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Pre- and postsynaptic actions of ATP on neurotransmission in rat submandibular ganglia

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Abstract

The pre- and postsynaptic actions of exogenously applied ATP were investigated in intact and dissociated parasympathetic neurones of rat submandibular ganglia. Nerve-evoked excitatory postsynaptic potentials (EPSPs) were not inhibited by the purinergic receptor antagonists, suramin and pyridoxal-phosphate-6-azophenyl-2′,4′-disulphonic acid (PPADS), or the desensitising agonist, α,β-methylene ATP. In contrast, EPSPs were abolished by the nicotinic acetylcholine receptor antagonists, hexamethonium and mecamylamine. Focal application of ATP (100 μM) had no effect on membrane potential of the postsynaptic neurone or on the amplitude of spontaneous EPSPs. Taken together, these results suggest the absence of functional purinergic (P2) receptors on the postganglionic neurone *in situ*. In contrast, focally applied ATP (100 μM) reversibly inhibited nerve-evoked EPSPs. Similarly, bath application of the non-hydrolysable analogue of ATP, ATPγS, reversibly depressed EPSPs amplitude. The inhibitory effects of ATP and ATPγS on nerve-evoked transmitter release were antagonised by bath application of either PPADS or suramin, suggesting ATP activates a presynaptic P2 purinoceptor to inhibit acetylcholine release from preganglionic nerves in the submandibular ganglia. In acutely dissociated postganglionic neurones from rat submandibular ganglia, focal application of ATP (100 μM) evoked an inward current and subsequent excitatory response and action potential firing, which was reversibly inhibited by PPADS (10 μM).

The expression of P2X purinoceptors in wholemount and dissociated submandibular ganglion neurones was examined using polyclonal antibodies raised against the extracellular domain of six P2X purinoceptor subtypes ($P2X_{1–6}$). In intact wholemount preparations, only the $P2X_5$ purinoceptor subtype was found to be expressed in the submandibular ganglion neurones and no P2X immunoreactivity was detected in the nerve fibres innervating the ganglion. Surprisingly, in dissociated submandibular ganglion neurones, high levels of $P2X_2$ and $P2X_4$ purinoceptors immunoreactivity were found on the cell surface. This increase in expression of $P2X_2$ and $P2X_4$ purinoceptors in dissociated submandibular neurones could explain the increased responsiveness of the neurones to exogenous ATP.

We conclude that disruption of ganglionic transmission *in vivo* by either nerve damage or synaptic blockade may up-regulate P2X expression or availability and alter neuronal excitability.

Author Keywords: acetylcholine; P2X receptors; synaptic transmission; electrophysiology; immunohistochemistry

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There is increasing evidence that ATP plays a role in neurotransmission in mammalian autonomic ganglia (for review see Ralevic and Burnstock, 1998). Extracellular ATP can activate a number of different receptors, termed P2 purinoceptors, which can be classified into two groups, P2X and P2Y purinoceptors. P2X purinoceptors are ligand-gated cation channels and P2Y purinoceptors are G protein-coupled receptors associated with intracellular second messengers. P2X receptors are widely distributed throughout the tissues of

the body, including some synapses where ATP can function as a neurotransmitter (Edwards; Silinsky and Galligan). Molecular cloning has confirmed the existence of at least seven distinct P2X subunits ($P2X_{1–7}$) (Hansen and Ralevic), although the putative $P2X_8$ has recently been demonstrated in avian skeletal muscle (Bo et al., 2000). Individual P2X purinoceptor subunits, however, are not expressed uniformly in all cell types (Dutton et al., 1999).

Purinergic P2 receptors have been shown to be located pre- and postysynaptically on neurones in the central and peripheral nervous systems (Sperlagh; Evans; Galligan; von and Le). Studies carried out on cultured mammalian autonomic neurones have shown that focal application of ATP evokes a rapid inward current (Fieber; Evans and Galligan). These observations have been extended in cultured guinea-pig submucosal and myenteric neurones to demonstrate that ATP mediates fast excitatory postsynaptic currents and acts as a neurotransmitter (Zhou and Galligan, 1996). Furthermore, P2X and nicotinic acetylcholine receptors (nAChRs) have been linked in a mutually inhibitory fashion in guinea-pig myenteric neurones (Zhou and Galligan, 1998). P2X purinoceptors and nAChRs have been localised on the same preganglionic nerve in chick ciliary ganglia (Sun and Stanley, 1996) and dissociated vagal preganglionic neurones in the rat (Nabekura et al., 1995).

The aim of the present study was to investigate ATP modulation of acetylcholine (ACh) release from the intact preganglionic nerve terminals of the rat submandibular ganglia, and to determine which, if any, P2X purinoceptors are located in the intact and dissociated parasympathetic ganglion neurones. A preliminary account of these results has been published in abstract form (Smith and Adams, 1998).

Experimental Procedures

Preparation – submandibular ganglia

Two–four week old rats (Central Animal Breeding House, University of Queensland, Qld., Australia) were anaesthetised with sodium pentobarbitone and killed by cervical fracture prior to removal of the submandibular ganglia. Experimental procedures were in accordance with the guidelines of the University of Queensland Animal Experimentation Committee and all efforts were made to minimise animal suffering and the number of animals used. The submandibular ganglia were exposed and removed as previously described (Kawa and Roper, 1984). Individual preparations were pinned to the Sylgard (Dow-Corning)-covered base of a 2-ml Perspex organ bath. Preparations were perfused with a Krebs solution of the following composition (mM): NaCl 118.4, $NaHCO_3$ 25.0, NaH_2PO_4 1.13, $CaCl_2$ 1.8, KCl 4.7, $MgCl_2$ 1.3 and glucose 11.1, gassed with a mixture of 95% O_2 and 5% CO_2 to pH 7.4, and maintained at 36–37°C.

