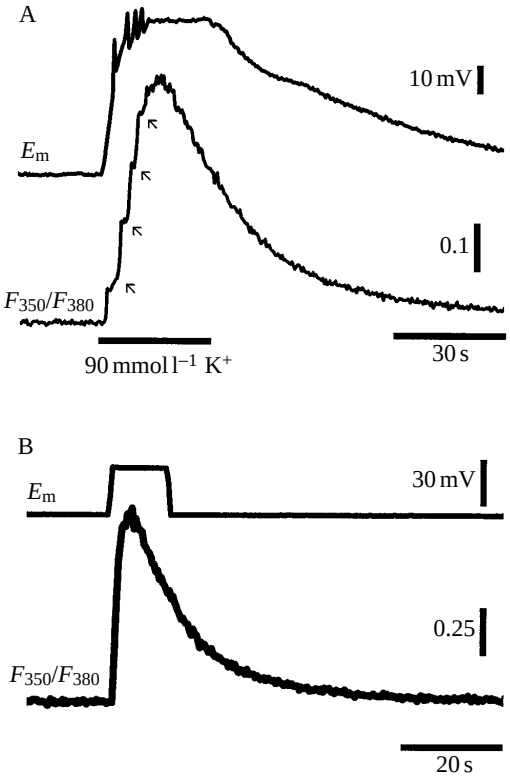

Fig. 1. Depolarization-induced transient elevations of $[Ca^{2+}]_i$. The membrane potential (E_m) and the intracellular Ca^{2+} signal (F_{350}/F_{380}) were simultaneously measured in two different cells. (A) A transient increase in $[K^+]_o$ to 90 mmol l^{-1} produced a depolarization of the cell membrane from −60 to −5 mV and initiated four action potentials (clipped). The arrows (lower trace) point to the discrete elevations in $[Ca^{2+}]_i$ associated with the four action potentials. (B) A depolarization step under voltage-clamp from −40 to −10 mV produced a large and monophasic increase in $[Ca^{2+}]_i$. As in A, $[Ca^{2+}]_i$ began to decline towards the resting level during the maintained depolarization.

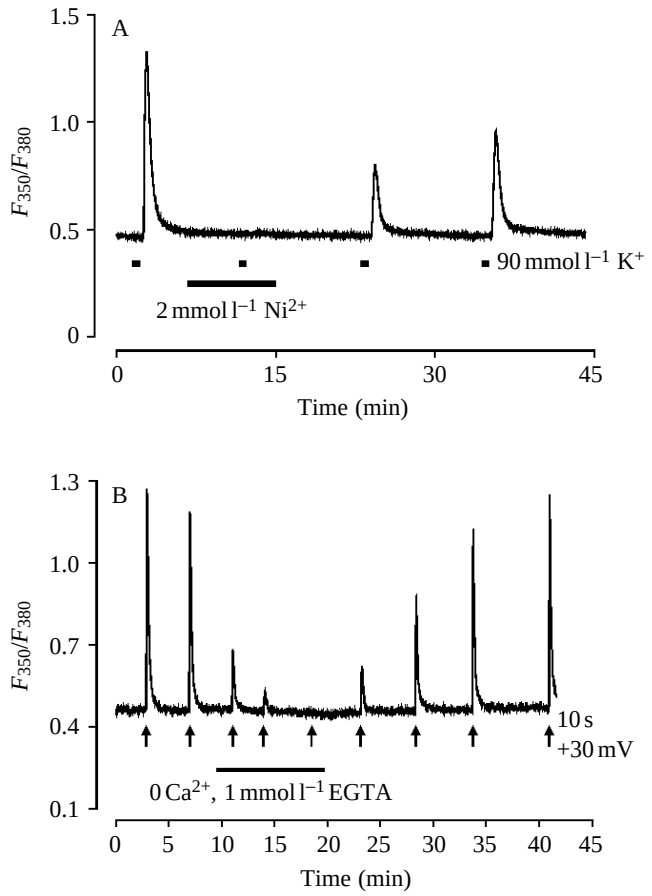

Fig. 2. Ca^{2+} transients are dependent on Ca^{2+} influx through voltage-gated Ca^{2+} channels. (A) Ca^{2+} transients, elicited by increasing $[K^+]_o$ to 90 mmol l^{-1} for 30 s, were completely inhibited by 2 mmol l^{-1} Ni^{2+}, an inorganic blocker of Ca^{2+} channels. The inhibition did not reverse readily after the removal of Ni^{2+} from the saline. (B) Ca^{2+} transients elicited by depolarizing steps (+30 mV for 10 s, arrows) under voltage-clamp were reversibly abolished by removal of external Ca^{2+} (0 Ca^{2+}, 1 mmol l^{-1} EGTA). The holding potential was −40 mV. The recordings shown in A and B are from different cells.

