Excitatory effect of ATP on rat area postrema neurons

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Abstract ATP-induced inward currents and increases in the cytosolic Ca\(^{2+}\) concentration ([Ca\(_{\text{in}}\)]\(_{\text{w}}\)) were investigated in neurons acutely dissociated from rat area postrema using whole-cell patch-clamp recordings and fura-2 microfluorometry, respectively. The ATP-induced current (I\(_{\text{ATP}}\)) and [Ca\(_{\text{in}}\)] \(_{\text{w}}\) increases were mimicked by 2-methylthio-ATP and ATP-\(\gamma\)S, and were inhibited by P2X receptor (P2XR) antagonists. The current–voltage relationship of the I\(_{\text{ATP}}\) exhibited a strong inward rectification, and the amplitude of the I\(_{\text{ATP}}\) was concentration-dependent. The I\(_{\text{ATP}}\) was markedly reduced in the absence of external Na\(^{+}\), and the addition of Ca\(^{2+}\) to Na\(^{+}\)-free saline increased the I\(_{\text{ATP}}\). ATP did not increase [Ca\(_{\text{in}}\)] \(_{\text{w}}\) in the absence of external Ca\(^{2+}\), and Ca\(^{2+}\)-channel antagonists partially inhibited the ATP-induced [Ca\(_{\text{in}}\)] \(_{\text{w}}\) increase, indicating that ATP increases [Ca\(_{\text{in}}\)] \(_{\text{w}}\) by Ca\(^{2+}\) influx through both P2XR channels and voltage-dependent Ca\(^{2+}\) channels. There was a negative interaction between P2XR- and nicotinic ACh receptor (nAChR)-channels, which depended on the amplitude and direction of current flow through either channel. Current occlusion was observed at V\(_{\text{h}}\) between −70 and −10 mV when the I\(_{\text{ATP}}\) and ACh-induced current (I\(_{\text{ACH}}\)) were inward, but no occlusion was observed when these currents were outward at a V\(_{\text{h}}\) of +40 mV. The I\(_{\text{ATP}}\) was not inhibited by co-application of ACh when the I\(_{\text{ACH}}\) was markedly decreased either by removal of permeant cations, by setting V\(_{\text{h}}\) close to the equilibrium potential of I\(_{\text{ACH}}\), or by the addition of d-tubocurarine or serotonin. These results suggest that the inhibitory interaction is attributable to inward current flow of cations through the activated P2XR- and nAChR-channels.

Keywords ATP · ACh · cytosolic Ca\(^{2+}\) concentration · Fura-2 microfluorometry · negative interaction between nicotinic and P2X channels · whole-cell patch-clamp recording

Introduction

The rat area postrema (AP) is a medullary circumventricular organ located in the hindbrain at the level of the obex, with a dense vascular supply devoid of a blood–brain barrier. The lack of a blood–brain barrier makes the AP ideally placed to act as a chemosensitive trigger zone involved in the control of vomiting in response to circulating emetic substances (Borison [1, 2]). Neurons in the AP are also responsive to changes in osmolarity or sodium concentration (Franchini et al. [3]), and can be activated by circulating vasoactive peptides such as angiotensin II (Fink et al. [4]), and arg-vasopressin (Undesser et al. ...
Anatomical studies have revealed that the AP sends dense efferent projections to the nucleus tractus solitarius, parabrachial nucleus, nucleus ambiguus, and the dorsal motor nucleus of the vagus, and receives afferent inputs from the hypothalamic paraventricular and dorsomedial nuclei, and from the caudal nucleus tractus solitarius (Morest [6]; Kooy and Koda [7]; Shapiro and Miselis [8]). Thus, the AP is not only capable of responding to circulating hormones, but is also anatomically well situated to regulate a range of other central neurons, including those important in cardiovascular control (Sun and Spyer [9]). The low intrinsic firing rates of AP neurons in vivo (Papas et al. [10]) suggests that understanding and modulating excitatory inputs to AP neurons could be particularly important in the functional output of AP neurons. A number of transmitters can evoke excitatory currents in the AP. Inward currents and increases in cytosolic Ca2+ ([Ca]i) via non-NMDA-glutamate receptors have been reported in rabbit and rat AP neurons, respectively (Jahn et al. [11]; Hay and Lindsley [12]). Our preliminary reports indicated that ATP also induces inward currents and [Ca]i increases via the activation of P2X receptor (P2XR) in isolated rat AP neurons (Sorimachi et al. [13, 14]). In addition, pre- and post-synaptic nicotinic ACh receptors (nAChR) have been demonstrated in the AP in rat brain slices (Funahashi et al. [15]), and we have recently reported the presence of nAChRs in dissociated rat AP neurons (Sorimachi and Wakamori [16]). During that study, we also noticed that many of these AP neurons also responded to ATP, which has prompted us to further investigate ATP responses in AP neurons, and potential interactions between nAChR and P2XR responses. In a variety of different peripheral neurons, including sympathetic neurons of bullfrog (Akasu and Koketsu [17]), rat (Nakazawa et al. [18]), and guinea-pig (Searl et al. [19]), cultured guinea-pig enteric and submucosal neurons (Zhou and Galligan [20] Barajas-Lopez et al. [21]), a negative interaction between P2XR- and nAChR responses has been reported. Such an interaction has also been observed for recombinant P2X2 and αβ4 nAChR channels in Xenopus oocytes and HEK cells (Khakh et al. [22, 23]; Boue-Grabot et al. [24]), where it has been recently suggested that this results from direct physical interactions between co-localized receptors (Khakh et al. [23]). In this study we more fully describe P2XR responses in AP neurons, demonstrate cross-inhibition between P2XRs and nAChRs and characterize some of the features of this cross-inhibition.