Intracellular recording and analysis

The lingual nerve was field-stimulated by rectangular voltage pulses via bare platinum wires delivered from a digital stimulator (Pulsar 7^+; Frederick Haer and Company, Brunswick, ME, USA) coupled to an optically isolated stimulation unit (Model DS2; Digitimer Ltd., Welwyn Garden City, UK). Intracellular recordings were made from individual ganglion cells using glass microelectrodes filled with 5 M potassium acetate (resistances 70–120 MΩ). Conventional intracellular recording techniques were used as described previously (Seabrook and Smith). Membrane potentials were recorded through a headstage connected to an Axoclamp-2A amplifier (Axon Instruments, Foster City, CA, USA) in bridge mode and stored on a digital tape recorder (DTR-1204; BioLogic Science Instruments, Claix, France). Evoked events were digitised at 5–10 kHz and transferred to a Pentium computer using an analogue-to-digital converter (TL-1 DMA interface) and Axotape software (Axon Instruments). The amplitude, frequency, rise time and latency of evoked and spontaneous events were analysed using the program, Axograph 2 (Axon Instruments). The mean resting membrane potential of the submandibular ganglion neurones was −66.3±0.6 mV (n=70). The mean baseline was determined by averaging the initial part of the digitised signal between the stimulus artifact and the onset of the response. Data are expressed as the mean±S.E.M. and n values refer to the number of preparations. Data were analysed statistically using Student's paired t-test with the level of significance being taken as $P<0.05$.

Whole-cell patch clamp recording of acutely dissociated neurones

Current and voltage recordings were made from isolated neurones 3–4 h after dissociation from submandibular ganglia from 2–4 week old rats. The procedure for isolation of the neurones from rat submandibular ganglia have been described in detail previously (Liu and Adams, 2001). Electrical access to the cell interior was obtained using the whole-cell recording configuration of the patch clamp technique (Hamill et al., 1981). Pipettes were pulled from thin wall borosilicate glass (Harvard Apparatus Ltd., Edenbridge, Kent, UK) using a Sutter Instruments P-87 pipette puller and following fire polishing had resistances of ~1 MΩ. The intracellular pipette solution contained (in mM): 140 CsCl, 2 MgATP, 2 Cs_4-1,2-bis-(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-

tetraacetic acid (BAPTA) and 10 HEPES–CsOH, pH 7.2. Access resistances using the dialysed whole-cell recording configuration were ≤2 MΩ following series resistance compensation.

Membrane currents and voltages were recorded using an Axopatch 200B patch clamp amplifier (Axon Instruments). Voltage and current protocols were applied using pClamp software (Version 6.1.2, Axon Instruments). Purinoceptor-mediated responses were evoked by focal application of a maximally effective concentration of ATP (100 μM) via pressure ejection (10 psi; Picospritzer II, General Valve, Fairfield, NJ USA) from an extracellular pipette positioned ~50 μm from the soma membrane. Signals were filtered at 1 kHz then digitised at 5 kHz (Digidata 1200 interface, Axon Instruments) and stored on the hard disc of a PC (Pentium II) for viewing and analysis.

Immunohistochemistry

The immunohistochemical protocol was based on previous specific P2X purinoceptor immunostaining procedures (Hansen and Hansen). Submandibular ganglia preparations, either wholemount or cell cultures, were fixed in 4% paraformaldehyde in borate–acetate buffer (pH 9.5) for 1 h. Preparations were placed in 0.1% dimethyl sulphoxide (DMSO) in phosphate-buffered foetal bovine serum (FBS) (100 ml phosphate-buffered saline (PBS), 2 ml FBS, 0.1 ml Triton X-100, 1 g bovine serum albumin) for 30 min. The preparation was then washed three times in PBS (10 min each) and immersed in 20% FBS in PBS for 1 h to block non-specific binding sites in the tissue. This was followed by incubation with 1:100 anti-synaptophysin antibody together with the relevant 1:100 anti-P2X antibody for 24 h at 25°C. Slides were rinsed in PBS followed by the addition of the relevant secondary fluorescent antibodies for 90 min at 25°C. The slides were washed three times in PBS (each for 10 min), coverslipped and sealed. Sections were viewed on a Bio-Rad M600 UV laser confocal microscope system, with standard settings.

Drugs

Drugs were dissolved in the Krebs solution perfusing the preparation. The following drugs were used: ATP, adenosine 5′-*O*-(3-thiotriphosphate) (ATPγS), α-bungarotoxin, κ-bungarotoxin (Calbiochem-Novabiochem, Croyden, Vic., Australia), hexamethonium chloride, mecamylamine and α,β-methylene adenosine 5′-triphosphate (α,β-MeATP Sigma-Aldrich Pty Ltd, Castlehill, NSW, Australia), 2-methylthioadenosine triphosphate tetrasodium salt (2-methylthio-ATP), suramin, pyridoxal-phosphate-6-azophenyl-2′, 4′-disulphonic acid (Research Biochemicals, Natick, MA, USA); and pentobarbitone sodium (Boehringer Ingelheim Pty., Artarmon, NSW, Australia). The P2X antibodies used were a gift from Dr. Julian Barden (University of Sydney) and have been characterised previously (Hansen and Hansen). Anti-mouse synaptophysin antibody specific for neuronal synaptic vesicle membranes was purchased from the DAKO Corporation (Carpinteria, CA, USA). All other immunohistochemical reagents were purchased from Sigma.