$[K^+]_o$ to 90 mmol l^{-1} depolarized the cell membrane to −9±3 mV (N=9) and initiated 2–6 action potentials. Each action potential was associated with a discrete elevation of $[Ca^{2+}]_i$, giving the overall increase a stepwise appearance.

In a different set of experiments, cells were voltage-clamped at a holding potential close to the resting membrane potential. Depolarizing voltage steps to −20 or −10 mV with a duration of 10–20 s resulted in monophasic elevations of $[Ca^{2+}]_i$ with an amplitude similar to those elicited by 90 mmol l^{-1} $[K^+]_o$ (Fig. 1B). Since the aim of using the voltage-clamp was to control membrane potential rather than to correlate Ca^{2+} inward current with Ca^{2+} transient amplitude, no attempts were made to block outward currents or to record clamp currents at a resolution that would allow a more detailed analysis.

Ca^{2+} transients are dependent on the influx of Ca^{2+} through voltage-gated channels

The Ca^{2+} transients are thought to result from activation of voltage-dependent Ca^{2+} channels allowing influx of Ca^{2+} from the external medium. To support this idea, cells were depolarized in the presence of Ni^{2+}, an inorganic Ca^{2+} antagonist that inhibits Ca^{2+} entry through Ca^{2+} channels, and in the absence of extracellular Ca^{2+}.

Many reports have shown that low concentrations (IC_{50}, approximately 50 μmol l^{-1}) of Ni^{2+} inhibit most LVA Ca^{2+} channels but have relatively little effect on almost all HVA Ca^{2+} channels (Fox *et al.* 1987; Bean, 1989; Zhang *et al.* 1993). Since the salivary cells of *Haementeria ghilianii* express both LVA and HVA Ca^{2+} channels (Wuttke and Berry, 1993), we used a relatively high concentration (2 mmol l^{-1}) of Ni^{2+} in order to block both Ca^{2+} conductances effectively. As shown in Fig. 2A, the Ca^{2+} transients were completely abolished by 2 mmol l^{-1} Ni^{2+} (N=2). They recovered only slowly and incompletely after removal of the Ni^{2+} from the saline.

Ca^{2+} transients were similarly abolished by removal of Ca^{2+} from the saline that contained 1 mmol l^{-1} EGTA (Fig. 2B,

N=2). The effects of Ca^{2+} removal were fully reversible. The results indicate that the Ca^{2+} transients depend entirely on Ca^{2+} influx from the external medium through voltage-activated channels.

