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SPECIAL REPORT

Full sensitivity of P_{2X_2} purinoceptor to ATP revealed by changing extracellular pH

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A full pharmacological characterization was carried out on a recombinant ATP-gated ion channel (P_{2X_2} purinoceptor) expressed in *Xenopus* oocytes. This slowly-desensitizing neuronal P_{2X_2} purinoceptor, activated by ATP ($EC_{50}=4.6\pm1$ μM at pH 7.4; $n=4$), showed the agonist potency order: ATP$\geqslant$2-MeSATP=ATPγS$\geqslant$ATPαS$>>$Bz-ATP. The receptor affinity for ATP was enhanced 5-10 fold by acidifying the bathing solution (to pH 6.5) but was diminished 4-5 fold in an alkaline solution (pH 8.0). The maximum activity of P_{2X_2} purinoceptors and the activity order of a series of nucleotides were unaltered by changing extracellular pH. Interestingly, ATP sensitivity at a recombinant P_{2Y_1} purinoceptor remained unaltered with changing extracellular pH. These results indicate that acidotic conditions in the synaptic cleft could strengthen purinergic transmission at neuronal P_{2X_2} purinoceptors.

Keywords: P_{2X} purinoceptor; recombinant receptors; PC12 cells; ATP; potentiation; pH; acidosis; *Xenopus* oocytes

Introduction Rat phaeochromocytoma PC12 cells have been used extensively as neuronal models to study fast excitatory nicotinic- and ATP-responses (Nakazawa *et al.*, 1991) and ATP-evoked vesicular release of catecholamines (Rhoads *et al.*, 1993). Recently, an ionotropic receptor gated by ATP (P_{2X_2} purinoceptor) was cloned from rat PC12 cells (Brake *et al.*, 1994). Adrenergic and cholinergic vesicles (which contain ATP) are known to possess a proton transporter which renders the vesicle lumen acidic (pH 5.5) (Johnson & Scarpa, 1976). Occasionally, ATP is involved in co-transmission with acidic transmitters (GABA and glutamate) in parts of the CNS (Edwards *et al.*, 1992) where, in rat hippocampus for example, synaptic transmission causes a transient acidic pH shift at the synapse (Krishtal *et al.*, 1987). Therefore, we decided to study ATP activation of two neuronal purinoceptors, P_{2X_2} (from rat PC12 cells) and P_{2Y_1} (from turkey brain), at different levels of extracellular pH. The results of this study show that the affinity of P_{2X_2} purinoceptors for ATP is selectively enhanced under acidic conditions, indicating that fast purinergic transmission at neuronal P_{2X} purinoceptors might be made more secure during 'synaptic acidosis' and co-release of ATP with acidic transmitters.

Methods Mature oocytes (stages V and VI) were taken from *Xenopus laevis*, defolliculated by a two-step process of collagenase treatment (Type I, 2 mg ml^{-1} in a Ca^{2+}-free Ringer solution) and mechanical stripping, and used for cytosol injections of cRNA (40 nl, 0.1-1 μg ml^{-1}) to express P_{2X_2} (Brake *et al.*, 1994) and P_{2Y_1} (Filtz *et al.*, 1994) purinoceptors. At 24-48 h post-injection, *Xenopus* oocytes were studied under voltage-clamp conditions with a twin-electrode amplifier (Axoclamp 2A). The voltage-recording and current-record microelectrodes (1-2MΩ tip resistance) were filled with 0.6 M K_2SO_4 and 3.0 M KCl, respectively. Oocytes were superfused with a salt solution (Ringer) containing (mM): NaCl, 110, KCl, 2.5, HEPES 5, $CaCl_2$ 1.8. Ringer pH was adjusted with HCl (1N) or NaOH (5N) to the levels described in the text. Inward currents (at $V_H=-30$ mV) were generated by stimulating recombinant purinoceptors with superfused nucleotides (applied for 1 min). Data were normalized to the maximum current (I_{max}) obtained with ATP at each pH level and dose-response data were transformed by the equation log (I/I-I_{max}) to yield Hill plots from which EC_{50} values were derived. The Hill coefficient (n_H) was taken from the slope of these plots. All nucleotides were dissolved in Ringer solution and pH re-adjusted (where necessary) to the appropriate level. Experiments were carried out at room temperature (18°-20°C). Data are expressed as the mean$\pm$s.e.mean, analyzed by ANOVA and compared by Student's t test using Instat V2.0 (Graphpad).