Results

Effects of purinoceptor antagonists on excitatory postsynaptic potential (EPSP)

Stimulation of the lingual nerve with trains of stimuli (0.1–50 Hz, 4–50 V, pulse width 0.05–0.25 ms) evoked EPSPs which could initiate action potentials in the cell bodies of the postsynaptic neurones of the rat submandibular ganglion (Seabrook and Adams, 1989). The postganglionic neurones studied (n>150) can be classified into three types by their responses to these trains of stimuli: (i) neurones in which supramaximal stimulation of the preganglionic nerve fibres at 0.1 Hz evokes a suprathreshold EPSP and action potential in response to every stimulus (strong input synapse, approximately 50% of total number of cells studied), (ii) neurones where the EPSP evoked by supramaximal stimulation does not usually reach threshold for the initiation of an action potential (weak input synapse, approximately 25% of total number of cells studied), and (iii) neurones that receive multiple synaptic inputs (approximately 25% of total number of cells studied), cell bodies being innervated by two or more preganglionic axons (Kawa and Smith).

To examine whether ATP was co-released with ACh, the effects of the following purinoceptor antagonists on the nerve-evoked postsynaptic response were investigated. The P2 receptor antagonists, suramin (10–300 μM) and pyridoxal-phosphate-6-azophenyl-2′,4′-disulphonic acid (PPADS, 100 μM) and the desensitising agonist, α,β-methylene ATP (10 μM) had no detectable effects on neurotransmitter release (Fig. 1) in any of the ganglion cell types studied or on the resting membrane potential of the postsynaptic neurone. EPSPs recorded from cells receiving strong or weak inputs were abolished by either hexamethonium (30–100 μM) or mecamylamine (10 μM; Fig. 2A) indicating that EPSPs were mediated by ACh acting at postsynaptic nAChRs. κ-bungarotoxin (100 nM) inhibited the nerve-evoked EPSP by ≥60% indicating that the nAChRs contain the α3β2 subunits (Luetje et al., 1990). In contrast, α-bungarotoxin (1 μM) produced no detectable

effects on EPSPs (Fig. 2B) suggesting that the nAChRs mediating neurotransmission at this synapse do not contain the α7 subunit.

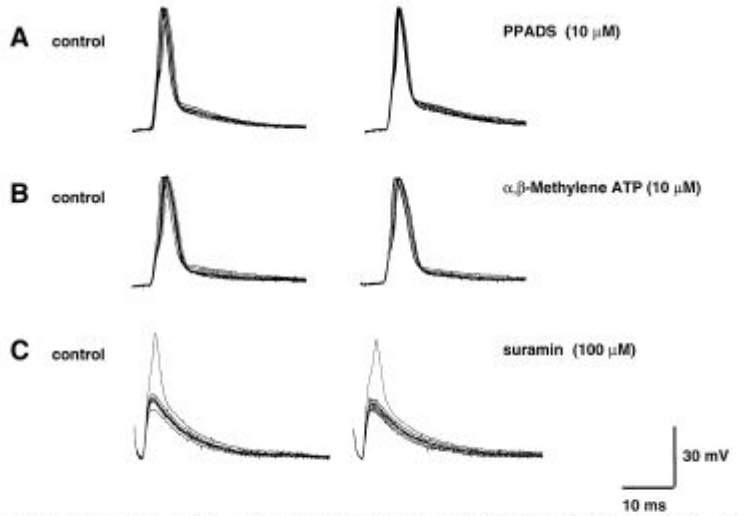

Fig. 1. Effect of P_2 purinoceptor antagonists, PPADS and suramin, and the desensitising agonist, α,β-methylene ATP on nerve-evoked responses in the submandibular ganglion. EPSPs and action potentials were evoked by trains of 10 stimuli at 1 Hz. (A) Ten superimposed traces of action potentials in the absence (control) and presence of PPADS (10 μM). Resting membrane potential, −68 mV. (B) Ten superimposed traces of action potentials in the absence (control) and presence of α,β-methylene ATP (10 μM). Resting membrane potential: −65 mV. (C) Ten superimposed traces of EPSPs in the absence (control) and presence of suramin (100 μM). Resting membrane potential, −67 mV.

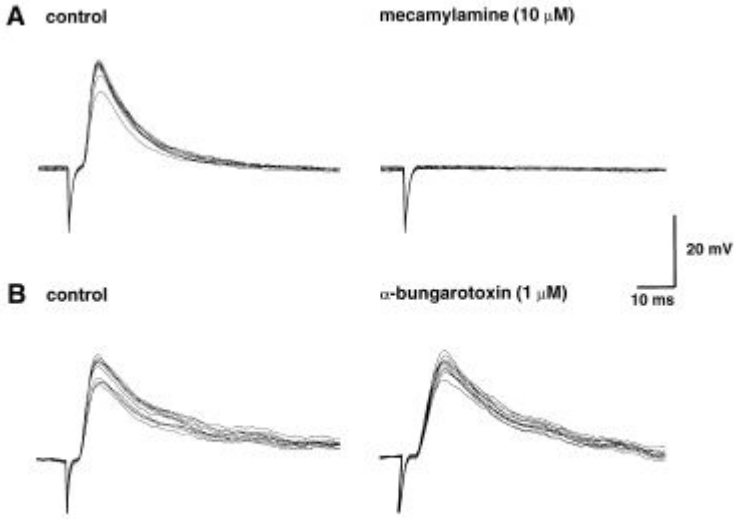

Fig. 2. Effect of the nicotinic receptor antagonists, mecamylamine and α-bungarotoxin, on nerve-evoked responses in the submandibular ganglion. EPSPs were evoked by trains of 10 stimuli at 0.5 Hz. (A) Ten superimposed traces of EPSPs in the absence (control) and presence of mecamylamine (10 μM). Resting membrane potential, −70 mV. (B) Ten superimposed traces of EPSPs in the absence (control) and presence of α-bungarotoxin (1 μM). Resting membrane potential, −64 mV.