Experimental procedures

Preparation of AP neurons

The study was approved by the Committee on Animal Experimentation, Kagoshima University. Wistar rats (13–18 days-old) were anaesthetized with ether and decapitated. The brain was quickly removed from the skull and placed in ice-cold HEPES-buffered saline containing 150 mM NaCl, 5 mM KCl, 2 mM CaCl2, 1 mM MgCl2, 10 mM HEPES and 5.5 mM glucose. The pH of the saline solution was adjusted to 7.4 by adding tris (hydroxymethyl) aminomethane. The brain was sliced at a thickness of 400 μm with a microslicer (DTK-1000, Dosaka, Kyoto, Japan), and the slices were kept in bicarbonate-buffered saline bubbled continuously with 95% O2–5% CO2 at room temperature (21–26 °C). The bicarbonate-buffered saline contained 120 mM NaCl, 5 mM KCl, 2 mM CaCl2, 1 mM MgCl2, 20 mM NaHCO3, 2 mM KH2PO4 and 5.5 mM glucose. Following 30–60 min incubation, the slices were treated with pronase (0.2 mg/ml) in bicarbonate-buffered saline for 20 min at 33 °C, followed by incubation for 30 min with bicarbonate-buffered saline containing thermolysin (0.12–0.15 mg/ml). The bilateral AP regions were identified with a binocular microscope (Zeiss, Germany), and were cut out using the tip of an injection needle, and subsequently mechanically triturated with fire-polished glass pipettes of decreasing diameters. Dissociated neurons were placed on the bottom of 35 mm culture dishes (Falcon, USA) for electrophysiological recordings, or on to glass coverslips (Matsunami, Japan) coated with poly-L-lysine for [Ca]i measurements.

Electrophysiological recordings

Electrical measurements were done using the whole-cell patch-clamp recording configuration under voltage-clamp conditions. Patch pipettes were fabricated from borosilicate glass tubes in five or six stages using a pipette puller (Model P-97, Sutter Instrument, San Rafael, CA, USA). The resistance between the recording electrode filled with the internal solution, and the reference electrode in the external solution, was 3–6 MΩ. Ionic currents were measured, and voltages controlled, using a patch-clamp amplifier (EPC-9, Heka, Darmstadt, Germany). All experiments were carried out at 24–26 °C. Culture dishes were placed on an inverted microscope (TE200, Nikon, Japan), and drugs were rapidly applied to single cells using a Y-tube perfusion device. The internal solution (patch
pH was adjusted to 7.2 with KOH. For measuring current–voltage (I–V) relationships, a Cs-based internal solution was employed, which contained 98 mM CsCl, 90 mM aspartate, 2 mM MgCl2, 5 mM HEPES, 5 mM EGTA, and 2 mM ATP. pH was adjusted to 7.2 with CsOH. The external solution was the HEPES-buffered saline, pH7.4, described above. When N-methyl-D-glucamine (NMDG) or sucrose was substituted for external Na+ or NaCl, respectively, the osmolarity of the solution was kept constant as measured using an osmometer (Vogel OM801, Germany). To obtain the Ca2+/Na+ permeability ratio (PCa/PNa), we measured the reversal potential of the ATP-activated current (EATP) in the presence of 1 or 110 mM of external Ca2+ by stepping the holding potential (Vh) between 10 and 30 mV, using increments of 10 mV. The 1 and 110 mM Ca2+ solutions contained 155 or 0 mM NaCl, respectively, in addition to 10 mM HEPES with pH adjusted to 7.4 with NaOH or Ca(OH)2, respectively. The values of PCa/PNa and PNa/PCa were calculated using the constant field equation as described by Lewis ([25]), taking the activity coefficients of Na+, Cs+, and Ca2+ as 0.75, 0.75, and 0.3, respectively.

Measurement of [Ca]in

Dissociated AP neurons on a glass coverslip were incubated with 1–2 μM fura-2 acetoxyethyl ester (fura-2/AM), 0.1% dimethyl sulfoxide, and 1% bovine serum albumin for 45 min at 37 °C. The coverslips were then mounted in a superfusion chamber and placed on the stage of an inverted microscope (Diaphot-TMD, Nikon, Japan). Cells were continuously superfused at a rate of 1 ml/min with HEPES-buffered saline at 24–26 °C via a polyethylene tube placed 1–2 mm away from the cells.

Cells were viewed with a 40× Fluor objective lens (Nikon), and a single cell (10–12 μm diameter) was fixed in a window positioned between the photomultiplier and the microscope. The changes in fluorescence ratios at 340 and 380 nm excitation wavelengths were measured using a CAM-200 spectrometer (Jasco, Japan). The absolute value of [Ca]in was calculated using the formulae as given by Grynkiewicz et al. [26]: [Ca]in = b × Kd(R – Rmin)/(Rmax – R). Calibration constants were determined in separate experiments with the same experimental set-up, as described previously (Sorimachi et al. [27]).

Student’s paired t test was used to evaluate differences between mean values obtained from the same cells, and Student’s unpaired t test was used for data obtained from different groups of cells.

