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Modulation of ATP-responses at recombinant rP2X$_4$ receptors by extracellular pH and zinc

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1 The modulatory effects of extracellular H^+ and Zn^{2+} were tested against ATP-responses at rat P2X$_4$ (rP2X$_4$) receptors expressed in *Xenopus* oocytes under voltage-clamp conditions.

2 ATP (0.1–100 μM, at pH 7.5), evoked inward currents *via* rP2X$_4$ receptors (EC_{50} value, 4.1±0.98 μM; n_H, 1.2±0.1). ATP potency was reduced 2 fold, at pH 6.5, without altering maximal activity. ATP potency was reduced by a further 4 fold, at pH 5.5, and the maximal activity of ATP was also reduced. Alkaline conditions (pH 8.0) had no effect on ATP-responses.

3 Zn^{2+} (100 nM–10 μM) potentiated ATP-responses at the rP2X$_4$ receptor by 2 fold, whereas higher concentrations (30 μM–1 mM) inhibited ATP-responses. Zn^{2+} potentiation was due to an increase in ATP potency, whereas its inhibitory action was due to a reduction in ATP efficacy.

4 Zn^{2+} modulation of ATP-responses was pH-dependent. At pH 6.5, the bell-shaped curve for Zn^{2+} was shifted to the right by 1 log unit. At pH 5.5, Zn^{2+} potentiation was abolished and its inhibitory effect reduced considerably.

5 Suramin (50 μM) also potentiated ATP-responses at rP2X$_4$ receptors. Neither H^+ (pH 6.5 and 5.5), Zn^{2+} (10–100 μM) or a combination of both failed to reveal an inhibitory action of suramin at rP2X$_4$ receptors.

6 In conclusion, H^+ and Zn^{2+} exerted opposite effects on the rP2X$_4$ receptor by lowering and raising agonist potency, respectively. H^+ ($\geqslant$3 μM) and Zn^{2+} ($\geqslant$30 μM) also reduces agonist efficacy by lowering the number of rP2X$_4$ receptors available for activation. The striking differences between the modulatory actions of H^+ and Zn^{2+} at rP2X$_4$ and rP2X$_2$ receptors are discussed.

Keywords: Extracellular pH; zinc; ATP; P2X receptor; *Xenopus* oocyte

Abbreviations: ATP, adenosine 5′-triphosphate; EC_{50}, agonist concentration producing 50% of the maximal response; I_{ATP}, ATP-activated membrane current; n_H, Hill co-efficient; pH_e, extracellular pH; PPADS, pyridoxal-α^5-phosphate-6-azophenyl-2′,4′-disulphonic acid; UTP, uridine 5′-triphosphate; V_h, holding potential

Introduction

Adenosine 5′-triphosphate (ATP) can act as a fast excitatory transmitter at neuronal P2X receptors in the central, peripheral and enteric nervous systems (Edwards *et al*., 1992; Evans *et al*., 1992; Silinsky & Gerzanich, 1993; Galligan & Bertrand, 1994; Sperlagh *et al*., 1995; Bardoni *et al*., 1997; Nieber *et al*., 1997). So far, seven P2X receptor subunits (P2X$_{1-7}$) have been identified (North & Barnard, 1997), although the recently-cloned human P2XM subunit may possibly represent the eighth member (Urano *et al*., 1997). Apart from P2X$_7$, transcripts for other P2X subunits have been localized in neuronal tissues.

The P2X$_4$ receptor subunit is concentrated in mammalian nervous systems and, along with P2X$_2$ and P2X$_6$, represent the more common P2X subunits found in adult neural tissues (Bo *et al*., 1995; Buell *et al*., 1996; Collo *et al*., 1996; Séguéla *et al*., 1996; Soto *et al*., 1996; Wang *et al*., 1996; Dhulipala *et al*., 1998; Lê *et al*., 1998). Homomeric P2X$_4$ receptors are characterized by a low sensitivity to P2 receptor antagonists, PPADS (pyridoxal-α^5-phosphate-6-azophenyl-2′,4′-disulphonic acid) and suramin. The blocking activity of PPADS and suramin is greater at human P2X$_4$ (hP2X$_4$) than the rat homologue (rP2X$_4$), yet still lower than at most other human and rat P2X receptor subtypes (Garcia-Guzman *et al*., 1997). The recombinant rP2X$_6$ receptor, however, is also insensitive to PPADS and suramin (Collo *et al*., 1996).

