Ionic selectivity of native ATP-activated (P2X) receptor channels in dissociated neurones from rat parasympathetic ganglia

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Abstract

1. The relative permeability of the native P2X receptor channel to monovalent and divalent inorganic and organic cations was determined from reversal potential measurements of ATP-evoked currents in parasympathetic neurones dissociated from rat submandibular ganglia using the dialysed whole-cell patch clamp technique.

2. The P2X receptor-channel exhibited weak selectivity among the alkali metals with a selectivity sequence of Na\(^+\) > Li\(^+\) > Cs\(^+\) > Rb\(^+\) > K\(^+\), and permeability ratios relative to Cs\(^+\) (P\(_X/P_{Cs}\)) ranging from 1.11 to 0.86.

3. The selectivity for the divalent alkaline earth cations was also weak with the sequence Ca\(^{2+}\) > Sr\(^{2+}\) > Ba\(^{2+}\) > Mn\(^{2+}\) > Mg\(^{2+}\). ATP-evoked currents were strongly inhibited when the extracellular divalent cation concentration was increased.

4. The calculated permeability ratios of different ammonium cations are higher than those of the alkali metal cations. The permeability sequence obtained for the saturated organic cations is inversely correlated with the size of the cation. The unsaturated organic cations have a higher permeability than that predicted by molecular size.

5. Acidification to pH 6.2 increased the ATP-induced current amplitude twofold, whereas alkalization to 8.2 and 9.2 markedly reduced current amplitude. Cell dialysis with either anti-P2X\(_2\) and/or anti-P2X\(_4\) but not anti-P2X\(_1\) antibodies attenuated the ATP-evoked current amplitude. Taken together, these data are consistent with homomeric and/or heteromeric P2X\(_2\) and P2X\(_4\) receptor subtypes expressed in rat submandibular neurones.

6. The permeability ratios for the series of monovalent organic cations, with the exception of unsaturated cations, were approximately related to the ionic size. The relative permeabilities of the monovalent inorganic and organic cations tested are similar to those reported previously for cloned rat P2X\(_2\) receptors expressed in mammalian cells.

Cell-surface receptors for extracellular purine nucleotides (P2 receptors) are found in numerous animal tissues where they regulate a broad range of physiological processes including synaptic transmission (see reviews by Fredholm, 1995; Ralevic & Burnstock, 1998). Adenosine 5'-triphosphate (ATP) receptors have been categorized into two major groups, P2X and P2Y, based on their pharmacological and electrophysiological properties, as well as their molecular structure. P2X receptors are direct ligand-gated ion channels, while P2Y receptors are G protein-coupled receptors (North & Barnard, 1997; Ralevic & Burnstock, 1998). ATP acting at P2X receptors has been shown to be a fast excitatory neurotransmitter in the central (Edwards et al. 1992) and peripheral (Silinsky & Gerzanich, 1993; Galligan & Bertrand, 1994) nervous systems. To date, at least seven subtypes of P2X receptor (P2X\(_{1-7}\)) have been cloned from mammalian species (North & Barnard, 1997; Soto et al. 1997). These show a broad expression pattern compared with other ligand-gated channels, with the various forms being found in central and peripheral nervous systems, different types of immune cells, glands, and smooth and skeletal muscles (Collo et al. 1996). The P2X receptor subunits can form channels as homomultimers or, in some cases, as heteromultimers (Lewis et al. 1995; Léet al. 1998; Torres et al. 1998). The properties of heterologously expressed P2X
purinoreceptors, however, do not always match those of receptors studied in native tissues, and the relationship between the properties of cloned P2X receptors and those studied in native cell types remains unclear.

Our current understanding about the structure and function of P2X receptors in vertebrate neurones remains limited. The properties of the ionic pore have been studied in several types of neurones by measuring ionic permeability. However, these studies were limited to a few alkali metal cations and Ca$^{2+}$ (rat and bullfrog sensory neurones: Bean et al. 1990; PC12 cells: Nakazawa et al. 1990; rat parasympathetic neurones: Fieber & Adams, 1991; guinea-pig coeliac neurones: Silinsky & Gerzanich, 1993; rat tuberomammillary nucleus neurones: Furukawa et al. 1994; NG108-15 cells: Kaiho et al. 1996) or a few organic monovalent cations (rat sensory neurones: Krishtal et al. 1983; PC12 cells: Nakazawa et al. 1990, 1991; rat nodose neurones: Virginio et al. 1998). No quantitative study of the ionic permeability properties of native neuronal P2X receptors has been undertaken.

In dissociated neurones of rat parasympathetic ganglia, the short latency of current activation and recording of single channel currents in excised membrane patches indicates that the ATP-evoked response is mediated by P2X receptors (Fieber & Adams, 1991). The agonist potency profile, very slow desensitization and relative sensitivity of ATP-evoked currents in these neurones to inhibition by suramin (IC$_{50}$, 6 µm) and Reactive Blue 2 (IC$_{50}$, 1 µm) (Fieber & Adams, 1991; Nutter & Adams, 1995a) is consistent with the pharmacological characteristics of a subset of P2X receptor types (Ralevic & Burnstock, 1998). The goal of the present study was to characterize the ionic permeability and pH sensitivity of P2X receptors and, using available anti-P2X antibodies, to identify the functional P2X purinoreceptors expressed in parasympathetic neurones of rat submandibular ganglia. The permeability of the ATP-activated channel of dissociated submandibular neurones to monovalent and divalent inorganic cations and monovalent organic cations was examined and the relative ionic permeabilities compared with those obtained for cloned P2X receptors expressed in mammalian cells (Evans et al. 1996; Virginio et al. 1998; Ding & Sachs, 1999). The ionic permeability and pH sensitivity of the ATP-activated receptor-channel in rat parasympathetic neurones are consistent with those of the cloned P2X$_2$ receptor. A preliminary report of some of these results has been presented in abstract form (Liu & Adams, 1997).