Results At pH 7.4, superfused ATP (1-100 μM) evoked fast inward cationic currents in *Xenopus* oocytes expressing P_{2X_2} purinoceptors. ATP and three related analogues (2-MeSATP, ATPαS and ATPγS) were full agonists. Their affinity constants (EC_{50} values) were: ATP, 4.6 ± 1 μM ($n=4$); 2-MeSATP, 7.1 ± 1 μM ($n=4$); ATPγS, 7.4 ± 1 μM ($n=4$); ATPαS, 13.2 ± 6.8 μM, ($n=3$). When Mg^{2+} (1 mM) was added to the superfusate, the affinity constant for ATP (25.8 ± 4.9 μM, $n=4$) was reduced significantly ($P<0.05$, by unpaired t test). The Hill coefficient (n_H) for each full agonist was 2.1 ± 0.1 (also in the presence of Mg^{2+}), indicating two molecules are required to activate this receptor. The activity order for agonists (at 30 μM, the EC_{100} for ATP) was: ATP$\geqslant$2-MeSATP=ATPγS$\geqslant$ATPαS$>>$2',3'-benzoyl benzoyl ATP (Bz-ATP)=CTP, while ADP, 2-MeSADP, ADPβS, AMP, adenosine, α,β-meATP, β,γ-meATP, UTP, ITP and GTP were inactive. The presence of extracellular Mg^{2+} (1 mM) did not alter the activity order. These data agreed with (and extended) the initial characterization (Brake *et al.*, 1994) of this cloned P_{2X_2} purinoceptor.

The amplitude of P_{2X_2} purinoceptor-mediated inward currents to a submaximal concentration of ATP (3 μM) was increased by the progressive acidification of the bathing medium, but decreased by alkalinization (Figure 1a). Full sensitivity was observed at pH 6.5; further acidification failed to increase the amplitude of ATP-responses. Neither acidification nor alkalinization altered the resting conductance of *Xenopus* oocytes, nor significantly altered the slope and reversal potential of the I-V relationship for ATP (Figure 1b). The concentration-response relationship for ATP was studied at four pH levels, 5.5, 6.5, 7.4 and 8.0 (Figure 1c). The resultant curves showed a leftwards displacement with acidification up to pH 6.5, but no difference in receptor affinity for ATP at pH 6.5 and pH 5.5. Alkalinization to pH 8.0 caused a rightward displacement of

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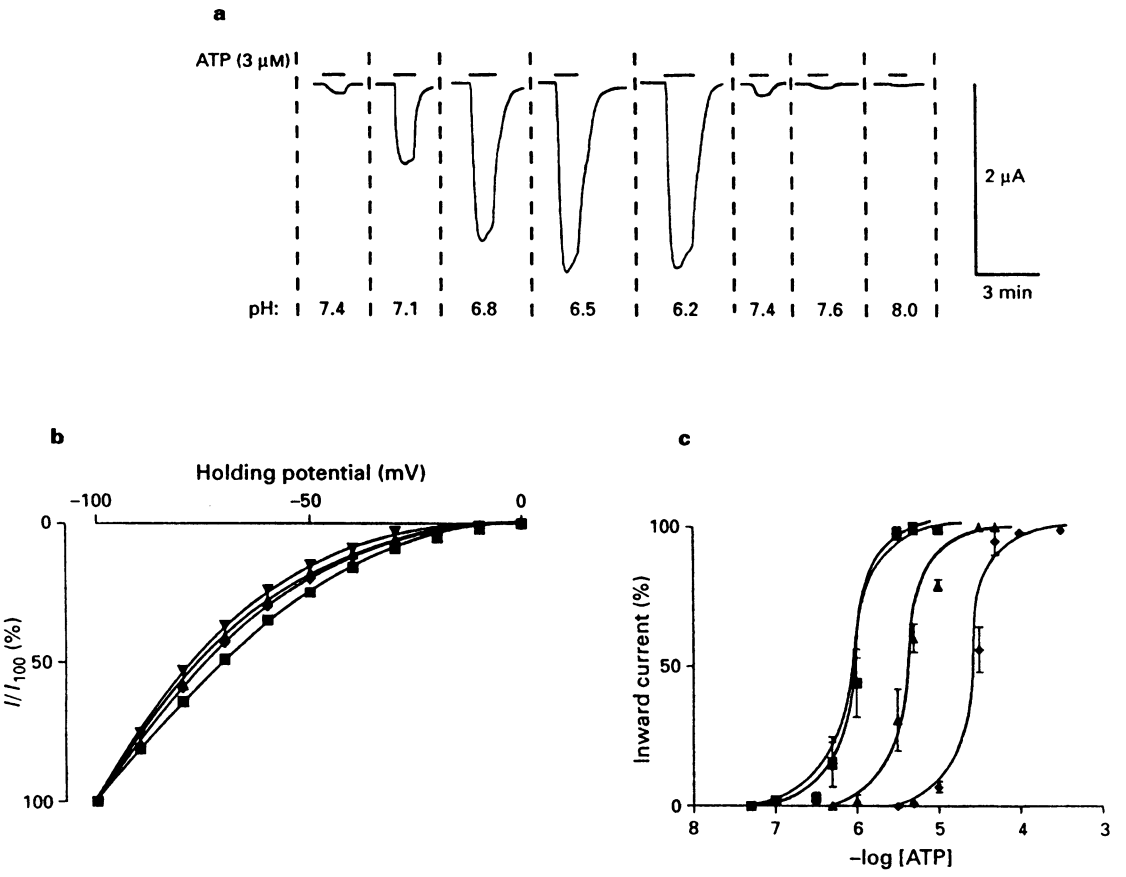

