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Potentiation of ATP-responses at a recombinant P_{2X_2} receptor by neurotransmitters and related substances

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1 The modulator effects of a series of neurotransmitters and related substances were tested on responses to adenosine 5′-triphosphate (ATP) at a recombinant P_{2X_2} receptor expressed in defolliculated *Xenopus* oocytes.

2 Nicotine, 5-hydroxytryptamine (5-HT), noradrenaline, adenosine, bradykinin and histamine (all 100 μM) potentiated the responses to ATP (3 μM), an effect found due to acidification of the bathing solution by these drugs.

3 Arachidonic acid, met-enkephalin, substance P, calcitonin gene-related peptide (CGRP) (all 100 μM) and nerve growth factor (NGF; 50 ng ml^{-1}) potentiated the responses to ATP (3 μM) through a largely or wholly pH-independent effect.

4 Small acidic and alkaline shifts, as little as 0.03 pH-units, enhanced or diminished the responses to ATP, respectively. A linear relationship existed between the degree of potentiation of the ATP-induced responses caused by nicotine, 5-HT, noradrenaline, adenosine, bradykinin and histamine and the potentiation of these responses induced by the addition of acid to the superfusate.

5 Since P_{2X} receptors on sensory neurones include P_{2X_2} subunits, the attendant acidosis and ATP-release associated with tissue injury may play a role in sensitizing sensory nerve fibres.

Keywords: P_{2X} receptor; ATP; potentiation; acidosis; *Xenopus* oocytes

Introduction

An adenosine 5′-triphosphate (ATP)-gated ion-channel (P_{2X_2} receptor) has been cloned from rat PC12 cells (Brake *et al*., 1994), this receptor/channel complex representing the second of seven members of the $P_{2X_{1-7}}$ receptor family (Burnstock & King, 1996). P_{2X_2} receptor transcripts are distributed widely throughout the central and peripheral nervous systems (Collo *et al*., 1996), including sensory dorsal root ganglion (DRG) cells where P_{2X} receptors are thought to be heteromultimeric assemblies of P_{2X_2} and P_{2X_3} subunits (Lewis *et al*., 1995).

We have shown in an earlier publication (King *et al*., 1996) that the sensitivity of recombinant P_{2X_2} receptors to ATP is dependent on extracellular pH, with acidic and alkaline shifts of the superfusate displacing the concentration-response curve for ATP to the left and right respectively. In this earlier paper, it was argued that acidic shifts might enhance purine signalling under physiological conditions, e.g., during exocytosis of acidified transmitter vesicles and secretory storage granules. Interestingly, a study of a similar nature has shown that ionic currents activated by ATP via native P_{2X} receptors in rat nodose ganglion cells (which, on anatomical and functional levels, are similar to sensory DRG cells) are potentiated by acidification and suppressed by alkalinization of the bathing medium (Li *et al*., 1996).

Nakazawa and Ohno (1996) have shown a different kind of potentiation of ATP-responses at the recombinant P_{2X_2} receptor, where high concentrations (100 μM) of 5-hydroxytryptamine (5-HT), dopamine and adenosine were effective modulators. The actions of these and other substances on recombinant P_{2X_2} receptors have been re-investigated, taking into account the effect of these high concentrations of drugs on extracellular pH. It was of particular interest to us to test neurotransmitters and paracrine substances that are contained in, or found near to, sensory neurones and their primary afferent nerve fibres, since P_{2X_2} subunits help form the native P_{2X} receptor. We find that potentiation by many substances can be explained by receptor protonation, although some substances also work through another mechanism.

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Methods

Defolliculated *Xenopus* oocytes (stages V and VI) were cytosolically-injected with P_{2X_2} receptor transcript (40 nl, 0.1 μg μl^{-1}; kindly given by Dr D. Julius (UCSF)) and, 24–48 h later, P_{2X_2}-mediated currents were recorded under voltage-clamp by use of twin-electrode amplifiers (Axoclamp 2A and 2B). The voltage-recording and current-recording electrodes (1–2 MΩ tip resistance) were filled with 0.6 M K_2SO_4 and 3 M KCl, respectively. Oocytes were superfused (5 ml min^{-1}) with Ringer salt solution containing (mM): NaCl 110, KCl 2.5, HEPES 5, $CaCl_2$ 1.8, adjusted to pH 7.45 or at the levels mentioned in the text.