Reagents

The following reagents were used: Fura-2/AM [Dojindo, Kumamoto, Japan], ACh [Horai Chem. Co., Japan], PPADS (pyridoxal-phosphate-6-azophenyl-2’, 4’-disulphonic acid) [RBI, USA.], pronase [Calbiochem., USA], thermolysin, ATP, 2-methylthio-ATP, ATPγS, α,β-methylene-ATP, β,γ-methylene-ATP, ADP, nitrendipine, nicardipine, suramin [all from Sigma, Aldrich, Tokyo, Japan], α-conotoxin-MVIIC, α-conotoxin-MVIIC and α-agatoxin-IVA [all from Peptide Institute, Osaka, Japan].

Results

ATP-induced current

Rapid application of ATP (100 μM) to isolated AP neurons voltage clamped at a Vh of −70 mV induced an inward current, which desensitized slowly (Fig. 1A). The ATP-induced current (IATP) was recorded at various Vhs between −70 and +50 mV (Fig. 1: N = 6), and the relative current amplitude was plotted against Vh exhibiting a strong inward rectification. The EATP, estimated from the intersection of the current response and the zero voltage-axis (Fig. 1B), was 22.7 ± 0.9 mV (N = 18).

To investigate the ATP concentration-response relationship, care was taken to adjust the pH of the solutions containing higher concentrations of ATP to 7.3, since addition of ATP reduced pH and a small decrease in pH to 7.1 increased the IATP to 189 ± 8% (N = 6) of control (pH7.3), as previously reported for recombinant P2X2 receptors (King et al. [28]). The responses to different ATP concentrations were flanked by responses to the control concentration of 100 μM, and normalized responses were expressed relative to the average of these control responses (Fig. 1C; Ca2mM). We also examined the effects of 0.1 and 10 mM Ca2+ on IATP. As summarized in Fig. 1C, an increase in the external concentration of Ca2+ shifted the concentration-response curve for ATP to the right, with the half-maximum effective concentration (EC50) values at 0.1, 2 and 10 mM Ca2+ being 30, 70, and 190 μM, respectively.
The purinergic agonists, 2-methylthio-ATP (50 μM) and ATPγS (50 μM) induced currents that were 41 ± 3% (N = 4), and 41 ± 4% (N = 5), respectively, of the I_{ATP} in response to 100 μM ATP. ADP (0.5 mM) did not produce any response. The I_{ATP} in response to 50 μM ATP was inhibited by the P2 antagonists; suramin (10 μM and 20 μM) and PPADS (50 μM), with the response being 35 ± 2% (N = 5), 25 ± 2% (N = 4), and 56 ± 5% (N = 5), of the control I_{ATP}, respectively.

ATP-induced [Ca]_{in} increase

ATP also increased [Ca]_{in} in a dose-dependent manner (Fig. 2A). These responses were all recorded in the presence of 2 mM external Ca^{2+}, and the [Ca]_{in} increases by various concentrations of ATP were normalized to that induced by 100 μM ATP. The normalized and averaged responses to ATP, 2-methylthio-ATP and ATPγS are shown in Fig. 2B. There was no ATP-induced [Ca]_{in} increase in the absence of external Ca^{2+} (N = 6; data not shown). Furthermore, neither ADP, α,β-methylene-ATP nor β,γ-methylene-ATP increased [Ca]_{in} when tested at concentrations of 200 μM(data not shown). The ATP-induced [Ca]_{in} increases were inhibited by suramin and PPADS (Table 1).

Effects of Ca^{2+} channel antagonists on ATP-induced [Ca]_{in} increase

Previous results, using cultured rabbit AP neurons, demonstrated the presence of a ω-conotoxin-M C-sensitive Ca^{2+} response, but that did not involve L- or N-type Ca^{2+} channels (Hay et al. [29]). We also investigated the effects of various Ca^{2+} channel antagonists on [Ca]_{in} increases induced by high KCl (110 mM) and ATP (100 μM). Antagonist for the L-type (nitrendipine, nicardipine), N-type (ω-conotoxin-M A), or P/Q-type (ω-conotoxin-M C) Ca^{2+} channels each substantially inhibited these [Ca]_{in} increases (Table 1). In contrast, the selective P-type Ca^{2+} channel antagonist, ω-agatoxin IVA (1 μM), did not have any inhibitory effect on the [Ca]_{in} increases (98 ± 5% of control, N = 4).

ATP-induced current in the absence of external Na^{+}

When external Na^{+} was completely replaced by NMDG^{+}, and in the absence of external Ca^{2+} (0 Ca^{2+} plus 0.5 mM EGTA), the I_{ATP} was markedly reduced to 8 ± 1% of the control I_{ATP} recorded in the presence of external Ca^{2+} and 150 mM Na^{+} (N = 9, Fig. 3A). This current was further reduced to 4 ± 1% (N = 3) in the presence of 20 μM suramin. The small
Fig. 2 Concentration-response relationships for the $[\text{Ca}]_{\text{in}}$ increases in response to ATP and ATP analogues. **A** Representative $[\text{Ca}]_{\text{in}}$ increases induced by various concentrations of ATP in the presence of 2 mM external Ca$^{2+}$. **B** Averaged concentration-response relationships for ATP and ATP analogues. All responses were normalized to the mean of two control responses induced by 100 μM ATP before and after the test response. Each point is the mean ± S.E.M. of data from five to nine neurons. MeS-ATP: 2-methylthio-ATP.