Figure 1 Effects of extracellular pH on ATP sensitivity at the P_{2X_2} purinoceptor. In (a), the activity of successive superfusions of ATP (3 μM) was increased by acidifying the bathing solution (from pH 7.4–6.2), but decreased by alkalinization (from pH 7.4–8.0) ($V_H = -30$ mV). In (b), the *I-V* relationship of the ATP-gated ion channel was not significantly altered by changing extracellular pH: (◆) pH 8.0; (▲) pH 7.4; (▼) pH 6.5; (■) pH 5.5. (Note: currents were normalized to the amplitude of currents evoked at -100 mV). In (c), the concentration-response curve for ATP (at $V_H = -30$ mV) was displaced to the left with acidic Ringer and rightwards with alkaline Ringer. Symbols as in (b). (Note: the concentration-response curves at pH 6.5 and pH 5.5 are superimposed, indicating an upper limit to the enhancement of receptor sensitivity).

the concentration-response curve. Under these conditions, EC_{50} values ($n=4$) for ATP were: pH 8.0, 28 ± 2 μM; pH 7.4, 7.3 ± 0.5 μM; pH 6.5, 1.2 ± 0.1 μM; pH 5.5, 1.1 ± 0.1 μM. The maximum activity to ATP was unaltered by changes in extracellular pH and the Hill co-efficient remained at 2. Also, the potency order of agonists at the P_{2X_2} purinoceptor was unaltered by changing extracellular pH (Table 1).

The activity of ATP was tested on a recombinant P_{2Y} purinoceptor derived from turkey brain (Filtz *et al.*, 1994); this metabotropic P_2 purinoceptor couples to phospholipase C and is identical (to within 1 residue) in sequence to another neuronal P_{2Y_1} purinoceptor cloned from chick brain. ATP (10 μM) evoked oscillatory chloride currents typical of expressed P_{2Y_1} purinoceptors in oocytes. The activity of ATP (normalized to ATP-responses at pH 7.4) was: pH 8.0, $95\pm10\%$ ($n=4$); pH 6.5, $113\pm12\%$ ($n=4$) (Figure 2). These two indices of activity were not significantly different (by paired *t* test) from the activity of P_{2Y_1} purinoceptors at pH 7.4.

Table 1 Agonist activity at the P_{2X_2} purinoceptor at varying pH levels

Agonist	*Activity*		
	pH 7.4	pH 8.0	pH 6.5
ATP	1	1	1
2-MeSATP	0.95 ± 0.09	0.95 ± 0.12	0.97 ± 0.06
ATPαS	0.85 ± 0.14	0.81 ± 0.9	0.80 ± 0.11
ATPγS	0.80 ± 0.09	0.78 ± 0.11	0.92 ± 0.06
ADP	0	0	0.02 ± 0.02
UTP	0	0	0

The activity of a series of nucleotides was tested on the P_{2X_2} purinoceptor at the three pH levels. ATP was applied at EC_{100} for each pH level and other nucleotides were given at the same concentration. The EC_{100} was taken as the lowest concentration of ATP (but not a supramaximal concentration) consistently giving a maximum response. The activity order for these agonists was unaffected by changing extracellular pH, indicating the effects of pH were not selective for any particular nucleotide. (Data: mean $\pm$ s.e. mean, $n=4$).