In cultured intracardiac ganglion neurones, focal application of ATP evokes a rapid depolarisation and action potentials (Fieber and Adams, 1991). In the intact submandibular ganglion preparation, focal application of ATP (100 μM) had no effect on the resting membrane potential of the postsynaptic neurone or on the amplitude or frequency of spontaneous EPSPs (n=11). However, ATP focally applied during a train of nerve stimulation caused potent inhibitory effects on neurotransmitter release in all neurones studied (strong, weak and multiple input neurones). Fig. 3 shows the effect of ATP (100 μM) on 12 consecutive responses to nerve stimulation at 0.4 Hz. During the focal application of ATP, the EPSP greatly decreased in amplitude, an effect that was rapidly reversible as the ATP is broken down and washed away. EPSPs returned to control amplitude approximately 10 s after the termination of ATP application. Measurements of EPSP amplitude were made at synapses where the nerve-evoked EPSP did not reach threshold for action potential firing. Control EPSPs had a

mean amplitude of 38.6±3.6 mV and after a 10 s focal application of ATP (100 μM), EPSPs had a mean amplitude of 15.7±3.3 mV (*n*=11), a 59% inhibition.

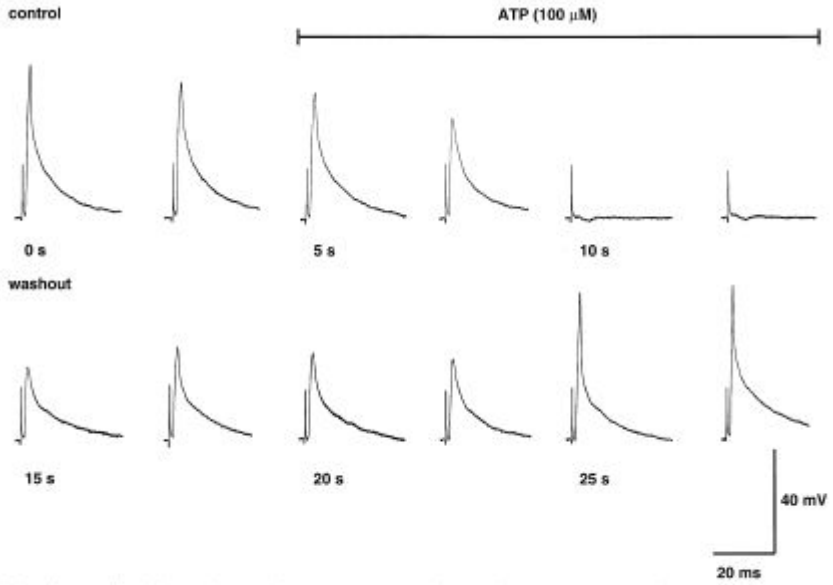

Fig. 3. Effect of focally applied ATP (100 μM) on 12 consecutive excitatory postsynaptic responses to nerve stimulation at 0.4 Hz. Focal application of ATP (10 s duration) is indicated by the black bar. EPSPs returned to control amplitude approximately 10 s after the termination of the agonist application. Resting membrane potential, −71 mV.

Similar experiments were carried out using the non-hydrolysable analogue of ATP, adenosine 5′-*O*-(3-thiophosphate) (ATPγS). ATPγS had no detectable effect on the resting membrane potential of the cell or on the frequency or amplitude of spontaneous EPSPs (*n*=9; data not shown). Fig. 4A shows the effect of ATPγS (100 μM) on 12 consecutive responses to nerve stimulation at 0.4 Hz. During the focal application of ATPγS, the EPSPs dramatically decreased in size, an effect that was more slowly reversible than ATP since ATPγS is not broken down. EPSP amplitude returned to control approximately 2 min after the termination of the application. Control EPSPs had a mean amplitude of 21.8±2.8 mV which decreased to 5.4±1.2 mV (*n*=9) after a 10 s focal application of ATPγS (100 μM), a 75% inhibition.

Bath application of ATPγS (10–100 μM) also caused an inhibition of neurally evoked transmitter release (Fig. 4B). Control EPSPs had a mean amplitude of 30.4±2.4 mV and 10 min after the bath application of ATPγS (10 μM) EPSPs had a mean amplitude of 18.0±2.9 mV (*n*=7), a 40% inhibition. 2-Methylthio-ATP (100 μM) caused a slight inhibition (~ 20%) of evoked transmitter release (not shown). Control EPSPs had a mean amplitude of 30.4±2.5 mV and 20 min after bath application of 2-methylthio-ATP (100 μM) EPSPs had a mean amplitude of 24.0±2.8 mV (*n*=5). UTP (100 μM) had no detectable effects on the amplitude of EPSPs or the membrane potential of the postsynaptic neurone (*n*=3, not shown).

The inhibitory effects of ATP and ATPγS on neurally evoked transmitter release could be reversibly antagonised by the bath application of either suramin (10 μM) or PPADS (10 μM; Fig. 5). These results suggest that ATP acts presynaptically through a P2 purinoceptor to inhibit ACh release from preganglionic nerves in rat parasympathetic ganglia.