**Table 1** Effects of P2 receptor antagonists and Ca$^{2+}$ channel blockers on the $[\text{Ca}]_{\text{in}}$ increases induced by ATP and high K$^+$-saline

| Stimulus   | Blockers                  | Percent of control |
|------------|----------------------------|--------------------|
| ATP100 M   | Suramin 10 M               | 55 ± 7 (N = 5)     |
|            | 20 M                       | 30 ± 5 (N = 6)     |
|            | 50 M                       | 13 ± 3 (N = 5)     |
|            | PPADS 10 M                 | 96 ± 6 (N = 3)     |
|            | 20 M                       | 70 ± 8 (N = 9)     |
|            | 50 M                       | 25 ± 5 (N = 6)     |
|            | 100 M                      | 11 ± 5 (N = 4)     |
| 110 mM KCl | Nitrendipine 2 M           | 44 ± 4 (N = 16)    |
|            | −CT.M C 2 M                | 61 ± 4 (N = 16)    |
|            | −CT.M A 2 M                | 62 ± 6 (N = 16)    |
|            | −CT.M A 2 M + −CT.M C 2 M  | 58 ± 5 (N = 10)    |
| ATP100 M   | Nitrendipine 2 M           | 56 ± 4 (N = 21)    |
|            | Nicardipine 2 M            | 47 ± 9 (N = 10)    |
|            | Cd$^{2+}$ 50 M             | 27 ± 3 (N = 14)    |
|            | −CT.M A 2 M + −CT.M C 2 M  | 77 ± 9 (N = 4)     |

The mean $[\text{Ca}]_{\text{in}}$ increase induced by the first and third applications of ATP (100 μM) or 110 mM KCl were averaged and referred to as 100%, and the response in the presence of the P2 antagonist or Ca$^{2+}$ channel blocker was expressed as a percentage of this control value. The cell was pre-treated for 30 s with the indicated agent before the second stimulation with 100 μM ATP or 110 mM KCl. Number of experiments is shown in parentheses. α-CT α-conotoxin.
suramin-sensitive, Na\(^+\)-independent current supports the previous suggestion that the P2XR channel is permeable to glucosamine (Nakazawa [30]). To confirm this, ATP did not induce a current at all when sucrose (0.25 M) was substituted for NaCl (again in the absence of external Ca\(^{2+}\), N = 5). Addition of Ca\(^{2+}\) to the NMDG\(^+\)-substituted saline further increased \(I_{ATP}\). The currents induced by 500 \(\mu\)M ATP in the presence of 2 and 10 mM Ca\(^{2+}\), but in the absence of external Na\(^+\) (replaced by NMDG\(^+\)) were 18 \(\pm\) 2% (N = 11) and 29 \(\pm\) 2% (N = 14, Fig. 3B) of the control \(I_{ATP}\), respectively. Similarly, the currents induced by 500 \(\mu\)M ATP in the presence of 2 and 10 mM Ca\(^{2+}\) added to the sucrose-substituted saline were 6 \(\pm\) 1% (N = 6), and 13 \(\pm\) 2% (N = 16; Fig. 3C) of the control \(I_{ATP}\), respectively.

We also measured the \(E_{ATP}\) of \(I_{ATP}\) in the presence of 1 mM external Ca\(^{2+}\) and 150 mM NaCl or 110 mM external Ca\(^{2+}\) and 0 mM NaCl. The \(E_{ATP}\) at 110 mM Ca\(^{2+}\) was 21.1 \(\pm\) 1.0 mV (N = 18), from which we calculated a \(P_{Ca}/P_{Cs}\) of 6.3. The \(E_{ATP}\) at 1 mM Ca\(^{2+}\) was 20.2 \(\pm\) 1.0 mV (N = 13), from which we calculated a \(P_{Na}/P_{Cs}\) ratio of 2.1. From these, we obtained a \(P_{Ca}/P_{Na}\) ratio of 3.0, confirming the substantial Ca\(^{2+}\) permeability of P2XR channels.

Negative interaction between P2XR and nAChR channels

Requirement of actual current flow through receptor channels for cross-inhibition.