Previously, we have shown that the activity of agonists and antagonists at one neuronal P2X receptor subtype, rP2X$_2$, is exceedingly sensitive to changes to extracellular pH (King *et al*., 1996, 1997). The concentration-response (C/R) curve for ATP (and other agonists) was shifted leftwards under acidic conditions and rightwards under alkaline conditions, without changing the maximal activity of the agonist. Even small changes in pH ($\geqslant$0.03 pH units) significantly altered the amplitude of ATP-responses at rP2X$_2$ (Wildman *et al*., 1997). Additionally, the blocking activity of suramin was greatly enhanced at rP2X$_2$ under acidic conditions and declined under alkaline conditions (King *et al*., 1997). Extracellular zinc (Zn^{2+}) also potentiated agonist and antagonist activity at rP2X$_2$ receptors (Brake *et al*., 1994; Nakazawa & Ohno, 1996, 1997; Wildman *et al*., 1998). However, Zn^{2+} modulation of agonist activity at rP2X$_2$ receptors is more complex than the corresponding H^+ modulation, since the former shows time-dependency and converts to inhibition after prolonged exposure while the latter is constant, time-independent and can overcome Zn^{2+} inhibition (Wildman *et al*., 1998).

ATP-responses at rat and human P2X$_4$ receptors are also affected by extracellular pH (Stoop *et al*., 1997; Clarke *et al*., 1998), but in a different way to rP2X$_2$ receptors. For these P2X$_4$ homologues, acidic and alkaline conditions respectively

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reduced and enhanced ATP-responses although the precise actions on the potency and efficacy of ATP remain to be determined. It has also been reported that ATP activity is potentiated by Zn^{2+} at rat and human $P2X_4$ receptors (Séguéla *et al*., 1996; Soto *et al*., 1996; Garcia-Guzman *et al*., 1997; Nakazawa & Ohno, 1997) although there is no information on how ATP potency and efficacy is altered. Additionally, it remains to be shown if Zn^{2+}-potentiation of agonist activity is time-dependent, as for $P2X_2$, and how Zn^{2+} and H^+ interact at $P2X_4$ receptors. In the present study, therefore, we describe the separate effects of extracellular pH and Zn^{2+} and their joint interaction on both agonist and antagonist activity at a neuronal P2X receptor subunit, the $rP2X_4$ subtype. The striking differences between the modulatory effects of H^+ and Zn^{2+} at $rP2X_4$ and $rP2X_2$ receptors are discussed.

Methods

Oocyte preparation

Xenopus laevis frogs were anaesthetized in Tricaine (0.2% w/v), killed by decapitation, and ovarian lobes surgically removed. Oocytes (stages V and VI) were defolliculated by a 2-step process involving collagenase treatment (Type IA, 2 mg ml^{-1} in a Ca^{2+}-free Ringer's solution, for 2–3 h) followed by stripping away the follicular layer with fine forceps. Defolliculated oocytes were stored in Barth's solution (pH 7.5, at 4°C) containing (mM): NaCl, 110; KCl, 1; $NaHCO_3$, 2.4; Tris HCl, 7.5; $Ca(NO_3)_2$, 0.33; $CaCl_2$, 0.41; $MgSO_4$, 0.82; gentamycin sulphate, 50 $\mu g\ l^{-1}$. Defolliculated oocytes were injected cytosolically with rat $P2X_4$ cRNA (40 nl, 1 $\mu g\ ml^{-1}$), incubated for 48 h at 18°C in Barth's solution then kept at 4°C for up to 12 days until used in electrophysiological experiments.

Electrophysiology

ATP-activated membrane currents (I_{ATP}) ($V_h = -60$ to -90 mV) were recorded from cRNA-injected oocytes using a twin-electrode voltage-clamp amplifier (Axoclamp 2B). The voltage-recording and current-recording microelectrodes (1–5 MΩ tip resistance) were filled with 3.0 M KCl. Oocytes were superfused with Ringer's solution (5 ml min^{-1}, at 18°C) containing (mM): NaCl, 110; KCl, 2.5; HEPES, 5; $BaCl_2$, 1.8, adjusted to pH 7.5. Where stated, the pH of the bathing solution was adjusted using either 1.0 N HCl or 1.0 N NaOH to achieve the desired level. Electrophysiological data were stored on a computer using a MP100 WSW interface (Biopac Systems Inc.) and analysed using the software package Acknowledge III (Biopac).