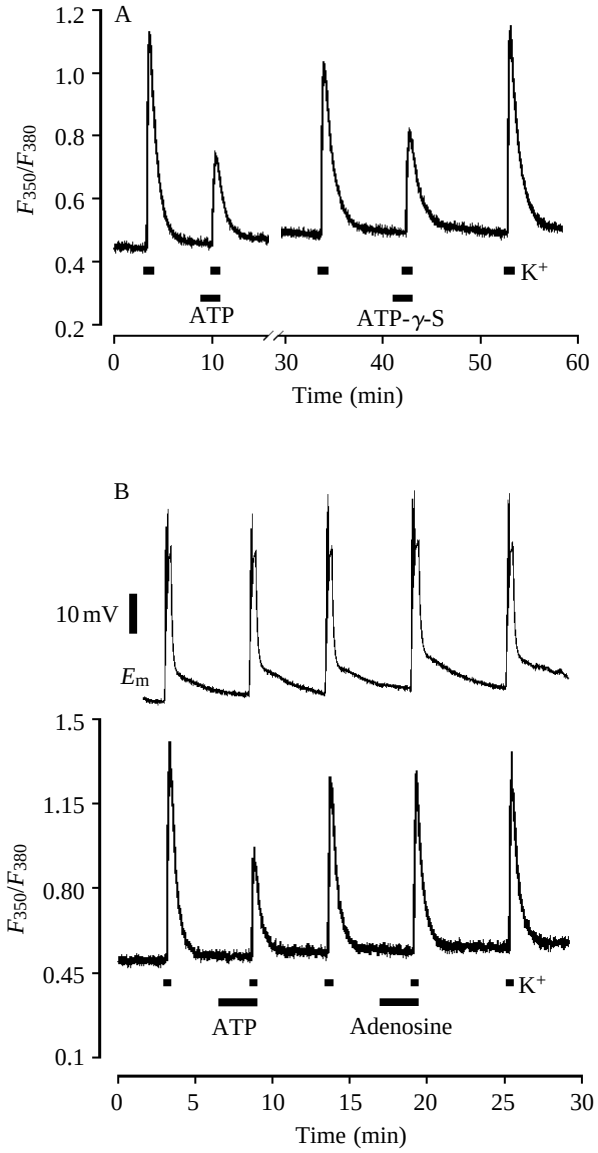

Fig. 3. Effects of ATP, ATP-γ-S and adenosine on Ca^{2+} transients. (A) ATP (1 μmol l^{-1}) and its hydrolysis-resistant analogue ATP-γ-S (1 μmol l^{-1}) attenuate Ca^{2+} transients evoked by membrane depolarizations in 90 mmol l^{-1} $[K^+]_o$ to a similar extent. The gap represents a time of 13.5 min. (B) The membrane potential (E_m, upper trace) and $[Ca^{2+}]_i$ (lower trace) were measured simultaneously. The first increase in $[K^+]_o$ depolarized the cell membrane from −60 to −5 mV and elicited five action potentials (which are not clearly resolved in this figure). ATP (1 μmol l^{-1}) reduced the number of action potentials to two without affecting the depolarization amplitude. The following control depolarization (in the absence of ATP) elicited four action potentials, and in the presence of adenosine (10 μmol l^{-1}) this number increased to five. Adenosine had little effect on the Ca^{2+} transients and did not affect the membrane potential changes induced by 90 mmol l^{-1} $[K^+]_o$.

Effects of extracellular ATP on Ca^{2+} transients, $[Ca^{2+}]_i$ and membrane potential

To test for its effects on Ca^{2+} transients, ATP (1 μmol l^{-1}) or adenosine (10 μmol l^{-1}) was added to the saline for 20–60 s before the membrane of a salivary cell was depolarized. ATP (1 μmol l^{-1}) reduced the amplitude of Ca^{2+} transients on average to 57±18 % (N=12; Fig. 3); this effect was reversible within a few minutes after the ATP had been washed off the preparation. ATP-γ-S, an analogue of ATP that is resistant to hydrolysis by ectonucleotidases, attenuated the Ca^{2+} transients to a similar extent as did ATP (Fig. 3A). Adenosine (10 μmol l^{-1}), an agonist for P_1-type purinergic receptors, had little or no effect on the Ca^{2+} transients (−5±7 %, N=4; Fig. 3B). This suggests that ATP, but not one of its dephosphorylated analogues, inhibits Ca^{2+} influx.