It has previously been reported that there is mutual occlusion between P2XR and nAChR in some neurons (Nakazawa et al. [18]; Nakazawa [30]; Searl et al. [19]; Zhou and Galligan [20]; Barajas-Lopez et al. [21]; Khakh et al. [22, 23]; Boue-Grabot et al. [24]). To examine whether there were negative interactions between the ACh-activated current (\(I_{ACh}\)) and \(I_{ATP}\), one receptor agonist was added in the presence of the other. As shown in Fig. 4A, the \(I_{ACh}\) (100 \(\mu\)M) was markedly reduced when activated in the presence of ATP (50 \(\mu\)M). We next examined \(I_{ACh}\) (100 \(\mu\)M) in the presence of various concentrations of ATP. With 2, 10, 20, and 100 \(\mu\)M ATP, \(I_{ACh}\) was reduced to 95 \(\pm\) 1% (N = 20), 84 \(\pm\) 2% (N = 24), 60 \(\pm\) 4% (N = 15), and 46 \(\pm\) 6% (N = 7), respectively, of control (\(P < 0.01\) except at 2 \(\mu\)M ATP). Thus, the \(I_{ACh}\) inhibition became stronger as the concentration of ATP was increased and with a higher agonist-receptor occupancy. In fact, when \(I_{ATP}\) at 100 \(\mu\)M ATP was markedly inhibited in the presence of 200 \(\mu\)M PPADS (8 \(\pm\) 2% of control, N = 6), \(I_{ACh}\) was not occluded (97 \(\pm\) 1% of control; N = 6). When the peak amplitude of \(I_{ATP}\), evoked by various concentrations of ATP, was plotted against the ratio of \(I_{ACh}\) in the presence and absence of ATP, there was an inverse correlation between the amplitudes of these responses (Fig. 4B). Conversely, when ATP (50 \(\mu\)M) was applied in the presence of ACh (100 \(\mu\)M), \(I_{ATP}\) was also occluded (Fig. 4C). Again, when \(I_{ACh}\) at 200 \(\mu\)M ACh was nullified in the presence of 1 mM hexamethonium, a nAChR antagonist (0.2 \(\pm\) 0.2% of control, N = 6), \(I_{ATP}\) was 96 \(\pm\) 2% (N = 6) of control. \(I_{ACh}\) at 10, 20, 50, and 200 \(\mu\)M ACh were 6 \(\pm\) 2% (N = 5), 20 \(\pm\) 4% (N = 5), 61 \(\pm\) 8% (N = 6), and 139 \(\pm\) 8% (N = 6) of \(I_{ACh}\) at 100 \(\mu\)M. There was also an inverse correlation between the peak amplitude of \(I_{ACh}\) evoked by various concentrations of ACh, and the ratio of \(I_{ATP}\) in the presence and absence of ACh (Fig. 4D). However, the inhibition of \(I_{ATP}\) by nAChR activation was weaker than that of \(I_{ACh}\) by P2XR activation. As the \(I_{ACh}\) desensitized faster than the \(I_{ATP}\), the weaker inhibition of \(I_{ATP}\) by ACh could be
due to the reduced amplitude of the $I_{\text{ACh}}$ at the time of ATP application.

To examine this possibility, $I_{\text{ATP}}$ was measured in the presence of both $\text{ACh}$ and d-tubocurarine (dTc, 10 µM) or serotonin (5HT, 50 µM), two experimental conditions which change the time course of the $I_{\text{ACh}}$ response. The former agent, dTc, has been shown to be both an open channel blocker and a competitive antagonist of nAChR (Manalis [31]), and, as expected, a low concentration (10 µM) slightly reduced the peak amplitude of $I_{\text{ACh}}$ (79 ± 4% of control, $N = 11$) and caused the $I_{\text{ACh}}$ to be terminated within 0.5 s (Fig. 5A, insert). Once the $I_{\text{ACh}}$ response had returned to baseline (in the presence of ACh and 10 µM dTc) and ATP was applied, there was no inhibition of $I_{\text{ATP}}$ (95 ± 1% of control, $N = 5$; Fig. 5A). The $I_{\text{ATP}}$ in the presence of ACh alone was 77 ± 4% of control ($N = 5$, $P < 0.01$; Fig. 5A). When we measured the current ($I_{\text{ACh} + \text{ATP} + \text{dTc}}$) induced by a combination of ACh, ATP and dTc, the peak amplitude of $I_{\text{ACh} + \text{ATP} + \text{dTc}}$ was occluded under these conditions, being 74 ± 2% ($N = 10$) of the predicted sum of $I_{\text{ATP}}$ and $I_{\text{ACh} + \text{dTc}}$ (Fig. 5B). The level of $I_{\text{ACh} + \text{ATP} + \text{dTc}}$ at 0.5 s following ligand application, which corresponds to a time when the nAChR channels are completely blocked (Fig. 5A, insert), was 96 ± 3% ($N = 8$) of the control $I_{\text{ATP}}$, measured immediately before and after (Fig. 5C). These results suggest that the inhibition of $I_{\text{ATP}}$ by $I_{\text{ACh}}$ disappears immediately after the $I_{\text{ACh}}$ is abolished, although ACh is still present and bound to the nAChRs. 5HT has been shown to also accelerate the decay of $I_{\text{ACh}}$ (Grassi et al. [32]; Garcia-Colunga and Miledi [33]; Sorimachi and Wakamori [34]), but not to the same extent as dTc. 5HT at 50 µM decreased the peak amplitude of $I_{\text{ACh}}$, slightly, to 88 ± 4% ($N = 6$) of control, and reduced the time constant (tau) of $I_{\text{ACh}}$ decay from 2.02 ± 0.18 to 1.06 ± 0.19 s ($N = 6$). As shown in Fig. 5D, the extent of inhibition of $I_{\text{ATP}}$ by
ACh was smaller (to 92 ± 2% of control, \( N = 6 \)) in the presence of ACh and 5HT compared to that in the presence of ACh alone (to 73 ± 3% of control, \( N = 6 \); \( P < 0.01 \)).