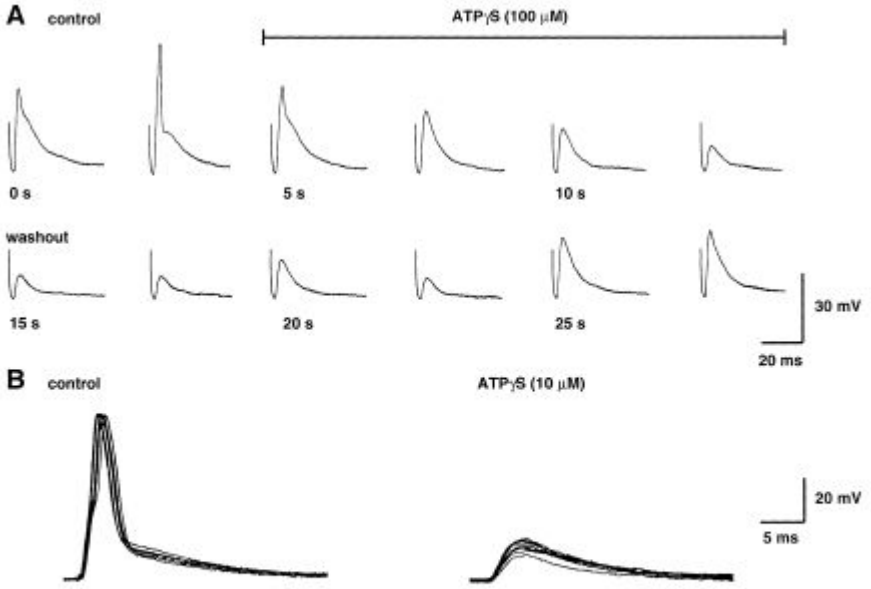

Fig. 4. (A) Effect of focally applied ATPγS (100 μM) on 12 consecutive excitatory postsynaptic responses to nerve stimulation at 0.4 Hz. Focal application of ATPγS (10-s duration) is indicated by the black bar. EPSPs returned to control amplitude approximately 2 min after the termination of the agonist application. Resting membrane potential, −68 mV. (B) Effect of bath-applied ATPγS on nerve-evoked action potentials in the submandibular ganglion. (i) Superimposed traces of control action potentials evoked by trains of stimuli at 0.3 Hz. (ii) Superimposed traces of postsynaptic responses evoked in the presence of 10 μM ATPγS. Resting membrane potential, −63 mV.

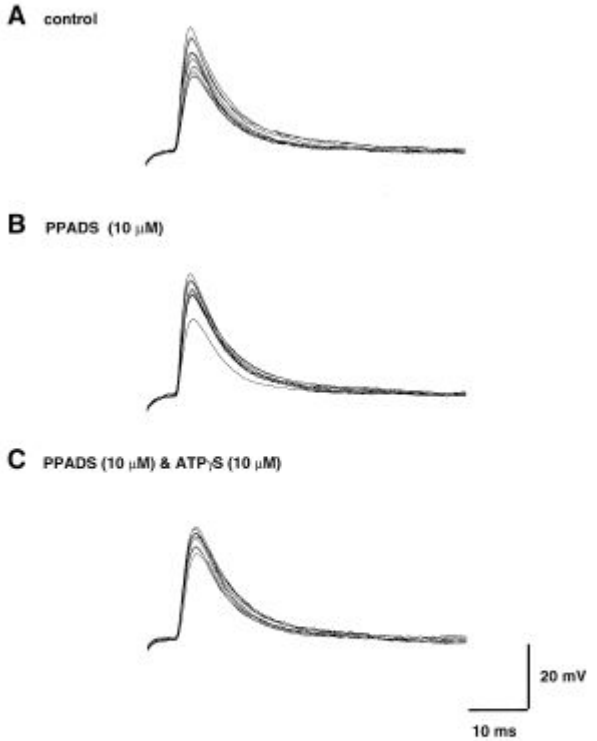

Fig. 5. Effect of PPADS and ATPγS on nerve-evoked responses in the submandibular ganglion. The nerve-evoked responses were evoked by trains of ten stimuli at 0.3 Hz. (A) Superimposed traces of postsynaptic responses evoked in the presence of 10 μM PPADS. (B) Superimposed traces of postsynaptic responses evoked in the presence of 10 μM PPADS and 10 μM ATPγS. Resting membrane potential, −63 mV.

ATP-evoked responses in acutely dissociated postganglionic neurones

Given that focal application of exogenous ATP to the postganglionic neurones of rat submandibular ganglia did not evoke a membrane response, a series of experiments were carried out on acutely dissociated submandibular ganglion neurones. Focal application of 100 μM ATP to isolated neurones within 3–4 h of dissociation from rat submandibular ganglia evoked an excitatory, depolarising response and action potential firing (control, Fig. 6A). In all acutely dissociated neurones studied, ATP (100 μM) evoked a membrane depolarisation >20 mV sufficient to reach threshold for action potential firing from a resting membrane potential of −52.1±2.4 mV (*n*=9). Bath application of PPADS (10 μM) reversibly inhibited the ATP-induced depolarisation and action potential firing (+PPADS, Fig. 6A) indicating that the excitatory response was mediated by the activation of P2 purinoceptors. Under voltage clamp conditions, brief application (10-ms pulse) of 100 μM ATP applied to soma membrane held at −60 mV evoked a rapid, transient inward current in >90% of the neurones studied (control, Fig. 6B). The peak current density of the ATP-evoked inward current at −60 mV was 9.6±0.9 pA/pF (*n*=6). The ATP-evoked inward current was also reversibly inhibited by bath application of 10 μM PPADS (+PPADS, Fig. 6B). The ATP-evoked inward current is consistent with activation of non-selective cation P2X receptor-channels described in detail previously in dissociated neurones of rat parasympathetic ganglia (Fieber and Liu).