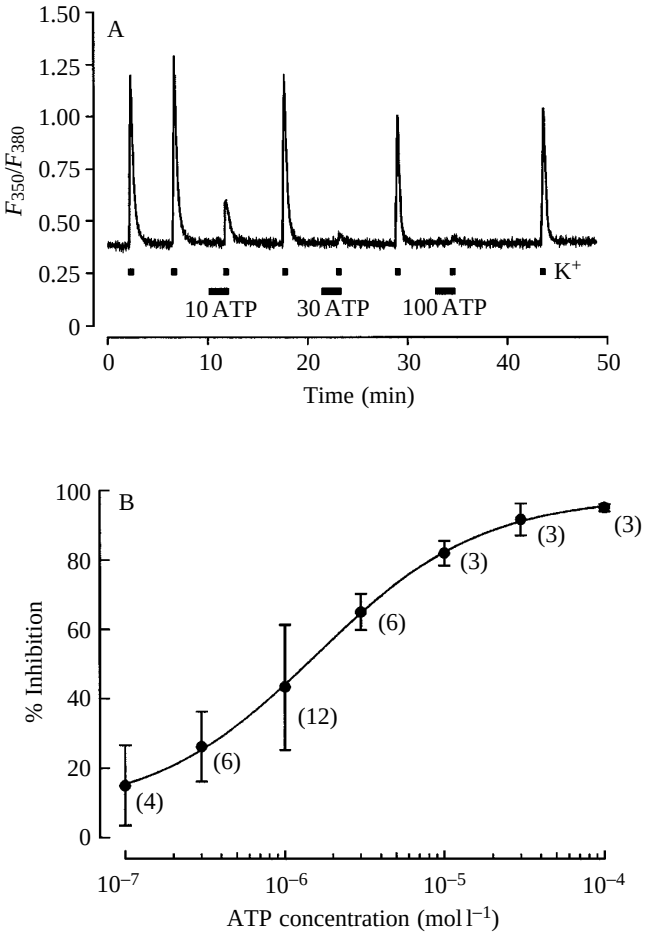

Fig. 4. The inhibition of Ca^{2+} influx by ATP is concentration-dependent. (A) Ca^{2+} transients, elicited by 90 mmol l^{-1} $[K^+]_o$, were greatly attenuated by 10 μmol l^{-1} ATP and nearly completely inhibited by 30 and 100 μmol l^{-1} ATP. The inhibitory effects of such high concentrations of ATP were not always completely reversible within the duration of the experiment. (B) Plot of Ca^{2+} transient inhibition (% of control) as a function of external ATP concentration. The points indicate mean values ± S.D. (N is given in parentheses) and have been fitted with a non-linear regression routine with a Hill coefficient of 0.84 and an apparent IC_{50} of 1.6 μmol l^{-1}.

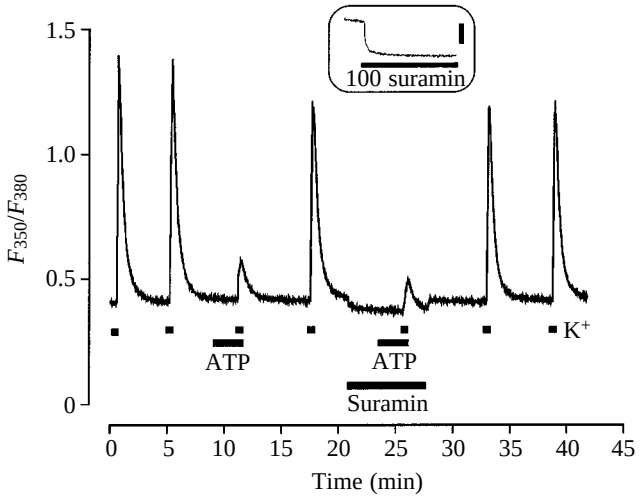

Fig. 5. Suramin does not block the inhibitory actions of ATP. Ca^{2+} transients were elicited by increases in $[K^+]_o$ to 90 mmol l^{-1}. ATP (1 μmol l^{-1}) was applied 2 min and suramin (25 μmol l^{-1}) 4.5 min before the membrane was depolarized. Suramin caused an apparent decrease in $[Ca^{2+}]_i$ that was due to a selective attenuation of the 350 nm fluorescence signal. The decrease of this signal induced by 100 μmol l^{-1} suramin is shown on an expanded scale in the inset, where the horizontal bar indicates the application of suramin (100 s) and the vertical bar represents 5×10^4 cts s^{-1} fluorescence emission at 350 nm excitation.

ATP, like adenosine, did not influence the resting $[Ca^{2+}]_i$, the resting membrane potential or the membrane potential changes induced by 90 mmol l^{-1} $[K^+]_o$ (Fig. 3). Therefore, ATP appears to inhibit Ca^{2+} influx directly by modulating Ca^{2+} channels rather than by acting indirectly *via* changes in membrane potential or $[Ca^{2+}]_i$. This modulatory action of ATP is probably mediated by a purinoceptor that appears to be similar to the vertebrate P_2 type in its insensitivity to adenosine.

ATP attenuated the Ca^{2+} transients in a dose-dependent manner. The threshold concentration was below 0.1 μmol l^{-1} (the lowest concentration tested) (Fig. 4), and the apparent IC_{50}, as determined from the dose–response curve (Fig. 4B), was 1.6 μmol l^{-1} ATP. Ca^{2+} transients were further reduced by higher ATP concentrations and almost completely inhibited by 30 μmol l^{-1} (6±1 %, *N*=3) and 100 μmol l^{-1} ATP (5±1 %, *N*=3) (Fig. 4). The generation of fully blown action potentials was completely suppressed in the presence of such high concentrations of ATP, although action potentials with a greatly reduced amplitude were sometimes observed. Lower concentrations of ATP reduced both the number of action potentials elicited and the influx of Ca^{2+} associated with an action potential. For example, 1 μmol l^{-1} ATP reduced the number of action potentials to 51±23 % (*N*=10) and the increase in $[Ca^{2+}]_i$ associated with the first action potential to 56±18 % (*N*=9) of the control values.