Inward currents (at $V_H = -90$ mV) were evoked by adding ATP (at a final concentration of 3 μM, approximate EC_{20} at pH 7.45) to the superfusate for a period of 60 s or until evoked currents reached a plateau. Data were normalized to responses to 3 μM ATP at pH 7.45. A supramaximal concentration of ATP (100 μM) was also applied to establish the maximum response (I_{max}) under control and test conditions. Modulators were added to the superfusate for a period of 2 min before and during ATP application, then both drugs were washed out for 5 min. The effect of modulators on P_{2X_2} receptor currents was tested first without adjusting pH, then retested with pH re-adjusted to 7.45. Modulators were tested on 3–4 oocytes and paired observations were made. Data in the text are expressed as means $\pm$ s.e.mean and compared by Student's t test on paired data, by use of Instat V2.0 (Graphpad).

Results

First, three submaximal responses of consistent amplitude were obtained to superfused ATP (3 μM) then each modulator (at 100 μM) tested for its ability to enhance or depress the ATP-induced responses at recombinant P_{2X_2} receptors. Modulators included: acetylcholine chloride and nicotine hydrogen tartrate; adenosine hemisulphate; γ-amino-n-butyric acid (GABA); arachidonic acid; bradykinin acetate; histamine dihydrochloride; 5-hydroxytryptamine creatinine sulphate (5-HT); noradrenaline bitartrate and dopamine hydrochloride;

met-enkephalin acetate; substance P amide; calcitonin gene-related peptide (CGRP) amide; nerve growth factor (NGF) (all from Sigma Chemicals, U.K.).

Nicotine, 5-HT, noradrenaline (NA), adenosine, bradykinin and histamine (all 100 μM) potentiated ATP-responses significantly ($P<0.05$), while acetylcholine, GABA and