**Methods**

Parasympathetic neurones from rat submandibular ganglia were dissociated and placed in tissue culture. Submandibular ganglia were dissected from 2- to 4-week-old rats, which were anaesthetized with sodium pentobarbitone (Nembutal) before being killed by cervical dislocation, in accordance with the guidelines of the University of Queensland Animal Experimentation Ethics Committee. Neurones providing parasympathetic innervation to the salivary glands lie in a thin triangular sheet of connective tissue stretching between the lingual nerve and the salivary ducts (Lichtman, 1977). Ganglia were removed and incubated in PSS solution containing 0.9 mg ml$^{-1}$ collagenase (Worthington Biochemical Corp., Freehold, NJ, USA) for 50 min at 37 °C. The tissue was transferred to a sterile culture dish containing culture medium (Dulbecco's modified Eagle's medium with 10 mm glucose, 10% (v/v) fetal calf serum, 100 U ml$^{-1}$ penicillin and 0.1 mg ml$^{-1}$ streptomycin), triturated with a fine-bore Pasteur pipette, then plated onto 18 mm glass coverslips coated with laminin. The dissociated cells were incubated at 37 °C under a 95% air-5% CO$_2$ atmosphere. Electrophysiological recordings were made from isolated neurones maintained in tissue culture for 12-60 h. At the time of experiments, the glass coverslip was transferred to a low volume (0.5 ml) recording chamber and viewed at ×400 magnification using an inverted phase contrast microscope. Experiments were conducted at room temperature (21-23 °C).

**Electrophysiological recording**

Agonist-evoked responses of dissociated submandibular neurones were studied under current and voltage clamp conditions using the whole-cell recording configuration of the patch clamp technique (Hamill et al. 1981). Patch pipettes (1-3 MΩ) were pulled from thin-walled borosilicate glass (GC150TF; Harvard Apparatus Ltd, Edenbridge, Kent, UK) and fire polished. Electrical access was achieved by rupturing the membrane patch and dialysing the cell. The series resistance ($R_s$) was usually < 4 MΩ and given that the maximum amplitude of the ATP-evoked currents near the reversal potential was < 50 pA, the voltage error due to $R_s$ would be < 0.2 mV. Membrane current and voltage were monitored using a patch clamp amplifier (Axopatch 200B, Axon Instruments Inc., Union City, CA, USA), filtered at either 2 or 5 kHz (-3 dB; 4-pole low-pass Bessel filter) and digitized at 10 kHz using a Digidata 1200A A-D/D-A interface (Axon Instruments Inc.). A Pentium/133 MHz PC running pCLAMP programs (Axon Instruments Inc.) was used to generate voltage pulses and to acquire and analyse data.
**Solutions**

The extracellular solution for whole-cell recording was physiological salt solution (PSS) containing (mm): 140 NaCl, 3 KCl, 2.5 CaCl₂, 1.2 MgCl₂, 7.7 glucose and 10 Hepes-NaOH, pH 7.2. The reference solution for reversal potential measurements contained (mm): 140 CsCl, 1 CaCl₂, 7.7 glucose and 10 histidine, pH 7.2. The relative permeability of the ATP-activated channel to inorganic and organic cations was investigated by replacement of CsCl with an osmotically equivalent amount of the chloride salt of the test cation. Inorganic salts included monovalent cations (140 mm) such as LiCl, KCl, RbCl, NaCl and NH₄Cl, and divalent cations (100 mm) such as MgCl₂, CaCl₂, SrCl₂, BaCl₂ and MnCl₂, all of analytical grade, and all solutions had a pH of 7.2. The following organic compounds were substituted for CsCl in the test solutions including different ammonium cations: hydroxylamine HCl, hydrazine HCl and methylamine HCl; unsaturated organic cations: guanidine HCl, acetamidine HCl and imidazole HCl; a series of monovalent organic cations: arginine HCl; Bis-Tris, methylamine HCl, dimethylamine HCl, trimethylamine HCl and tetramethylammonium HCl; ethylamine HCl, diethylamine HCl, triethylamine HCl and tetraethylammonium HCl; ethanolamine HCl, diethanolamine HCl and triethanolamine HCl; and 2-aminoethanethiol HCl. All chemicals were purchased from Sigma Chemical Company (St Louis, MO, USA) with the exception of hydrazine HCl and guanidine HCl which were purchased from Eastman Kodak (Rochester, NY, USA). When the test cation source compound was a free amine, it was adjusted to the desired pH with HCl. All organic solutions had a pH of 7.2, with the exception of hydrazine chloride (pH 6.6) and hydroxylamine chloride (pH 5.6), which were studied at a slightly more acidic pH to increase the ionized concentration of the test ion. The osmotic pressure of the solutions was monitored with a vapour pressure osmometer (Wescor 5500, Logan, UT, USA). Reversal potential measurements were corrected for differences in junction potential between the bath solution and the indifferent electrode (0.15 m KCl-agar bridge). Liquid junction potential measurements were calculated theoretically using JPCalc (Barry, 1994).