Next, we measured the current induced by the concomitant applications of ACh and ATP (\( I_{ACh+ATP} \)) at a \( V_h \) of \(-70 \text{ mV}\). As shown in Fig. 6A, \( I_{ACh+ATP} \) was 75 ± 1% (\( N = 44; P < 0.01 \)) of the predicted sum of \( I_{ACh} \) and \( I_{ATP} \). The decay phase of \( I_{ACh} \), \( I_{ATP} \) and \( I_{ACh+ATP} \) were fit to a single exponential function, giving tau of 3.0 ± 0.2, 7.5 ± 0.6 and 3.7 ± 0.2 s, respectively. When the decay of \( I_{ACh} \) was accelerated by the presence of 100 µM 5HT, the tau of \( I_{ACh} \), \( I_{ATP} \), and \( I_{ACh+ATP} \) were 0.8 ± 0.3 s (\( N = 7 \)), 7.4 ± 1.4 s (\( N = 7 \)) and 2.6 ± 0.8 s (\( N = 7 \)), respectively (the amplitude of \( I_{ACh+ATP} \) under these conditions was 76 ± 2% of the predicted sum of \( I_{ACh} \) and \( I_{ATP} \)). Thus, \( I_{ACh+ATP} \) desensitization occurs with kinetics that cannot be
explained by the desensitization kinetics of $I_{ACh}$ or $I_{ATP}$ alone, suggesting that $I_{ACh + ATP}$ is mediated via both receptors.

When Ca$^{2+}$ was omitted from extracellular solution, the negative interaction between the two receptor channels was still obtained; with $I_{ACh + ATP}$ being 77 ± 1% ($N = 5$; $P < 0.01$) of the predicted sum of $I_{ACh}$ and $I_{ATP}$. Hence negative interaction is not mediated by a Ca$^{2+}$ influx-dependent mechanism, although both nAChR and P2XR are Ca$^{2+}$-permeable cation channels (Fieber and Adams [35]; Rogers and Dani [36]). This result, however, does not necessarily rule out the possible involvement of [Ca]$^{2+}$ in negative interaction.

Occlusion was not only observed at a $V_h$ of −70 mV; $I_{ACh + ATP}$ at $V_h$s of −20, and −10 mV were 80 ± 2% ($N = 12$; $P < 0.01$), and 76 ± 2% ($N = 8$; $P < 0.01$), respectively, of the predicted sum of $I_{ACh}$ and $I_{ATP}$. In sharp contrast, such occlusion was not observed at a positive potential; the outward current caused by co-application of ACh and ATP at a $V_h$ of +40 mV was 100 ± 1% ($N = 10$) of the predicted sum of individual current (Fig. 6B).

We next investigated the possibility that the combined addition of ACh and ATP alters the driving force for Na$^+$ by attempting to measure $E_{ACh + ATP}$. In these studies, currents in response to ACh, ATP, and both ligands were measured at various fixed potentials close to the reversal potential for $I_{ACh}$ and $I_{ATP}$. The $E_{ACh}$ and $E_{ATP}$ were 8.7 ± 1.5 mV ($N = 5$) and 21.0 ± 1.6 mV ($N = 7$), respectively, and $I_{ACh + ATP}$ measured at a potential between these two reversal potentials (e.g.10 mV, Fig. 6C) showed a combination of both the ACh-induced outward current and the ATP-induced inward current. These results suggest that the driving force for permeant ions during combined ACh and ATP is similar to that during the application of each ligand separately.

The question arises as to whether occlusion is specific just for Na$^+$ ions or whether inward currents carried by cations other than Na$^+$ can also contribute to occlusion during co-activation of nAChRs and P2XRs. To address this question, we measured $I_{ACh}$, $I_{ATP}$, and $I_{ACh + ATP}$ in the presence of Na$^+$-free (NMDG$^+$) saline containing 10 mM Ca$^{2+}$ (Fig. 7). NMDG$^+$ does not permeate through nAChR (Sorima-

**Fig. 6** Voltage dependence of $I_{ACh}$ and of $I_{ATP}$ occlusion. A) At a $V_h$ of −70 mV, co-application of ACh (200 μM) and ATP (100 μM) induced an inward current that was smaller in amplitude than the predicted sum of the individual $I_{ACh}$ and $I_{ATP}$. B) At a $V_h$ of +40 mV, the amplitude of the outward current induced by the co-application of ACh (500 μM) and ATP (200 μM) was equal to the predicted sum of the individual $I_{ACh}$ and $I_{ATP}$. C) Co-application of ATP and ACh does not markedly change $E_{ACh}$ or $E_{ATP}$. Representative currents induced by ACh, ATP, and by co-application of ACh and ATP at a $V_h$ of +10 mV. Note that $I_{ACh + ATP}$ is composed of early outward and delayed inward current, corresponding to the ACh component and the ATP component, respectively.

**Fig. 7** Occlusion of $I_{ACh + ATP}$ in the presence of Na$^+$-free saline containing 10 mM Ca$^{2+}$. $I_{ACh}$, $I_{ATP}$ or $I_{ACh + ATP}$ was recorded 20 s after switching to Na$^+$-free (replaced by NMDG$^+$) saline containing 10 mM Ca$^{2+}$. The sequence of applications was repeated multiple times. Following each ligand application, the external solution was changed back to normal saline containing 150 mM Na$^+$ before the next ligand application. $V_h$: −70 mV.
chi and Wakamori [16]), and hence $I_{\text{ACh}}$ would be mediated by only Ca$^{2+}$ influx, whereas $I_{\text{ATP}}$ would be mediated via both NMDG$^+$ and Ca$^{2+}$ influx (Fig. 3). Under these conditions, $I_{\text{ACh}} + ATP$ was again occluded, being 70 ± 3% of the predicted sum of $I_{\text{ACh}}$ and $I_{\text{ATP}}$ ($N = 11; P < 0.01$; Fig. 7).