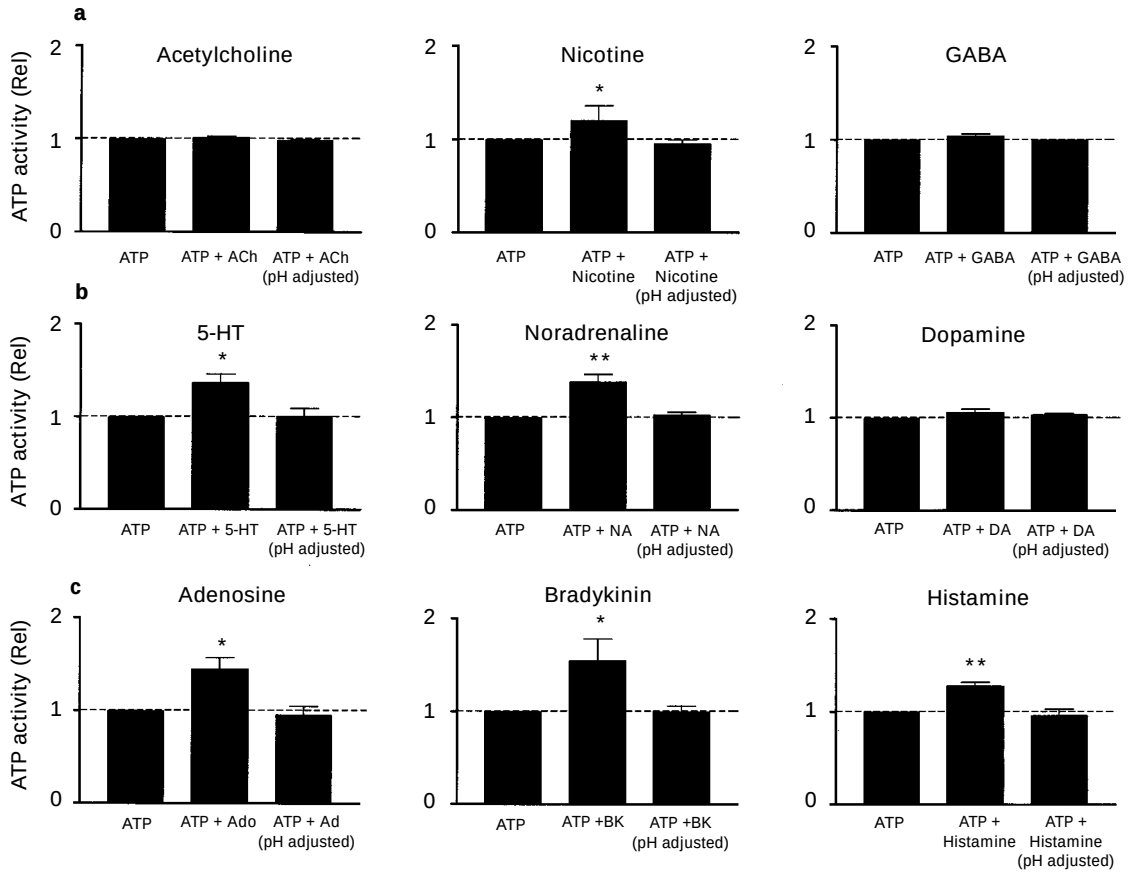

Figure 1 pH-dependent modulation of responses to ATP. A series of transmitters (a and b) and related substances (c) were tested for their ability to enhanced submaximal responses to ATP (3 μM). The first column of each histogram shows the amplitude of the response to ATP (normalized to 1) at pH 7.45, the second column shows the effect of a test substance on the amplitude of the ATP-induced responses, and the third column shows the effect of pH readjustment to 7.45 on the modulator effect of the test substances. Each column shows the mean ± s.e.mean of 3–4 observations. $^*P<0.05$, $^{**}P<0.01$, by paired t test.

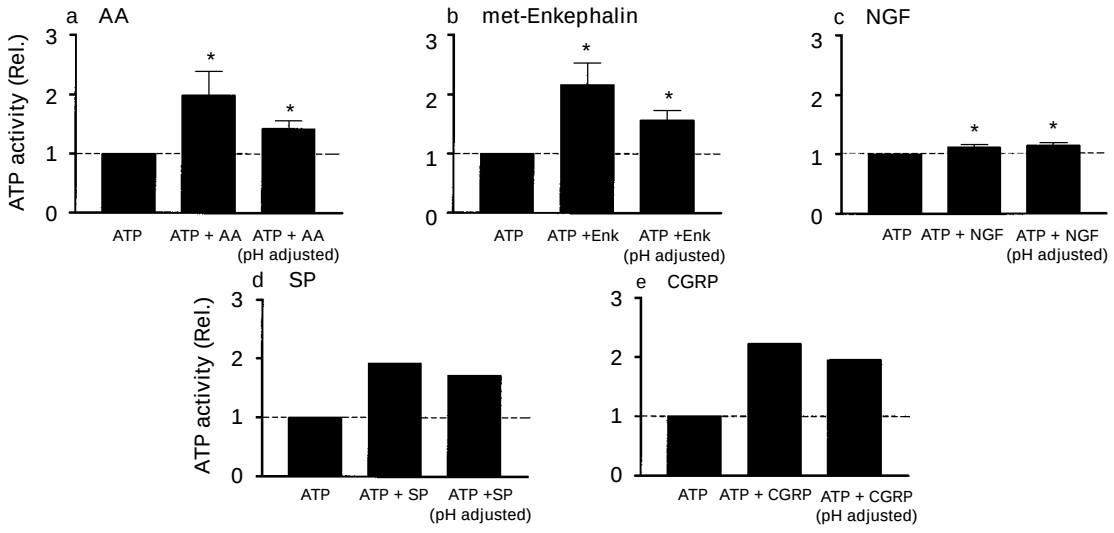

Figure 2 Another series of transmitters and paracrine substances (a, arachidonic acid (AA); b, met-enkephalin (Enk); c, nerve growth factor (NGF); d, substance P (SP); e, calcitonin gene-related peptide (CGRP)) were tested for their ability to enhanced submaximal responses to ATP (3 μM). The first column of each histogram shows the amplitude of the response to ATP (normalized to 1) at pH 7.45, the second column shows the effect of a test substance on the amplitude of the ATP-induced responses, and the third column shows the lack of effect of pH readjustment to pH 7.45 on the modulator effect of the test substance. Each column shows the mean ± s.e.mean of 3–4 observations (a–c), while data for substance P and CGRP were taken from single experiments. $^*P<0.05$, by paired t test.

dopamine had no effect (Figure 1). Of the substances potentiating the responses to ATP, nicotine was the least active (20±10% enhancement) while bradykinin was the most active (55±20% enhancement). The rank order of potency was (at 100 μM): bradykinin > adenosine > 5-HT = noradrenaline > histamine > nicotine, with acetylcholine, dopamine and GABA being inactive. On measuring the pH of the superfusate containing active modulators, each of the above potentiators caused a small acidic shift (0.03–0.06 pH-unit) in the pH level of the bathing solution. When this pH shift was corrected, all of the above potentiators failed to enhance the responses to ATP (Figure 1). Inactive substances which had failed to potentiate the responses to ATP caused little change (0–0.02 pH-units) in the pH of the superfusate. Thus, ATP (3 μM) elicited submaximal responses of consistent amplitude when the extracellular pH was tightly controlled, regardless of the presence of these neurotransmitters and related substances.