The intracellular pipette solution contained (mm): 140 CsCl, 2 MgATP, 2 Cs₄BAPTA and 10 Hepes-CsOH, pH 7.2. ATP-evoked currents were investigated in response to pressure application (Picospritzer II, General Valve Corp., Fairfield, NJ, USA) of 100 μM Na₂ATP, added to the appropriate test solution, from an extracellular micropipette (3-5 μm tip diameter). The pressure ejection pipette was positioned ≥50 μm from the soma membrane to evoke maximal responses to agonist under control conditions (10 ms, 10 p.s.i.). A maximally effective concentration (≥ 100 μm) determined from ATP dose-response relations was used for purinoceptor activation (EC₅₀, 8 μm; Nutter & Adams, 1995a). To minimize receptor desensitization, a delay of > 60 s between agonist applications was maintained. Agonist was applied during continuous bath perfusion at a rate of 2 ml min⁻¹. To ensure total exchange of the external reference and test solutions during the course of an experiment, the recording chamber was perfused with a minimum of 20 vol. (10 ml) of solution. Pyridoxal phosphate-6-azophenyl-2′,4′-disulphonic acid (PPADS; Research Biochemicals International, Natick, MA, USA) was bath applied at the concentration indicated.

Specific polyclonal antibodies raised in rabbit against highly purified peptides corresponding to residues 382-399 of rat P2X₁, with additional N-terminal cysteine and serine, 457-472 of rat P2X₂, with additional N-terminal cysteine, and 370-388 of rat P2X₄, with additional N-terminal cysteine, were used in the patch pipette solution at a concentration of 1:200 in the presence of bovine serum albumin (0.5 %). The antibodies were affinity purified on immobilized P2X₁, P2X₂ and P2X₄ and the epitopes specific for P2X₁, P2X₂ and P2X₄ are not present in any other known protein. Anti-P2X antibodies specific for the P2X receptor subtypes were supplied by Alomone Laboratories (Jerusalem, Israel). Given the large molecular weight of these antibodies, 15-20 min dialysis was required to allow adequate transfer of the antibodies into the cell from the back-filled patch pipette (Pusch & Neher, 1988). ATP-evoked currents were recorded within 1 min of establishing the whole-cell recording mode and approximately 15 min after dialysis with the antibody. The use of antibodies as tools to study the subunit composition and function of nicotinic acetylcholine receptors (nAChRs) in rat autonomic neurone function has been described previously (see Skok et al. 1999).

**Data analysis**

The reversal (zero-current) potential, E<sub>rev</sub>, for ATP-evoked currents in the various test solutions was determined from peak current amplitude evoked in response to ATP during steps to different membrane potentials in 5 mV increments. Responses obtained in test solutions were preceded and followed by recordings in the reference solution. Pronounced inward rectification of the ATP-evoked currents required measurements of ΔE<sub>rev</sub> to be based on clearly defined ATP-evoked inward and outward currents recorded around the reversal potential. Test measurements were
rejected if $E_{\text{rev}}$ in the reference solution changed by 3 mV or more, or if distinct inward and outward currents could not be resolved. Reversal potentials were determined by interpolation of polynomial fits of at least six peak current values obtained at membrane potentials on either side of $E_{\text{rev}}$. Relative permeability estimates for cations were calculated using the Goldman-Hodgkin-Katz (GHK) voltage equation (see Hille, 1992). The form of the equation used to determine the relative permeabilities from shifts in reversal potential ($\Delta E_{\text{rev}}$) was:

$$P_X/P_{\text{Cs}} = \exp(\Delta E_{\text{rev}}F/RT)\{[\text{Cs}^+]_i/[\text{X}^+]_o\},$$

where $RT/F$ have their usual meanings and equal 25.4 mV at 22 °C, $P_X/P_{\text{Cs}}$ is the permeability ratio for $X^+$ relative to $\text{Cs}^+$, and $[\text{Cs}^+]_i$ and $[\text{X}^+]_o$ are the ion concentrations of the internal and external solutions, respectively. To determine the relative permeability to divalent cations, the GHK equation was modified to include divalent cations and activity coefficients (see Fieber & Adams, 1991). Activity coefficients of the salts were obtained from Butler (1968) and Robinson & Stokes (1965).

For Ca$^{2+}$ and other divalent cations, the form of equation is modified to:

$$P_{\text{Cs}}/P_{\text{X}} = [\text{Cs}^+]\exp(\Delta E_{\text{rev}}F/RT\{1 + \exp(\Delta E_{\text{rev}}F/RT)\})/[\text{X}^{2+}]_o.$$

All numerical data are presented as means ±s.e.m., with the number of experiments in parentheses.

Results

Excitatory response of rat parasympathetic neurones to exogenous ATP

Under current clamp, a brief pulse (10 ms) of 100 μm ATP applied to the soma of an isolated parasympathetic neurone evoked a rapid depolarization (< 10 ms latency) and action potential firing (Fig. 1a). The ATP-induced depolarization was 22.3 ± 2.2 mV in amplitude and 5.6 ± 0.4 s in duration from a resting membrane potential of -51.0 ± 3.3 mV ($n = 9$). The ATP-evoked response was reversibly inhibited by bath-applied PPADS (10 μm) (Fig. 1a).

Focal application of ATP to the voltage-clamped neurone activated a transient inward current in > 85 % of the neurones examined. Whole-cell currents evoked by ATP at different holding potentials are shown in Fig. 1B. The ATP-induced current-voltage ($I$-$V$) relationship exhibited marked inward rectification and the reversal (zero-current) potential obtained in PSS was +0.7 ± 2.3 mV ($n = 14$; Fig. 1C). ATP-evoked current density averaged -34.7 ± 3.4 pA pF$^{-1}$ ($n = 14$) at a membrane holding potential of -100 mV. The inward rectification was observed in the absence of divalent cations in either the intra- or extracellular solution suggesting that the reduced outward current observed at positive membrane potentials is unlikely to be due to divalent cation block of the channel (Nutter & Adams, 1995a).
Figure 1. Excitatory response of rat submandibular neurones to exogenous ATP