Suramin does not block the effects of ATP

The trypanocidal compound suramin has been reported to be a selective antagonist of vertebrate P_2 receptors (vas deferens, Dunn and Blakeley, 1988; phaeochromocytoma cells, Nakazawa *et al.* 1990; bladder and taenia coli, Hoyle *et al.* 1990; coeliac ganglion neurones, Evans *et al.* 1992; medial habenula neurones, Edwards *et al.* 1992; ventricular myocytes, Qu *et al.* 1993; megakaryocytes, Uneyama *et al.* 1994). To test for its effects on the salivary cells of *Haementeria ghilianii*, suramin (25 μmol l^{-1} or 100 μmol l^{-1}) was added to the saline 2–3 min before the ATP (1 μmol l^{-1}). As illustrated in Fig. 5, suramin reduced the fluorescence ratio F_{350}/F_{380} by selectively attenuating the 350 nm fluorescence (see inset of Fig. 5). It did not, however, antagonize the inhibitory actions of ATP (*N*=3).

Discussion

Validity of the fluorescence measurements

In this paper, we show that measurements of $[Ca^{2+}]_i$ in the salivary cells of *Haementeria ghilianii* can be performed over long periods (i.e. >2 h) using the Ca^{2+} indicator Fura-2. This fluorimetric technique may be hampered by photobleaching (Becker and Fay, 1987) or loss of dye from the cell (Mitsui *et al.* 1993), including diffusion of dye into cells electrically coupled to the cell under investigation (Munsch and Deitmer, 1995). In the majority of our experiments, the Ca^{2+}-independent fluorescence decreased after dye injection by about 50 % before it stabilized. Diffusion of dye into neighbouring cells is unlikely to have contributed to the decrease. The salivary cells are not electrically coupled (Wuttke and Berry, 1988) and we did not observe a spread of fluorescence into neighbouring cells. It is also unlikely that continuous leakage of dye across the cell membrane or photobleaching of the dye are major factors causing the decrease, because both should have resulted in a rapid and nearly complete loss of fluorescence (Munsch and Deitmer, 1995). We think, therefore, that diffusion of the dye away from the point of injection into the large cell was the main cause for the observed changes in absolute fluorescence. Considering the enormous size of the salivary cells (up to 1200 μm in diameter), it is likely that local changes in dye concentration due to diffusion continue for some time after dye injection. This could either decrease or increase the fluorescence, depending on the relative positions of the field of view (which covers only part of the cell soma) and the insertion point of the dye-filled microelectrode. We observed an initial increase in fluorescence immediately after finishing the dye injection in some of our experiments.

We used the ratiometric approach for the Ca^{2+} imaging and found in all our experiments that $[Ca^{2+}]_i$ was stable from the beginning of the fluorescence measurements (which began after dye injection) throughout the experiments. This is in agreement with the theory of the ratiometric fluorescence measurement, which predicts that the ratio of the Ca^{2+}-independent and Ca^{2+}-dependent fluorescence is sufficient to measure $[Ca^{2+}]_i$ independently of variable factors such as the dye concentration (Grynkiewicz *et al.* 1985).

Receptor type

ATP attenuated the amplitude of Ca^{2+} transients in a rapid and concentration-dependent manner with a threshold concentration below $0.1\,\mu mol\,l^{-1}$, while adenosine was without effect. This suggests the presence of a P_2-type purinoceptor in the salivary cells of *Haementeria ghilianii*.

The two major classes of P_2 purinoceptors, P_{2X} and P_{2Y}, may be distinguished on the basis of the relative potencies of ATP analogues and differing functional responses (Burnstock, 1990). The P_{2X} receptors belong to the superfamily of transmitter-gated ion channels, whereas the P_{2Y} receptors are thought to be metabotropic and members of the G-protein-coupled receptor superfamily. We did not test for potency orders of ATP analogues to distinguish between P_{2X} and P_{2Y}. However, our experiments provide no evidence for the coupling of the purinoceptor to a ligand-gated ion channel. These channels are usually permeable to Na^+, K^+ and Ca^{2+}, and their opening has a depolarizing, excitatory effect (Bean, 1992). ATP, however, did not affect the resting membrane potential of salivary cells (Fig. 3) nor did it produce a change in membrane resistance or, if the membrane potential was controlled by voltage-clamp, in holding current (Wuttke and Berry, 1993).