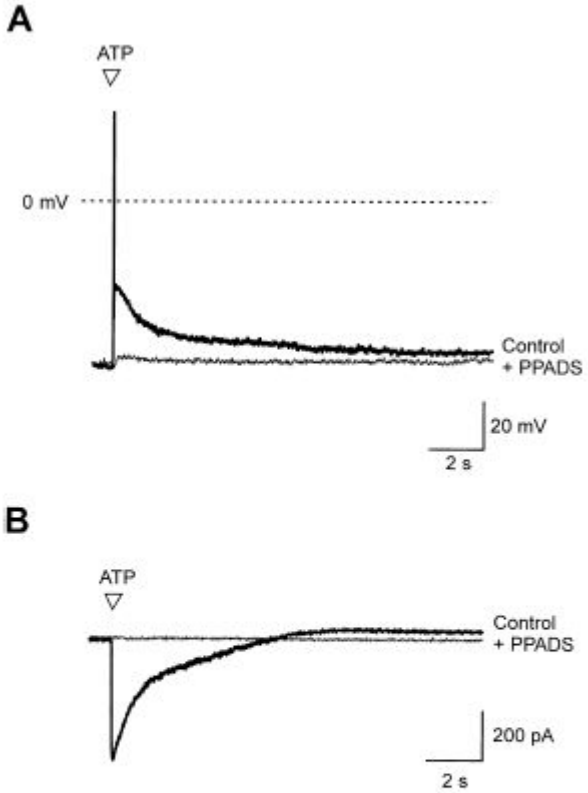

Fig. 6. Excitatory response of acutely dissociated postganglionic neurones from rat submandibular ganglia to exogenous ATP. (A) Superimposed traces of responses evoked by a brief pulse (10 ms) of 100 μM ATP (arrow) from an extracellular pipette in the absence (control, dark trace) and presence of 10 μM PPADS (+PPADS, light trace). The ATP-induced depolarisation and action potential firing is inhibited in the presence of PPADS. Resting membrane potential, −57 mV. (B) Superimposed traces of ATP-evoked currents obtained at −60 mV in the absence (control, dark trace) and presence of 10 μM PPADS (+PPADS, light trace). The slow outward current (< 50 pA) is most likely due to activation of P2Y purinoceptors in these neurones (Liu et al., 2000). Arrow indicates application (10-ms pulse) of 100 μM ATP.

Expression of P2X receptors in wholemount and cultured preparations

A monoclonal antibody against synaptophysin was used to identify nerves and ganglia in the tissue. Synaptophysin is a protein component of synaptic vesicles that are found in almost all neurones (Navone et al., 1986). Double labelling experiments conducted with anti-synaptophysin and anti-P2X antibodies allowed P2X immunoreactivity to be described in relation to the ganglia and surrounding nerves.

Antibodies raised against individual P2X receptor subtypes ($P2X_{1-6}$) were used to determine if any P2X receptor subtypes were expressed in the submandibular ganglia. These P2X receptor antibodies have been shown previously to be specific for each P2X receptor subtype (Dutton and Worthington). Control antibody protocols were undertaken to check for any non-specific immunoreactivity within the tissue. No immunoreactivity was seen in any structures examined when normal serum was incubated in place of the primary antibody. Therefore any immunoreactivity observed would indicate the expression of a specific ligand-gated P2X purinoceptors in postganglionic neurones. Only one P2X subtype, $P2X_5$, was found expressed within the submandibular ganglion intact wholemount preparation (n=41, Fig. 7C,D). The $P2X_5$ immunoreactivity demonstrated was predominantly of the small punctate variety that has been previously found in other tissues such as the bladder (Dutton et al., 1999) and heart (Hansen et al., 1999b). It is expected that labelling would be localised to the submandibular ganglion cells only, as the $P2X_5$ antibody is raised to the extracellular domain of the P2X receptor. Furthermore, little cellular permeabilisation was carried out and no P2X immunoreactivity was detected in associated synaptophysin-labelled nerve fibres to the individual ganglia (Fig. 7A,B).

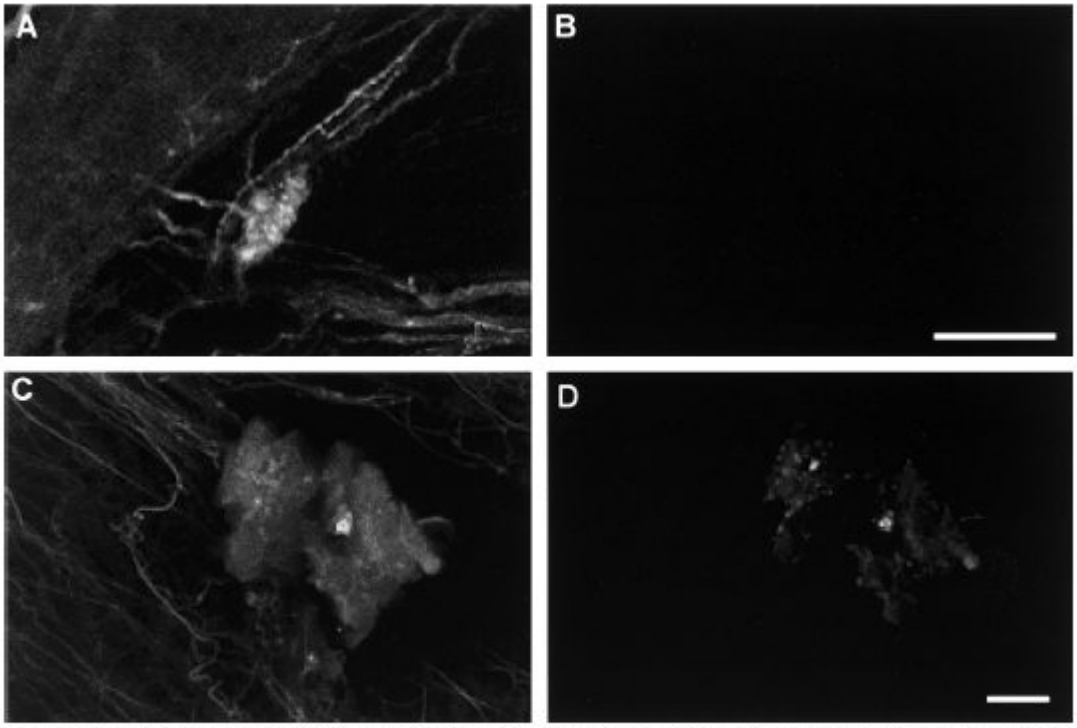

Fig. 7. (A, B) A confocal micrograph of a wholemount rat submandibular ganglion. The section is double-labelled with antibodies against synaptophysin and $P2X_2$. (A) Demonstrates synaptophysin labelling, and (B) demonstrates the lack of label for $P2X_2$ purinoceptors. A similar amount of labelling is also seen in wholemount preparations labelled with antibodies for $P2X_1$, $P2X_3$, $P2X_4$ and $P2X_6$. (C, D) A confocal micrograph of a wholemount rat submandibular ganglion seen at a higher power of magnification, and double labelled with antibodies against synaptophysin and $P2X_5$. (C) demonstrates the extensive network of nerves labelled with synaptophysin antibodies. (D) demonstrates that the only P2X immunoreactivity found in the submandibular ganglion, is that with $P2X_5$ immunostaining. Scale bars = 10 μm.