A small subset of modulators was identified which exerted their potentiating effect through a largely or wholly pH-independent effect. Arachidonic acid and met-enkephalin (100 μM) potentiated the responses to ATP significantly ($P<0.05$), although their effect was blunted slightly with pH adjustment (Figure 2). After pH adjustment, arachidonic acid and met-enkephalin increased the responses to ATP by 42±13% and 56±17%, respectively. We also tested NGF (at 50 ng ml^{-1}) which caused a modest but significant increase (12±5%) in the responses to ATP without altering extracellular pH (Figure 2). Substance P and CGRP (at 100 μM) were more potent modulators, increasing submaximal responses to ATP by 92% and 113% respectively, and exerting a similar effect after pH adjustment (Figure 2). These two peptides were tested only in one experiment, the cost of retesting peptides at a high concentration (100 μM) being prohibitive.

We questioned whether the very small pH changes caused by some false-positive potentiators could enhance the responses to ATP, in spite of knowing that pH adjustment had reversed their potentiating effects. So, the amplitude of the responses to ATP was monitored after small changes to the pH of the bathing solution had been made with hydrochloric acid (HCl, 0.1 M) and sodium hydroxide (NaOH, 0.5 M). From pH 7.45, responses to ATP were measured over a limited range of pH 7.1–7.5 (Figure 3a). Small acidic pH shifts as low as 0.03 pH-units enhanced the responses to ATP while a shift of 0.1 pH-units doubled the size of the responses to ATP. On the other hand, an alkaline shift of 0.05 pH-units reduced the responses to ATP by 50%. The change in responsiveness to ATP over small changes (±0.05 pH-units) in extracellular pH was relatively linear (Figure 3b). When the mean pH shift of false-positive potentiators was plotted against their potentiating activity, these data points followed the linear relationship between extracellular pH and ATP-response amplitude (Figure 3b).

Finally, it was confirmed that none of the pH-independent and pH-dependent potentiators altered the maximum response to ATP (100 μM). The absence of a change in the maximum response (data not shown) agreed with results an earlier study (King *et al.*, 1996) where extracellular pH was shown to alter P_{2X_2}-affinity for ATP without changing the maximum response.

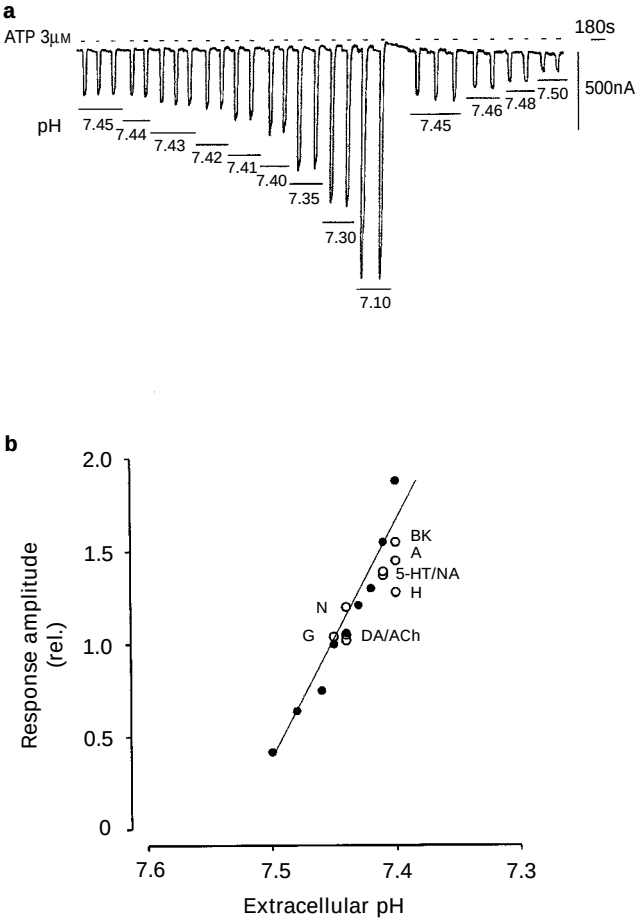

Figure 3 In (a), submaximal responses to ATP (3 μM) were enhanced by acidification (from pH 7.45 to pH 7.1) and reduced by alkalinization (pH 7.45 to pH 7.5) of the bathing medium. In (b), the relationship between the amplitude of the ATP-induced responses and extracellular pH was linear over a limited range of pH 7.5 to 7.4 (see ●). The potency of the modulator substances listed in Figure 1 appear to follow this linear relationship (see ○). Abbreviations: A, adenosine; ACh, acetylcholine; BK, bradykinin; DA, dopamine; G, GABA; H, histamine; 5-HT, 5-hydroxytryptamine; N, nicotine; NA, noradrenaline. Each point represents the mean of 3–4 measurements.