A, superimposed traces of responses evoked by a brief pulse (10 ns) of 100 μM ATP (open arrowhead) from an extracellular pipette in the absence (Control) and presence of 10 μM PPADS. The ATP-induced depolarization and action potential firing was inhibited in the presence of PPADS. Resting membrane potential, −69 mV. B, family of ATP-evoked currents obtained in physiological salt solution (PSS) at the membrane potentials indicated. Open arrowhead indicates application (10 ns pulse) of 100 μM ATP. C, current–voltage (I–V) relationship for peak current amplitude evoked by ATP in PSS. Each point represents the mean ± S.E.M. current density (pA pF⁻¹) from a minimum of 14 cells.
Inorganic cation selectivity of the ATP-activated channel

ATP receptor-channels of rat parasympathetic neurones exhibit weak selectivity among the monovalent alkali metal cations. Families of currents traces recorded at 5 mV increments around the reversal potential in the reference solution and after isosmotic replacement of Cs⁺ with the test cations Li⁺, Na⁺, Rb⁺, and K⁺ are shown in Fig. 2a. Shifts in reversal potential, ΔE<sub>rev</sub>, were determined from plots of peak current amplitude as a function of membrane potential in test solutions relative to that obtained in the Cs⁺ reference solution. The average I-V relationships obtained for Li⁺, Na⁺, Rb⁺ and K⁺ are shown in Fig. 2B. The crystal diameter of the test ions, the observed shifts in E<sub>rev</sub> compensated for the liquid junction potential (Barry, 1994) and permeabilities relative to Cs⁺ (P<sub>X</sub>/P<sub>Cs</sub>) calculated by the equation given in Methods, are listed in Table 1. The selective sequence of the neuronal ATP receptor-channel for monovalent cations is Na⁺ > Li⁺ > Cs⁺ > Rb⁺ > K⁺, with permeability ratios of 1.11, 1.02, 1.00, 0.91 and 0.86, respectively.

### Table 1. ΔE<sub>rev</sub> and relative permeabilities for monovalent alkali metal cations

<table>
<thead>
<tr>
<th>X</th>
<th>Diameter * (Å)</th>
<th>ΔE&lt;sub&gt;rev&lt;/sub&gt; ± S.E.M. (mV)</th>
<th>n</th>
<th>P&lt;sub&gt;X&lt;/sub&gt;/P&lt;sub&gt;Cs&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Na⁺</td>
<td>1.90</td>
<td>1.4 ± 0.0</td>
<td>10</td>
<td>1.11</td>
</tr>
<tr>
<td>Li⁺</td>
<td>1.20</td>
<td>0.4 ± 0.2</td>
<td>5</td>
<td>1.02</td>
</tr>
<tr>
<td>Cs⁺</td>
<td>3.88</td>
<td>0.0 ± 0.0</td>
<td>45</td>
<td>1.00</td>
</tr>
<tr>
<td>Rb⁺</td>
<td>2.06</td>
<td>-3.7 ± 0.4</td>
<td>5</td>
<td>0.91</td>
</tr>
<tr>
<td>K⁺</td>
<td>2.60</td>
<td>-4.6 ± 0.5</td>
<td>5</td>
<td>0.86</td>
</tr>
</tbody>
</table>

* Diameters determined from ionic radii for alkali metal cations (Pauling, 1960). Reference solution (mm): 140 CsCl, 4 CsCl₂, 7.7 glucose and 10 histidine, pH 7.2. Internal solution (mm): 140 CsCl, 2 MgATP, 2 Ca, BAPTA and 10 Hepes, pH 7.2.

Figure 2. Reversal potential measurements of ATP-evoked currents obtained in the presence of monovalent alkali metal cations. A. Whole-cell ATP-evoked currents obtained at 5 mV increments around the reversal potential in an isotonic Cs⁺ extracellular solution and following isosmotic replacement with Na⁺, Li⁺, K⁺ or Rb⁺. B. I-V relations in the reference Cs⁺ solution and in isotonic Na⁺, Li⁺, K⁺ and Rb⁺ test solutions (symbols as indicated in A). The shift in reversal potential (ΔE<sub>rev</sub>) corrected for the junction potential was +3.7 mV in Li⁺, +7.3 mV in Na⁺, −2.8 mV in K⁺ and −4.9 mV in Rb⁺ test solutions with respect to the Cs⁺ reference solution. Each point represents the mean ± S.E.M. peak current amplitude of at least 5 cells.
The ATP receptor-channel of parasympathetic neurones was also permeable to the divalent alkaline earth metal cations but to a lesser extent than to monovalent metal cations. ATP-induced currents obtained after isosmotic substitution of Ca\(^{2+}\), Sr\(^{2+}\), Ba\(^{2+}\) or Mg\(^{2+}\) for Cs\(^{+}\) are shown in Fig. 3a. The ATP-induced I-V relationships obtained in the presence of various alkaline cations are shown in Fig. 3B. Substitution of the divalent cations for Cs\(^{+}\) shifted \(E_{rev}\) to more negative values and reduced peak current amplitude. The crystal diameter for test ions, the shifts in \(E_{rev}\) and corresponding relative permeabilities are listed in Table 2. The selectivity of divalent cations was also weak with the sequence \(Ca^{2+} > Sr^{2+} > Ba^{2+} > Mn^{2+} > Mg^{2+}\), and with permeability ratios \((P_X/P_{Cs})\) of 1.03, 0.85, 0.78, 0.54 and 0.43, respectively.