In cultured submandibular ganglion neurones, immunoreactivity could still be discerned for $P2X_5$, at a level similar to that in the intact preparation (Fig. 8E), however, immunoreactivity for the other P2X receptor subtypes examined (i.e. $P2X_{1-4}$ and $P2X_6$) could also be detected. High levels of $P2X_2$ and $P2X_4$ immunoreactivity were found in the cultured preparations. The $P2X_2$ immunoreactivity was localised at the cell membranes (Fig. 8B), whereas the $P2X_4$ immunoreactivity was seen diffusely throughout the cells (Fig. 8D). Very low levels of $P2X_1$, $P2X_3$ and $P2X_6$ were detected in some cultured preparations, with the immunoreactivity confined to the membranes of the individual cells (Fig. 8A,C,F).

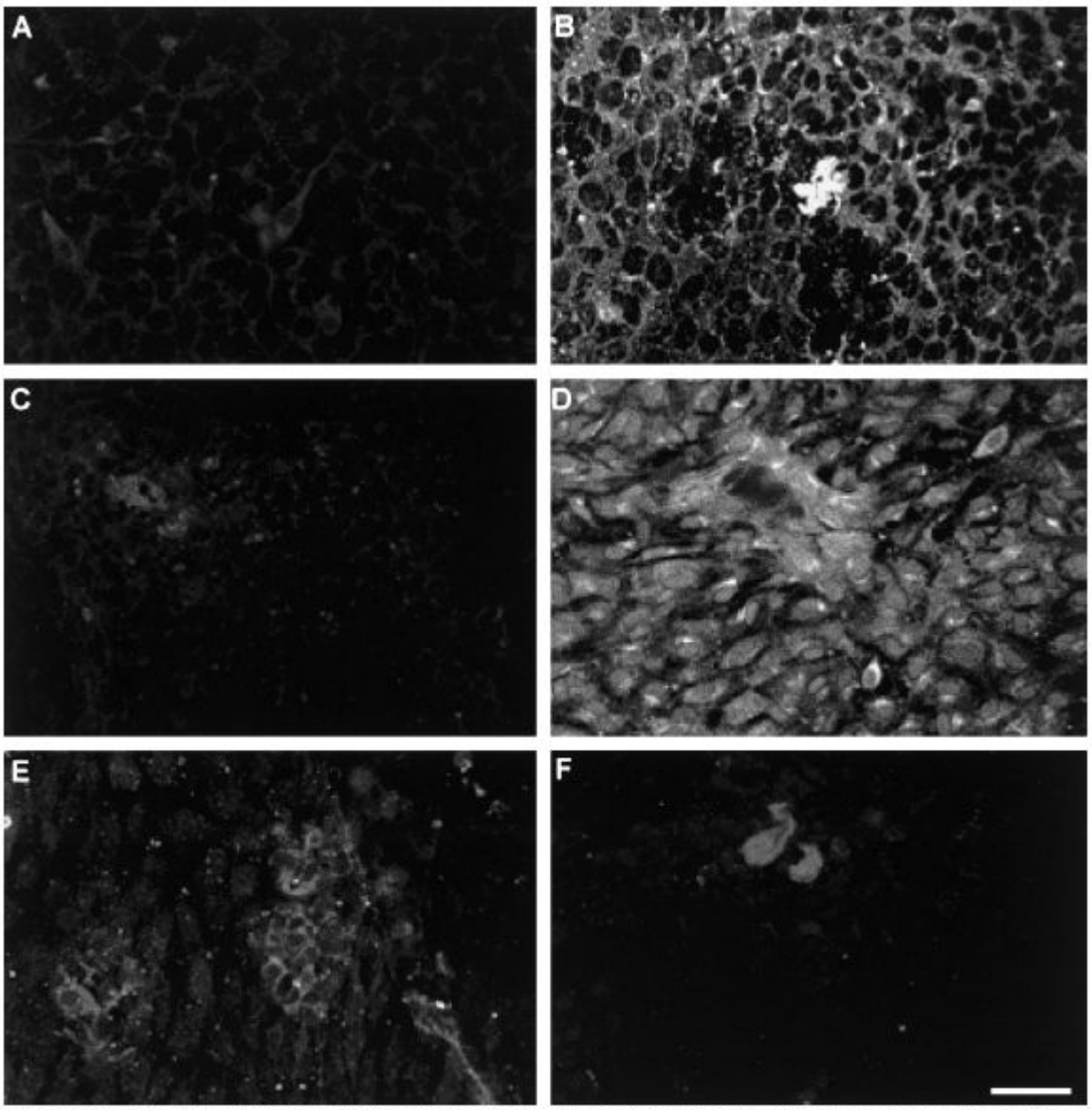

Fig. 8. (A–F) Confocal micrographs of P2X purinoceptor immunoreactivity in cultured neurones from rat submandibular ganglia. Low levels of immunoreactivity were seen in (A) ($P2X_1$), (C) ($P2X_3$) and (F) ($P2X_6$). Medium levels of immunoreactivity were demonstrated in (E) ($P2X_5$). High levels of P2X immunoreactivity were seen in (B) ($P2X_2$) and (D) ($P2X_4$). Scale bar = 50 μm.