In a further attempt to distinguish whether channel activation or ion permeation was primarily responsible for occlusion of one channel by the other, we compared $I_{\text{ATP}}$ with $I_{\text{ACh}} + ATP$ under two experimental conditions, in which a higher concentration of ACh should activate nAChR but produce very little current. In the absence of Na$^+$ and Ca$^{2+}$ (replaced by NMDG$^+$), application of ACh induces a negligible current (Sorimachi and Wakamori [16]), while ATP induces a substantial current under the same conditions (Fig. 3A). As shown in Fig. 8B, there was no occlusion in these conditions; the amplitude of $I_{\text{ATP}} + \text{ACh}$ was 105 ± 1% ($N = 10$) of the predicted sum of $I_{\text{ACh}}$ and $I_{\text{ATP}}$. We also investigated currents in response to the ligands at a potential close to the $E_{\text{ACh}}$ ($7 ± 2$ mV in this experiment, $N = 13$), taking advantage of the more positive $E_{\text{ATP}}$ ($22.7 ± 0.9$ mV), so that at this potential only the $I_{\text{ATP}}$ was observed. The amplitude of $I_{\text{ATP}} + \text{ACh}$ was 105 ± 3% ($N = 13$) of that of $I_{\text{ATP}}$, and $I_{\text{ACh}}$ was close to zero (Fig. 8D). These results are, however, in contrast to that obtained in sympathetic neurons (Nakazawa [30]), in which $I_{\text{ATP}}$ in the absence of both Na$^+$ (replaced by glucosamine) and Ca$^{2+}$ was inhibited in the presence of ACh, which caused no current (Nakazawa [30]). In these experiments, we only included data in which $I_{\text{ATP}}$ was large enough to be clearly resolved, greater than 50 pA (corresponding to a control $I_{\text{ATP}}$ at a $V_h$ of $-70$ mV larger than 1.5 nA). These results suggest that actual current flow through both nAChR and P2XR is responsible for occlusion.

**Discussion**

In this study, we found that ATP induced an inward current and increased [Ca]$_{\text{in}}$ in isolated rat AP neurons. The ATP-induced current and [Ca]$_{\text{in}}$ increase were mimicked by ATP/S and 2-methylthio-ATP, but not by $\alpha$, $\beta$-methylene-ATP, $\beta$, $\gamma$-methylene-ATP nor ADP, and was inhibited by suramin and PPADS, suggesting that it was mediated by P2XRs. These
results are in good agreement with previous histo-
chemical findings, which demonstrated the presence of
P2X2, P2X4, and P2X6 receptor mRNAs (Collo et al.
[37]), and of P2X2 receptor immunoreactivity in AP
neurons (Kanjhan et al. [38]; Atkinson et al. [39]).

The $I_{\text{ATP}}$ showed strong inward rectification and the
$E_{\text{ATP}}$ was $22.7 \pm 0.9$ mV (Fig. 1B). The amplitude of
$I_{\text{ATP}}$ varied inversely with the extracellular Ca$^{2+}$
concentrations (Fig. 1C). The inhibitory effect of
increasing extracellular Ca$^{2+}$ has also been reported in
rat sensory neurons (Krishtal et al. [40]), PC-12 cells
(Nakazawa et al. [18]), ventromedial hypothalamic
neurons (Sorimachi et al. [41]), and in cells expressing
recombinant P2X2 receptors (Evans et al. [42]; Vir-
ginio et al. [43]), where an allosteric alteration of the
ATP binding sites has been suggested to be the
underlying mechanism.

The $I_{\text{ATP}}$ was markedly reduced, but still persisted
when NMDG$^+$ was substituted for external Na$^+$ even
in the absence of Ca$^{2+}$ (Fig. 3A). Since ATP did not
induce a current when succrose was substituted for
external NaCl, our result suggests that NMDG$^+$ could
permeate through P2XR. A substantial $I_{\text{ATP}}$ has been
similarly reported in PC-12 cells and sympathetic
neurons when glucosamine was substituted for Na$^+$
(Nakazawa et al. [18]; Nakazawa [30]). The addition of
Ca$^{2+}$ to NMDG-Cl- and sucrose-substituted saline
increased the $I_{\text{ATP}}$ (Fig. 3B and C, respectively),
indicating that Ca$^{2+}$ also permeates through P2XR
channel. We quantified the relatively high Ca$^{2+}$ per-
meability, obtaining a $P_{\text{Ca}}/P_{\text{Na}}$ ratio of 3.0. A relatively
high permeability of P2XR to Ca$^{2+}$ has been reported in
previous studies (Nakazawa et al. [18]; Sorimachi
et al. [41]; Evans et al. [42]; Virginio et al. [43]).
Although direct influx of extracellular Ca$^{2+}$ through
P2XR channel may thus contribute to the ATP-
induced [Ca]$^{2+}$ increase, membrane depolarization and
the secondary activation of voltage-dependent Ca$^{2+}$
channels could also make a significant contribu-
tion to the [Ca]$^{2+}$ increase. In fact, the high K$^+$ and
ATP-induced [Ca]$^{2+}$ increases were substantially
inhibited by a range of Ca$^{2+}$ channel antagonists,
including those which block L- and N-type Ca$^{2+}$
channels (Table 1). This is in contrast to a previous
study, which reported an absence of the L- and N-type
Ca$^{2+}$ channels in rabbit AP neurons (Hay et al. [12]).
The discrepancies between their results and ours could
be, at least in part, accounted for by the use of
different AP preparations (cultured rabbit neurons
vs. acutely dissociated rat neurons).