Table 2. \(\Delta E_{rev}\) and relative permeabilities for divalent metal cations

<table>
<thead>
<tr>
<th>X (100 mM)</th>
<th>Diameter* (Å)</th>
<th>(\Delta E_{rev} \pm \text{S.E.M.}) [mV]</th>
<th>(n)</th>
<th>(P_X/P_{Cs})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ca(^{2+})</td>
<td>1.98</td>
<td>-0.2 ± 1.1</td>
<td>5</td>
<td>1.03</td>
</tr>
<tr>
<td>Sr(^{2+})</td>
<td>2.26</td>
<td>-3.2 ± 0.4</td>
<td>4</td>
<td>0.88</td>
</tr>
<tr>
<td>Ba(^{2+})</td>
<td>2.70</td>
<td>-4.6 ± 0.8</td>
<td>6</td>
<td>0.78</td>
</tr>
<tr>
<td>Mn(^{2+})</td>
<td>1.60</td>
<td>-11.2 ± 0.4</td>
<td>4</td>
<td>0.54</td>
</tr>
<tr>
<td>Mg(^{2+})</td>
<td>1.30</td>
<td>-15.6 ± 0.7</td>
<td>4</td>
<td>0.43</td>
</tr>
</tbody>
</table>

*Diameters determined from ionic radii of divalent alkaline earth and transition metal cations (Pauling, 1960). Reference solution (mm): 140 CsCl, 1 CaCl\(_2\), 7.7 glucose and 10 histidine, pH 7.2. Internal solution (mm): 140 CsCl, 2 MgATP, 2 Cs\(_2\)BAFFTA and 10 Hepes, pH 7.2.

Figure 3. Reversal potential measurements of ATP-evoked currents obtained in the presence of alkaline earth divalent cations.

A. Whole-cell ATP-evoked currents recorded in the presence of 100 mM Ca\(^{2+}\), Sr\(^{2+}\), Ba\(^{2+}\) and Mg\(^{2+}\) in response to a 10 ms pulse of 100 µM ATP at 5 mV increments around the reversal potential. B. I-V relations obtained in the presence of isotonic Ca\(^{2+}\), Ba\(^{2+}\), Sr\(^{2+}\) and Mg\(^{2+}\) extracellular solutions (symbols as indicated in A). The shift in reversal potential (\(\Delta E_{rev}\)) was +0.2 mV in Ca\(^{2+}\), -3.2 mV in Sr\(^{2+}\), -4.6 mV in Ba\(^{2+}\) and -15.6 mV in Mg\(^{2+}\) test solutions with respect to the Ca\(^{2+}\) reference. Substitution of divalent metal cations for Cs\(^{+}\) in the external solution reduced ATP-evoked current amplitude and shifted the reversal potential to more negative membrane potentials. Each point represents the mean ± S.E.M. peak current amplitude of at least 4 cells.
Organic cation permeability of the ATP-activated channel

The permeability of the ATP-activated channel to organic cations of varied molecular dimensions was assessed by shifts in $E_{\text{rev}}$ relative to that in the presence of Cs$^+$. ATP-evoked currents obtained at different membrane potentials around the reversal potential after replacement of Cs$^+$ with monovalent organic cations including hydroxylammonium, ammonium and hydrazinium are shown in Fig. 4a. The $I$-$V$ relationships obtained in the presence of the ammonium cations are plotted in Fig. 4B. A positive shift in reversal potential was observed for all test ammonium cations indicating a higher permeability than Cs$^+$. ATP-evoked currents obtained in the presence of isotonic (140 mm) solutions of the unsaturated organic cations guanidine, acetamidine and imidazole are shown in Fig. 4a. The $I$-$V$ relationships obtained in the presence of these cations exhibited a relatively positive reversal potential for their molecular size and are shown in Fig. 4B.

Figure 4. Reversal potential measurements in the presence of highly permeant organic cations substituting for Cs$^+

A, ATP-evoked currents obtained at 5 mV intervals around the reversal potential in test solutions containing isotonic ammonium cations (hydroxylammonium, ammonium and hydrazinium) and unsaturated monovalent organic cations (guanidine, imidazole and acetamidine). B, $I$-$V$ relationship obtained after isotonic replacement of Cs$^+$ with hydroxylammonium, ammonium, hydrazinium, guanidine, imidazole and acetamidine (symbols as indicated in A). The shift in reversal potential was +25.1 mV in hydroxylammonium, +18.0 mV in ammonium, +12.4 mV in hydrazinium, +20.5 mV in guanidine, +9.8 mV in imidazole and +7.6 mV in acetamidine test solutions with respect to the Cs$^+$ reference solution. Each point represents the mean ± S.E.M. peak current amplitude of at least 4 cells.
ATP-evoked currents obtained in the presence of organic cations of increasing size including methylammonium, dimethylammonium, trimethylammonium, ethylammonium, diethylammonium, triethylammonium, ethanolammonium, diethanolammonium and triethanolammonium are shown in Fig. 5a. The corresponding I-V relationships obtained for methylammonium, dimethylammonium, trimethylammonium, ethylammonium and ethanolammonium are shown in Fig. 5b. The reversal potentials shifted to more negative values with an increase in molecular size. No inward current was evoked by ATP application in either isotonic arginine or Bis-Tris solutions (n = 4; data not shown), suggesting that arginine and Bis-Tris are impermeant and an upper limit (∼7 Å diameter) to the size of permeant cations. The shifts in $E_{rev}$ (compensated for liquid junction potential) and relative permeabilities of all monovalent organic cations tested are listed in Table 3 as a function of the molecular diameter of the ions examined.