Discussion

The effects of exogenously applied ATP on intact and dissociated parasympathetic neurones were investigated. In the intact rat submandibular ganglia, synaptic transmission was unaffected by P2X purinoceptor antagonists but abolished by nAChR antagonists. These data suggest that ACh is the sole excitatory neurotransmitter at synapses within this ganglia which is consistent with that reported previously in intact guinea-pig sympathetic ganglia (Inokuchi and McLachlan, 1995).

Focal application of ATP or the non-hydrolysable analogue ATPγS (100 μM) had no effect on the membrane potential of the postganglionic neurone or on the amplitude of spontaneous EPSPs. Taken together, these results suggest the absence of functional purinergic receptors on the postsynaptic neurone *in situ*. However, focal application of ATP and ATPγS and bath application of ATPγS potently and reversibly inhibited nerve-evoked transmitter release in the submandibular ganglia. The absence of any effect on either the resting membrane potential of the postsynaptic neurone or the amplitude and frequency of spontaneous EPSPs suggests that the inhibitory effect is presynaptic in nature and that there are no functional P2 purinoceptors postsynaptically.

The P2 purinoceptor antagonists, suramin and PPADS blocked the presynaptic inhibition of evoked transmitter release by ATP and ATPγS. This result suggests that ATP acts through presynaptic P2 purinoceptors. The P2 purinoceptor agonist, 2-methylthio-ATP, caused a slight inhibition (20%) of EPSP

amplitude but UTP had no detectable effects on evoked transmitter release. Taken together, the agonist potency profile for inhibition of neurotransmitter release suggests a presynaptic P2Y purinoceptor is activated by the P2 agonists, possibly a $P2Y_1$ or a $P2Y_{11}$ receptor subtype (Ralevic and Burnstock, 1998).

In acutely dissociated postganglionic neurones from rat submandibular ganglia, focal application of ATP evoked an inward current and subsequent excitatory depolarising response and action potential firing, which was reversibly inhibited by PPADS, consistent with activation of P2X purinoceptors (Ralevic and Burnstock, 1998). This result is in contrast to the results from the intact submandibular ganglia preparation but consistent with previous studies on cultured neurones from rat intracardiac ganglia (Fieber and Adams, 1991). The question arises whether P2X purinoceptor expression is up-regulated or becomes functionally available when the postganglionic neurones are dissociated.

To determine which P2X receptor subtypes are present in submandibular ganglion cells, immunohistochemistry, using specific polyclonal antibodies, was undertaken. $P2X_5$ subunit immunoreactivity was detected in the intact preparation, however, it was only found intracellularly. In dissociated ganglion neurones the levels of $P2X_2$ and $P2X_4$ immunoreactivity are substantially increased. Up-regulation of $P2X_4$ expression has been demonstrated previously in parasympathetic ganglion preparations (Tenneti et al., 1998). $P2X_4$ up-regulation in rat parotid cells occurs following parasympathetic denervation suggesting that there are presynaptic factors regulating P2X expression. Inhibition of neurotransmitter release with tetrodotoxin and cadmium or blockade of the postsynaptic ACh response with tetrodotoxin and mecamylamine was found to up-regulate P2X purinoceptor expression in the wholemount submandibular ganglion preparation (M.A. Hansen and D.J. Adams, unpublished observations). Further investigation is warranted to determine the conditions necessary to up-regulate P2X purinoceptor activation *in vivo*.

Purinoceptors have been localised on presynaptic nerves (Le and Li) and P2X receptors have been implicated in the presynaptic modulation of other neurotransmitters. It has been demonstrated in rat dorsal horn neurones that PPADS depresses the amplitude of evoked glutamatergic EPSCs indicating that endogenous ATP acts on presynaptic P2X receptors to enhance the amount of neurotransmitter released (Li et al., 1998). Recent reports also describe P2X receptor modulation of adrenergic transmission in rat superior cervical ganglion explant cultures (Boehm, 1999) and GABAergic transmission in cultured dorsal horn neurones from rat spinal cord (for review see Khakh and Henderson, 2000). It may be hypothesised that there is some factor down-regulating the expression of the P2X purinoceptor in the intact nerve terminal. Thus, by regulating P2X purinoceptor expression or availability, sensitive control of neurotransmitter release could be effected. It has recently been demonstrated that hormones may affect P2X receptor expression in the bladder (Yunaev et al., 2000) suggesting that hormones or another intrinsic factor may down-regulate the expression of P2X receptors *in vivo*.

In conclusion, in the intact submandibular ganglia preparation, ATP inhibits neurotransmitter release through the activation of presynaptic P2Y purinoceptors but has no effect on the postsynaptic neurones. However, in dissociated parasympathetic ganglion neurones, ATP evokes a transient inward current and increased neuronal excitability due to an up-regulation of P2X purinoceptors on the postganglionic neurones. Disruption of ganglionic transmission *in vivo* by either nerve damage or synaptic blockade may cause an increase in expression or availability of a different class of cell-surface receptors and mediate a membrane response. For example, ischaemic injury and release of ATP may up-regulate P2X receptor expression or availability and alter neuronal excitability in the heart and vasculature (for review see Armour, 1999). These observations may illustrate the inherent plasticity of the peripheral nervous system.

Abbreviations

ACh, acetylcholine; ATPγS, adenosine 5′-*O*-(3-thiophosphate); α,β-MeATP, α,β-methylene adenosine 5′-triphosphate; BAPTA, 1,2-bis-(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid; DMSO, dimethyl sulphoxide; EPSP, excitatory postsynaptic potential; FBS, foetal bovine serum; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-(2-ethanesulphanic acid); 2-methylthio-ATP, 2-methylthioadenosine triphosphate tetrasodium salt; nAChR, nicotinic acetylcholine receptor; PBS, phosphate-buffered saline; PPADS, pyridoxal-phosphate-6-azophenyl-2′,4′-disulphonic acid

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