Discussion The ATP-gated ionotropic receptor, or P_{2X_2} purinoceptor, cloned from PC12 cells was activated fully by ATP, 2-MeSATP, ATPαS and ATPγS, but partially activated by Bz-ATP. This agonist profile accords precisely with the range of nucleotides evoking secretion via native P_2 purinoceptors in rat PC12 cells (Rhoads *et al.*, 1993), indicating that the recombinant P_{2X_2} purinoceptor retained its pharmacological identity. ATP-affinity was reduced 4–7 fold by extracellular Mg^{2+} (which did not change extracellular pH), consistent with earlier observations on the effects of Mg^{2+} and other divalent cations on single-channel conductance of native P_2 purinoceptors in rat PC12 cells (Nakazawa & Hess, 1993). Acidification of the extracellular solution increased ATP affinity at the P_{2X_2} purinoceptor 5–10 fold, while alkalinization decreased ATP affinity 4–5 fold. The affinity of the P_{2X_2} purinoceptor for ATP also can be increased 4 fold by Zn^{2+} ions

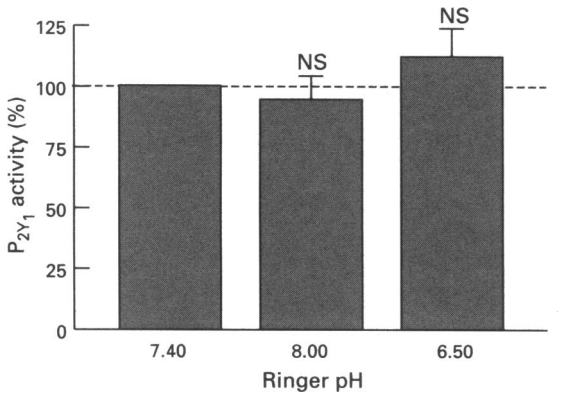

Figure 2 Effects of extracellular pH on ATP sensitivity at a P_{2Y_1} purinoceptor. Activity of ATP (10 μM) at turkey P_{2Y_1} purinoceptor at three pH levels, 6.5, 7.4 and 8.0. ATP-activity (at pH 7.4) was normalized to 100% and ATP-activity at other pH levels was calculated relative to this reference. ATP-activity was not affected by either acidification or alkalinization (NS = no significant difference by paired t test). (Data: mean ± s.e.mean, $n = 4$).

(Brake *et al.*, 1994) but the combined effects of Zn^{2+}, other divalent cations and pH remained to be tested. Although agonist affinity can be modified in a number of ways at the P_{2X_2} purinoceptor, the potency order of nucleotides, the number of molecules activating this receptor and the maximum response to receptor stimulation appeared to remain unaltered.

A comparison of recombinant P_{2X_2} and P_{2Y_1} purinoceptors revealed that changing extracellular pH levels altered the sensitivity of only the P_{2X_2} purinoceptor. This fundamental difference between ionotropic and metabotropic ATP receptors may play an important role in purinergic signalling at neurones, particularly in the CNS where ATP and acidic transmitters (GABA and glutamate) appear to be co-released. Acidification has been shown to enhance the facilitation of synaptic transmission by histamine in mouse hippocampus (Yanovsky *et al.*, 1995). Interestingly, a new ionotropic ATP-receptor (P_{2X_4} purinoceptor) has been cloned from rat hippocampus in our laboratory (Bo *et al.*, 1995), although the effects of pH (and other modulators) remain to be tested on this new P_2 purinoceptor.

It has proved difficult to demonstrate purinergic transmission in the CNS (Edwards *et al.*, 1992), although there is good evidence for high-affinity binding sites for ATP and other nucleotides throughout the neuraxis (Bo & Burnstock, 1994). Much attention has been given to the role of ecto-ATPases in the CNS in influencing the activity of neurally-released ATP. The observed acidic pH shift at the synaptic cleft (Krishtal *et al.*, 1987) may represent an additional factor if the sensitivity of ATP at ionotropic receptors is enhanced as the local concentration of acid (as amino acids and from vesicle protons) builds up during neurotransmitter release.

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