![Figure 5](https://example.com/figure5.png)
pH dependence of ATP-evoked currents

The effect of external pH on ATP-evoked current amplitude and reversal potential was examined. The permeability of several organic compounds with $pK_a < 9$ was examined below pH 7 in order that the test ions were predominantly in the ionized form. Furthermore, it has been shown that different cloned P2X receptors exhibit different pH sensitivities (King et al. 1996; Stoop et al. 1997). Therefore, the pH sensitivity of the ATP-evoked current amplitude was also investigated to determine the P2X receptor subtypes expressed in native cells. The effect of external pH on ATP-evoked currents was examined using histidine base ($pK_a = 1.8, 6.0$ and 9.2) as the buffer, and the reference solution was adjusted to the test pH value (5.2-9.2) with HCl. ATP-induced inward currents obtained at a holding potential of -80 mV in the same cell at pH 5.2, 6.2, 7.2, 8.2 and 9.2 are shown in Fig. 6a. Acidification to pH 6.2 increased the peak current amplitude twofold, whereas alkalization to 8.2 and 9.2 reduced the current amplitude by ≥ 50%. Lowering the extracellular pH to 5.2 did not potentiate the ATP-evoked current amplitude, which was similar to that observed at pH 7.2. The reversal potential of the ATP-evoked current current, however, did not change with external pH (data not shown). Another series of experiments were carried out using Mes ($pK_a = 6.15$)-buffered external solutions, which produced similar results to those shown in Fig. 6b. These data indicate that protonation of the extracellular histidine buffer is unlikely to be responsible for the inhibition of ATP-mediated currents. Proton potentiation and pH sensitivity of the ATP-activated current over the range pH 6.2-8.2 in rat submandibular neurones is similar to that reported in rat nodose (Li et al. 1996) and coeliac (Zhong et al. 2000) ganglion neurones.
anti-P2X2 and anti-P2X4 antibodies inhibit ATP-evoked currents

To confirm the predominant subtype(s) of P2X purinoceptors mediating ATP-evoked currents, cells were dialysed with antibodies directed against specific P2X receptor subtypes. Cell dialysis with either anti-P2X2 or anti-P2X4 antibodies (diluted 1:200) attenuated the ATP-evoked currents within 10-15 min of establishing the whole-cell recording configuration (Fig. 7a). In contrast, anti-P2X1 antibodies had no effect on the ATP-evoked current amplitude, even after > 20 min cell dialysis (n = 7; Fig. 7a). The peak current amplitude obtained in response to 100 μM ATP was depressed by 43.6 ± 9.1 % by anti-P2X2 antibodies, by 37.4 ± 7.3 % by anti-P2X4 antibodies and by 69.4 ± 4.6 % (n = 5) by anti-P2X2+ anti-P2X4 antibodies relative to neurones dialysed with control pipette solution. When anti-P2X1 and heat-inactivated anti-P2X2+ anti-P2X4 antibodies were included in the pipette solution, the ATP-evoked current amplitude was similar to that observed in neurones dialysed with control pipette solution. Dialysis with anti-P2X1, anti-P2X2, or anti-P2X4 antibodies did not change the holding current nor appreciably affect the rate of decay of the ATP-evoked currents. Mean data for the relative peak ATP-evoked current amplitude obtained for at least five cells (anti-P2X1, anti-P2X2, anti-P2X4, anti-P2X2+ anti-P2X4 antibodies and heat-inactivated anti-P2X2+ anti-P2X4 antibodies) are shown in Fig. 7B.
Figure 7. Inhibition of ATP-induced currents by cell dialysis with anti-P2X<sub>2</sub> and anti-P2X<sub>3</sub> antibodies. A, superimposed ATP-evoked currents obtained at a holding potential of -80 mV immediately after whole-cell recording was established (control, thick trace) and approximately 15 min after dialysis with pipette intracellular solutions containing anti-P2X<sub>2</sub>, anti-P2X<sub>3</sub>, anti-P2X<sub>2</sub> + anti-P2X<sub>3</sub> and heat-inactivated anti-P2X<sub>2</sub> + anti-P2X<sub>3</sub> antibodies (thick trace). Antibodies diluted 1:200. B, bar graph of relative peak current amplitude obtained following intracellular dialysis with anti-P2X<sub>2</sub> (n = 7), anti-P2X<sub>3</sub> (n = 5), anti-P2X<sub>2</sub> + anti-P2X<sub>3</sub> (n = 6) and heat-inactivated anti-P2X<sub>2</sub> + anti-P2X<sub>3</sub> (n = 5) antibodies expressed relative to that obtained in the absence of anti-P2X antibodies (* P < 0.05, ** P < 0.005). Data were normalized with respect to those obtained immediately upon establishing the whole-cell recording configuration.

Discussion

The transient inward current evoked at negative membrane potentials in response to exogenous application of ATP to dissociated neurones from rat parasympathetic ganglia is due to the activation of cation-selective P2X receptor-channels, which exhibit negligible anion permeability (Fieber & Adams, 1991). Furthermore, the short latency of current activation in dialysed neurones in response to ATP and inhibition by PPADS is consistent with a direct ligand-gated ion channel of the P2X purinoceptor class (Ralevic & Burnstock, 1998). ATP has previously been shown to activate cation-selective channels in isolated rat sensory neurones (Krishtal et al. 1983; Bean et al. 1990; Virginio et al. 1998), rat sympathetic neurones (Nakazawa et al. 1994), rat parasympathetic neurones (Fieber & Adams, 1991) and guinea-pig coeliac neurones (Evans et al. 1992; Silinsky & Gerzanich, 1993). These and the present study used the measurement of shifts in reversal potential upon ion substitution to calculate the relative permeability using the GHK voltage equation.