Discussion

It has been proposed that native P_{2X} receptors on DRG sensory neurones with C-fibre axons are heteromultimeric assemblies of P_{2X_2} and P_{2X_3} subunits (Lewis *et al.*, 1995). We have shown that responses to ATP at recombinant P_{2X_2} receptor are potentiated by acidic shifts in the bathing medium up to pH 6.5, while lower pH values do not potentiate the responses further (King *et al.*, 1996). However, we have not been able to demonstrate the same pH effect on responses to ATP at recombinant P_{2X_3} receptors (King: personal observation). Thus, extracellular acidic shifts may alter the ATP-affinity for P_{2X_2} subunits in P_{2X} receptors on sensory neurones, a conclusion drawing support from the results of Li *et al.* (1996) who have shown that ATP-responses at nodose ganglion sensory neurones are also potentiated by receptor protonation.

Previously, we found that nanomolar concentrations of diadenosine pentaphosphate (Ap_5A) potentiate ATP-responses at P_{2X_2} receptors (Pintor *et al.*, 1996), while others have shown that submillimolar (100 μM) concentrations of 5-HT, dopamine and adenosine also potentiate ATP-responses at P_{2X_2} receptors (Nakazawa & Ohno, 1996). We were interested in this new form of P_{2X_2} receptor modulation through the allosteric effects of neurotransmitters and related substances. In particular, it was interesting to test neurotransmitters found in sensory neurones (e.g. enkephalin, substance P and CGRP) and in dorsal spinal horn (e.g. ACh, GABA and NA), paracrine substances released (especially during injury) near nerve endings of sensory neurones (e.g. adenosine, arachidonic acid, bradykinin and histamine), and substances that modulate P_{2X} receptors at PC12 cells from which P_{2X_2} was cloned (e.g. nicotine, dopamine and 5-HT). All of these substances were tested at 100 μM since Nakazawa and Ohno (1996) found this concentration of 5-HT, dopamine and adenosine to be highly effective under similar circumstances with P_{2X_2} receptors expressed in oocytes. We also

tested NGF in the present study because it has been shown to enhance nociception by sensory nerves when administered subcutaneously (Andreev *et al*., 1995).

The results of this study show that most substances potentiated ATP-responses at the relatively high concentration of 100 μM. In many cases, the commercially-available salts of these substances generated weak acids and the resultant pH shift often was the sole mechanism for potentiation of ATP-responses at P_{2X_2} receptors. Other substances such as peptide neurotransmitters found in small C-fibre sensory neurones and an eicosanoid (arachidonic acid) which is generated during tissue injury were reasonably effective modulators of P_{2X_2} receptors but their actions were pH-independent. The concentration threshold for these modulator substances at P_{2X_2} remains to be determined and it will be interesting to see how little of these pH-independent modulators is required to cause maximal potentiation of the ATP-induced responses. To date, the modulator effect of Ap_5A ($EC_{50} = 3$ nM) remains the most notable in potentiating P_{2X_2}-affinity for ATP (Pintor *et al*., 1996).

The recombinant P_{2X_2} receptor has been shown to be exquisitely sensitive to pH and small changes of ± 0.05 units or more had significant effects on receptor affinity for ATP. Interestingly, ATP is weak acid (pH shift $= 0.06 \pm 0.02$ in Ringer solution at 100 μM) and it has been necessary to adjust the pH of stock solutions in our experiments. It is equally important to recognize that ATP and other neurotransmitters and hormones are stored under acidic conditions in synaptic vesicles and secretory storage granules (Johnson & Scarpa, 1976). Thus, exocytosis of acidified neurotransmitters and acidified paracrine substances may upregulate ATP-signalling at P_{2X} receptors on the central and peripheral nerve endings of sensory neurones. Tissue injury and inflammation often result in localized acidosis as well as release of ATP and other substances. The extracellular pH in tissues can fall markedly during ischaemia (pH 5.7), at the site of bone fracture (pH 4.7) and at sites of inflammation (pH 5.4) with attendant hyperalgesia in all cases (see: Steen *et al*., 1992). Thus, the sequelae to tissue injury may play an important role in the sensitization of primary afferent nerve terminals following damage by the combination of acidic conditions, ATP-release and release of allosteric modulators of P_{2X_2} receptors.

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