The present results, in combination with our
previous demonstration of nAChR on AP neurons
(Sorimachi and Wakamori [16]), suggest that both
ATP and ACh may act as excitatory neurotransmitters
in AP neurons, although their release from presyn-
aptic nerve terminals has not yet been reported. We
also report a negative functional interaction between
P2XR and nAChRs in AP neurons, as has been ob-
erved in a variety of peripheral neurons and in re-
combinant expression systems (Nakazawa et al. [18];
Nakazawa [30]; Zhou and Galligan [20]; Barajas-
Lopez et al. [21]; Searl et al. [19]; Khakh et al. [22,
23]; Boue-Grabot et al. [24]). This is the first report,
that we are aware of, of such interactions occurring
in central neurons. When ACh was applied in the pres-
ence of ATP, there was a positive correlation between
the peak amplitude of inward $I_{\text{ATP}}$ and the amount of
occlusion of inward $I_{\text{ACh}}$ (Fig. 4A and B). The
converse was also true when ATP was applied in the
presence of ACh (Fig. 4C and D). Non-additivity of
the $I_{\text{ACh,ATP}}$ was observed even when the inward
current was carried by NMDG$^+$ and/or Ca$^{2+}$ (Fig. 7).
Co-application of two agonists did not seem to change
the driving force for Na$^+$, because at a $V_h$ between
$E_{\text{ACh}}$ and $E_{\text{ATP}}$ an outward current due to the
activation of nAChR, followed by an inward current due
to the activation of P2XR, was observed (Fig. 6C).
Occlusion was also observed at a $V_h$ of $-10$ mV, which
is closer to $E_{\text{ACh}}$ or $E_{\text{ATP}}$. Furthermore, the removal of
external Ca$^{2+}$ did not alter the occlusion, and thus a
Ca$^{2+}$-mediated mechanism does not contribute to the
current occlusion.

The $I_{\text{ACh,ATP}}$ occlusion was observed at all nega-
tive holding potentials when the current was inward
but was not observed for outward currents at a $V_h$
of $+40$ mV. Here the outward $I_{\text{ACh,ATP}}$ was not different
from the predicted sum of $I_{\text{ACh}}$ and $I_{\text{ATP}}$ (Fig. 6B).
The same dependence on current direction has been
reported in guinea-pig enteric and submucosal neurons
(Zhou and Galligan [20]; Barajas-Lopez et al. [21]),
suggesting that the current occlusion was triggered by
the inward movement of cations through two channels.

Some investigators have shown that the amplitude
of $I_{\text{ACh,ATP}}$ is equal to or even smaller than that of
either $I_{\text{ACh}}$ or $I_{\text{ATP}}$. For instance, the concentration of
one agonist causing little or no inward current pro-
duced dramatic occlusion of the inward current gen-
erated by the other agonist (Searl et al. [19]). Khakh
et al. [22] using Xenopus oocytes co-expressing
P2X2 and αβδ nAChR channels, provided several
lines of evidence which indicated that occlusion was
mostly mediated by the inhibition of the nAChR
channel by activation of the P2XR. However, we
found that concomitant application of two agonists
always caused a larger current than either agonist
(Fig. 6A), and that the amount of occlusion of one


channel current in the presence of the other channel agonist was correlated with the amplitude of current through the other channel (Fig. 4). Furthermore, we found that $I_{\text{ACH}+\text{ATP}}$ desensitizes faster than $I_{\text{ATP}}$, but more slowly than $I_{\text{ACH}}$, and $I_{\text{ACH}+\text{ATP}}$ at a $V_h$ between $E_{\text{ACH}}$ and $E_{\text{ATP}}$ showed a combination pattern of early outward $I_{\text{ACH}}$ and delayed inward $I_{\text{ATP}}$ (Fig. 6C). These results both strongly suggest that in AP neurons, $I_{\text{ACH}+\text{ATP}}$ are carried through both nAChR and P2XR channels, and that inhibition between these channels is reciprocal.

The $I_{\text{ATP}}$ was not inhibited by co-application of ACh when the inward $I_{\text{ACH}}$ was markedly reduced in the absence of permeant cations (Fig. 8B) or at a $V_h$ very close to $E_{\text{ACH}}$ (Fig. 8D), and the inhibited $I_{\text{ATP}}$ in the presence of ACh (Fig. 5B) recovered as soon as the nAChR channel closed in the presence of dTc (Fig. 5C). These results thus suggest that the inhibitory interaction not only requires the activation of both receptor channels, but also requires a substantial current to flow through these channels. These results are in contrast to that obtained in sympathetic neurons, in which the ATP-induced glucosamine influx was inhibited in the presence of ACh (Nakazawa [30]). The cause of this inconsistency remained unknown, but may reflect a real difference in the underlying mechanisms of occlusion in these two types of neurons.

Altogether, our results have characterized the P2XR responses in AP neurons and the cross-inhibition between P2XRs and nAChRs in AP neurons. We show that the current flow through one receptor channel hinders the current flow through the other channel. These interactions support the notion that these channels are positioned very close to each other, as has been previously considered (Zhou and Galligan [20]; Barajas-Lopez et al. [21]; Khakh et al. [22]; Boue-Grabot et al. [24]) and more recently demonstrated for recombinant channels (Khakh et al. [23]). These results will be important to consider when designing ligands to modify excitability of ATP neurons and may have some physiological function during co-activation of P2XRs and nAChRs by synaptically released transmitters.

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