There was no simple correlation between the shifts in reversal potential and the amplitude of whole-cell ATP-evoked currents observed with monovalent inorganic and ammonium cations. The peak amplitudes of ATP-evoked currents obtained in isotonic Na<sup>+</sup>, NH<sub>4</sub><sup>+</sup>, N<sub>2</sub>H<sub>5</sub><sup>+</sup> and NH<sub>3</sub>OH<sup>+</sup> were similar to that obtained in isotonic Cs<sup>+</sup>, whereas the current amplitudes were smaller in isotonic solutions of the other monovalent alkali metal cations (see Fig. 2). The ATP-activated channel in rat parasympathetic neurones exhibited weak selectivity among the alkali metals with a (E<sub>rev</sub>) selectivity sequence of Na<sup>+</sup> > Li<sup>+</sup> > Cs<sup>+</sup> > Rb<sup>+</sup> > K<sup>+</sup>, and permeability ratios relative to Cs<sup>+</sup> (P<sub>Na</sub>/P<sub>Cs</sub>) ranging from 1.11 (Na<sup>+</sup>) to 0.86 (K<sup>+</sup>). The permeability sequence approximately follows Eisenman sequence XI, indicating that the narrow region of the ion-conducting pore is probably a high field strength site (Eisenman, 1962). The selectivity sequence of the neuronal P2X receptor channel expressed in rat parasympathetic ganglia differs from that reported in neuroblastoma × glioma hybrid NG108-15 cells (Na<sup>+</sup> > Li<sup>+</sup> > Rb<sup>+</sup> > K<sup>+</sup> > Cs<sup>+</sup>; Kaiho et al. 1996), dissociated neurones from the rat tuberomammillary nucleus (Na<sup>+</sup> > K<sup>+</sup> > Rb<sup>+</sup> > Li<sup>+</sup> > Cs<sup>+</sup>; Furukawa et al. 1994) and cloned P2X<sub>3</sub> purinoceptors expressed in HEK293 cells (K<sup>+</sup> > Na<sup>+</sup> > Cs<sup>+</sup>, Evans et al. 1996; K<sup>+</sup> > Rb<sup>+</sup> > Cs<sup>+</sup> > Na<sup>+</sup> > Li<sup>+</sup>, Ding & Sachs, 1999). Differences in the ionic selectivities reported for both
native and cloned P2X receptors may be due to a change in cation permeability during prolonged exposure (> 10 s) to ATP in whole-cell recordings (Khakh et al. 1999; Virginio et al. 1999) or to different P2X receptor subtypes. ATP-evoked currents were strongly inhibited when the extracellular divalent cation concentration was increased as previously described in rat autonomic neurones (Nakazawa, 1994; Nutter & Adams, 1995a) and have been attributed to a saturable binding site for divalent cations within the ion conducting pore of the channel (Nakazawa & Hess, 1993; Ding & Sachs, 1999). The selectivity for the divalent alkaline earth cations was also weak with the sequence Ca$^{2+} > $Sr$^{2+} > $Ba$^{2+} > $Mg$^{2+}$, and GHK permeability ratios ($P_{Ca}/P_{Na}$) of 1.03, 0.85, 0.78, 0.54 and 0.43, respectively. The relative Ca$^{2+}$ permeability of ATP-activated channels has been described previously in rat and bullfrog sensory neurones ($P_{Ca}/P_{Na}$ = 0.3 in 113 mm Ca$^{2+}$; Bean et al. 1990), rat tuberomammillary neurones ($P_{Ca}/P_{Na}$ = 2.55 in 23 mm Ca$^{2+}$; Furukawa et al. 1994), rat nodose ganglion neurones ($P_{Ca}/P_{Na}$ = 1.5 in 5 mm Ca$^{2+}$; Virginio et al. 1998), cultured rat retinal ganglion cells ($P_{Ca}/P_{Na}$ = 2.2 in 20 mm Ca$^{2+}$; Taschenberger et al. 1999) and PC12 cells ($P_{Ca}/P_{K}$ = 1.0 in 48.6 mm Ca$^{2+}$; Nakazawa et al. 1990). In cloned P2X receptors expressed in mammalian cells, $P_{Ca}/P_{Na}$ was estimated to be 3.9 for P2X$_1$ and 2.2 for P2X$_2$ in 112 mm Ca$^{2+}$ (Evans et al. 1996). The results obtained are difficult to compare because of different extracellular Ca$^{2+}$ concentrations, different intracellular cations used (Na$^+$, Cs$^+$, or K$^+$), ambiguities about whether and how corrections were made for the ionic activities of Na$^+$ and Ca$^{2+}$, and differences in the relative permeability of the ‘impermeant’ cation used to substitute for the extracellular Ca$^{2+}$. Considering the underlying errors and assumptions, the value obtained for native P2X receptors in rat parasympathetic neurones ($P_{Ca}/P_{Na}$ = 0.93) is similar to that for cloned P2X$_2$ receptors (Evan et al. 1996; Virginio et al. 1998). The relative Ca$^{2+}$ permeability of P2X receptors is typically higher than those reported for nAChRs in frog skeletal muscle ($P_{Ca}/P_{Na}$ = 0.16 in 80 mm Ca$^{2+}$; Adams et al. 1980) and rat parasympathetic neurones ($P_{Ca}/P_{Na}$ = 0.65 in 100 mm Ca$^{2+}$; Nutter & Adams, 1995b).