The purinoceptor thus seems to resemble the vertebrate P_{2Y} type (for a recent description and newly proposed subclassification, see Barnard *et al.* 1994). Because UTP has a similar potency compared with ATP (Everill and Berry, 1995), the purinoceptor may at present best be compared with the P_{2U} type (O'Connor *et al.* 1991), although the presence of two independent receptors for UTP and ATP has not been excluded.

Besides certain similarities, there are also important differences in the properties of vertebrate P_{2Y} receptors and the purinoceptor of the salivary cells. For example, activation of vertebrate P_{2Y} receptors usually results in the release of Ca^{2+} from intracellular stores (Barnard *et al.* 1994). In the salivary cells, however, even high concentrations of ATP failed to increase $[Ca^{2+}]_i$ (Fig. 4A). The receptors are also different in their sensitivity to suramin, which has been shown to block P_2 receptors in many different vertebrate preparations. In the salivary cells, however, suramin did not antagonize the inhibitory actions of ATP on the Ca^{2+} transients (Fig. 5; Everill and Berry, 1995). The receptor shares this insensitivity with other invertebrate P_2-type purinoceptors, expressed by neurones in the central nervous system of the medicinal leech (Backus *et al.* 1994).

ATP inhibition of Ca^{2+} transients

The depolarization-induced Ca^{2+} transients depend primarily on the influx of Ca^{2+} through voltage-gated Ca^{2+} channels (Fig. 2). This influx is consistently and rapidly inhibited by ATP. At low ATP concentrations and with $90\,mmol\,l^{-1}$ K^+ as the depolarizing agent, this reduction is due to a decrease in both the number of action potentials elicited and the Ca^{2+} influx associated with each action potential. Higher concentrations of ATP ($30–100\,\mu mol\,l^{-1}$) block the generation of action potentials and almost completely inhibit the Ca^{2+} transients without altering the resting $[Ca^{2+}]_i$, the resting membrane potential or the depolarization amplitude. This rules out the involvement of a Ca^{2+}-induced inactivation of Ca^{2+} current (e.g. Xiong *et al.* 1991) and suggests that the Ca^{2+} influx is inhibited by a direct modulation of voltage-gated Ca^{2+} channels. A previous voltage-clamp study supports the view that an HVA Ca^{2+} conductance is selectively modulated by ATP, reducing the Ca^{2+} current amplitude and increasing its rate of inactivation in these salivary cells (Wuttke and Berry, 1993).

A similar inhibition by ATP of a Ca^{2+} conductance has been found in some vertebrate preparations. In bovine chromaffin cells, ATP slows the activation kinetics of an HVA Ca^{2+} current (Gandía *et al.* 1993), whereas in ferret ventricular myocytes, it inhibits an L-type Ca^{2+} current by altering its inactivation kinetics and by slowing the recovery from inactivation (Qu *et al.* 1993). In both preparations, the inhibitory actions of ATP are likely to be mediated by P_2-type purinergic receptors that appear to have a similar affinity for ATP (IC_{50} $1\,\mu mol\,l^{-1}$ and $0.56\,\mu mol\,l^{-1}$, respectively) compared with that of the purinoceptor on the leech salivary cells (IC_{50} $1.6\,\mu mol\,l^{-1}$). Experimental evidence suggests that the two vertebrate receptors are coupled to G-proteins. No such evidence is yet available for the purinoceptor of *Haementeria ghilianii*.

P_2 receptors are not usually coupled to adenylyl cyclase (Burnstock, 1990). Recent evidence, however, suggests that activation of certain P_{2U} receptors not only activates phospholipase C, but also changes the concentration of cyclic nucleotides. For example, a reduction in the cyclic AMP concentration has been reported for hamster smooth muscle (Sipma *et al.* 1994) and rat glioma cells (Boyer *et al.* 1993), whereas in mouse neuroblastoma × rat glioma hybrid cells the cyclic GMP synthesis is activated (Reiser, 1995). In the salivary cells, changes in intracellular cyclic AMP concentration are unlikely to be involved in the inhibition of Ca^{2+} transients because cyclic AMP has no apparent effect on Ca^{2+} currents. Injection of cyclic GMP into a salivary cell, however, increases action potential duration by enhancing the inward Ca^{2+} current (Everill and Berry, 1995). Whether ATP, which has an opposite effect on Ca^{2+} currents, acts by reducing the intracellular cyclic GMP concentration remains to be investigated.

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