The permeability ratios calculated for the different ammonium cations including the saturated combinations of hydroxyl, amino and methyl ammonium derivatives are higher than those of the alkali metal cations. The permeability sequence obtained for the saturated organic cations is inversely correlated to the size of the cation. The unsaturated organic cations guanidine, imidazole and acetamide have a higher permeability than that predicted by their molecular size, which may result from the restricted planar conformation of the unsaturated cations. The permeability ratios for the series of measurably permeant monovalent organic cations, with the exception of unsaturated cations, were approximately related to the geometric mean of the diameters (see Fig. 8). The relative permeabilities of the monovalent inorganic and organic cations tested are similar to those recently reported for ATP-gated ion channels (P2X$_2$ receptors) expressed in mammalian cells (Evans et al. 1996). The measureable permeability to large organic cations such as Tris, tetraethylammonium, and N-methyl-d-glucamine (NMDG) of the cloned P2X$_2$ receptor (Evans et al. 1996) and native neuronal P2X receptors (Krishtal et al. 1983; Nakazawa et al. 1990, 1991; Virginio et al. 1998) is consistent with the findings of the present study. Recent single channel studies of cloned P2X$_2$ receptors expressed in HEK293 cells, however, indicated that the open channel was virtually impermeant to organic ions such as NMDG, Tris, tetramethylammonium and tetraethylammonium (Ding & Sachs, 1999), suggesting that large organic cations interact with the channel pore. Assuming the open channel as a cylindrical pore and fitting the permeability data to the model suggests that organic cations with a mean diameter exceeding 0.7 nm would not permeate and provides an estimated minimum pore diameter. Although the permeability of heterologously expressed P2X receptor-channels to large organic cations has recently been reported to increase during prolonged (> 10 s) application of ATP (Khakh et al. 1999; Virginio et al. 1999), this is unlikely to occur in the present study in which ATP was applied for a maximum of 100 ms.

Identification of the P2X purinoceptor subtype(s) expressed in rat parasympathetic neurones was assisted by examining the pH sensitivity of the ATP-evoked current and through the use of specific anti-P2X antibodies added to the patch pipette ‘intracellular’ solution. Lowering the extracellular pH to 6.2 increased the ATP-evoked current amplitude approximately twofold, whereas alkalization to pH 8.2 and 9.2 markedly reduced current amplitude. Acidification of the extracellular pH has been shown to shift the ATP concentration-response curve to the left without changing the maximal response to ATP in rat coeliac and sensory ganglion neurones (Li et al. 1996; Zhong et al. 2000). The pH sensitivity profile obtained over the range pH 6.2-8.2 in rat parasympathetic neurones is consistent with that reported for the cloned rat P2X$_2$ and P2X$_{3/2}$ receptor expressed in either Xenopus oocytes (King et al. 1996) or HEK293 and CHO cells (Stoop et al. 1997). The apparent lack of potentiation of the ATP-evoked current at pH 5.2 may be due to superimposed proton block of the channels at pH < 6.2 or a heterogeneous channel population consistent with the presence of the P2X$_4$ phenotype (Wildman et al. 1999). A more direct determination of the P2X receptor subtypes expressed in rat parasympathetic neurones was obtained by cell dialysis with anti-P2X antibodies to unique cytoplasmic sequences of the P2X receptor subtypes. Cell dialysis with either anti-P2X$_2$ and/or anti-P2X$_4$ antibodies attenuated the ATP-evoked current amplitude. In contrast, anti-P2X$_1$ and heat-inactivated anti-P2X$_2$ and anti-P2X$_4$ antibodies had no effect on ATP-evoked currents. Dialysis with both anti-P2X$_2$ and anti-P2X$_4$...
antibodies, however, could not inhibit completely the ATP-induced current. Although antibodies to the other P2X receptors were not available for testing, these data are consistent with results of RT-PCR studies of mRNA expression in dissociated neurones from rat submandibular ganglia. Using specific primers for P2X1,5 subunits, PCR and restriction digests confirmed the predominance of P2X2 and P2X4 subtypes in these neurones (M. Stafford & D. J. Adams, unpublished observations). Taken together, these data indicate that the P2X purinoceptors expressed in rat parasympathetic neurones are predominantly either homomeric and/or heteromeric P2X2 and P2X4 receptors. Recently, biochemical evidence has suggested that among the seven P2X receptor subtypes, six can form homomeric complexes, the exception being P2X6 (Torres et al. 1999), and apart from P2X7, all are able to form heteromeric assemblies with P2X2 combining with P2X1, P2X3, P2X5 and P2X6 subunits. At least two different P2X receptors (P2X2 and P2X3) have been shown to coexist in individual neurones from rat nodose ganglia (Thomas et al. 1998) and rat dorsal root ganglia (Grubb & Evans, 1999). Similarly, P2X4 and P2X6 subunits co-assemble to form multimeric channels with unique functional properties that reflect those found in native neurones in the central nervous system (Lè et al. 1998).

The molecular architecture of the pore of a P2X receptor (Rassendren et al. 1997) is substantially different from that of the nAChR (see Corringer et al. 2000) but it appears to form an ion-conducting pore with several properties in common with the nAChR including: (1) a weak selectivity among monovalent and divalent metal cations, (2) a similar rank order for organic cation permeability, and (3) block upon raising the extracellular divalent cation concentration. Although state-dependent cross-inhibition between α3β4 nicotinic and P2X2 channels expressed in Xenopus oocytes has recently been demonstrated (Khakh et al. 2000), open channel block of neuronal nAChRs but not P2X receptors in rat parasympathetic neurones by local anaesthetics (Cuevas & Adams, 1994) is consistent with the ionic pore of the two classes of direct ligand-gated ion channel being functionally distinct.

In conclusion, the present study of the relative monovalent and divalent cation permeability of ATP-activated channels in dissociated neurones of rat submandibular ganglia provides a more extensive characterization of the ionic selectivity of the pore of the native P2X receptor-channel(s) in mammalian parasympathetic neurones. The inorganic and organic cation permeability of the ATP-gated receptor-channel is similar to that of the cloned P2X2 receptor (Evans et al. 1996) with a minimum pore diameter of ≥ 0.7 nm. However, the pH sensitivity and inhibition of the ATP-evoked current by specific anti-P2X antibodies are consistent with homomeric and/or heteromeric P2X2 and P2X4 receptor subtypes expressed in rat submandibular neurones.

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References