Solutions

All solutions were nominally Ca^{2+}-free to avoid the activation of a Ca^{2+}-dependent Cl^- current ($I_{Cl,Ca}$) in oocytes (Bo *et al*., 1995). ATP was prepared in a Ca^{2+}-free Ringer's solution (concentrations as stated in the text) and superfused by a gravity-feed continuous flow system which allowed rapid addition and washout. ATP was added for 120 s or until the current reached a peak, then washed out for a period of 15 min. Data were normalized to the maximum current (I_{max}) evoked by ATP at pH 7.5 for agonist concentration-response (C/R) relationships studied at all pH levels. The agonist concentration required to evoke 50% of the maximum response (EC_{50}) was taken from Hill plots, constructed using the formula $\log(I/I_{max} - I)$ where I is the current evoked by each concentration of ATP. High concentrations of ATP (300 μM–3 mM) can activate an inward Na^+-current (I_{Na}) in a small proportion of defolliculated oocytes and this current is inhibited by UTP (300 μM) (Kupitz & Atlas, 1993). To avoid such endogenous currents, UTP (300 μM) was added to the superfusate in experiments (mainly at pH 5.5) where it was necessary to use high concentrations of ATP (>300 μM). UTP (300 μM) had no effect on ATP potency at $rP2X_4$ receptors at pH 7.5 (EC_{50} values: 3.4 ± 1.0 μM *vs* 4.0 ± 1.3 μM, paired data, $n = 3$).

The effects of extracellular zinc were investigated on agonist activity in two ways. First, Zn^{2+} was added to ATP solutions and C/R curves for ATP were constructed (data normalized to the maximal ATP-response at pH 7.5). Pre-incubation with Zn^{2+} for 15 min prior to adding ATP solutions had the same effect on agonist responses as did the simultaneous application of Zn^{2+} and ATP. Second, C/R curves for Zn^{2+} were constructed using a submaximal concentration of ATP (EC_{20}) (data normalized to responses to the respective EC_{20} concentration for ATP at pH 8.0, 7.5, 6.5 and 5.5).

Statistics

Data are presented as means ± s.e.mean of four sets of data from different oocyte batches. Significant differences were determined by either unpaired Student's *t*-test or one-way analysis of variance (ANOVA) followed by Dunnett's test, using commercially available software (Instat v2.05a, GraphPad).

Drugs

All common salts and reagents were AnalaR grade (Aldrich Chemicals, U.K.). Adenosine 5′-triphosphate disodium salt (ATP), uridine 5′-triphosphate sodium salt (UTP) and zinc chloride were purchased from Sigma Chemical Co. (Poole, Dorset, U.K.). Suramin was a gift from Bayer plc (Newbury, Berkshire, U.K.).

Results

Effect of extracellular pH on I_{ATP}

At pH 7.5, ATP (100 nM–100 μM) evoked inward membrane currents in defolliculated oocytes expressing $rP2X_4$ receptors (EC_{50} value, 4.1 ± 0.98 μM; Hill co-efficient (n_H), 1.2 ± 0.1, $n = 4$). At pH 8.0, there was no significant change in ATP potency or maximal activity (EC_{50} value, 2.8 ± 0.6 μM; n_H, 1.1 ± 0.1; $n = 4$) (see Figure 1). Acidification of the superfusate significantly reduced ATP potency ($P < 0.01$) (pH 6.5: EC_{50} value, 8.4 ± 1.2 μM; n_H, 1.0 ± 0.1, $n = 5$; pH 5.5: EC_{50} value, 31.7 ± 4.9 μM; n_H, 1.0 ± 0.1, $n = 6$). The efficacy of ATP was diminished only at pH 5.5 (37 ± 4% of maximal ATP-responses at pH 7.5) (see Figure 1). The modulatory effects of H^+ (at either pH 6.5 or 5.5) were reversed after readjusting the superfusate to pH 7.5. Water-injected (control) defolliculated oocytes failed to respond to ATP (100 μM).

Zn^{2+} potentiation of I_{ATP}

Zn^{2+} (0.1–10 μM) potentiated membrane currents to ATP (3 μM) at $rP2X_4$ receptors by approximately 2 fold (EC_{50} value,

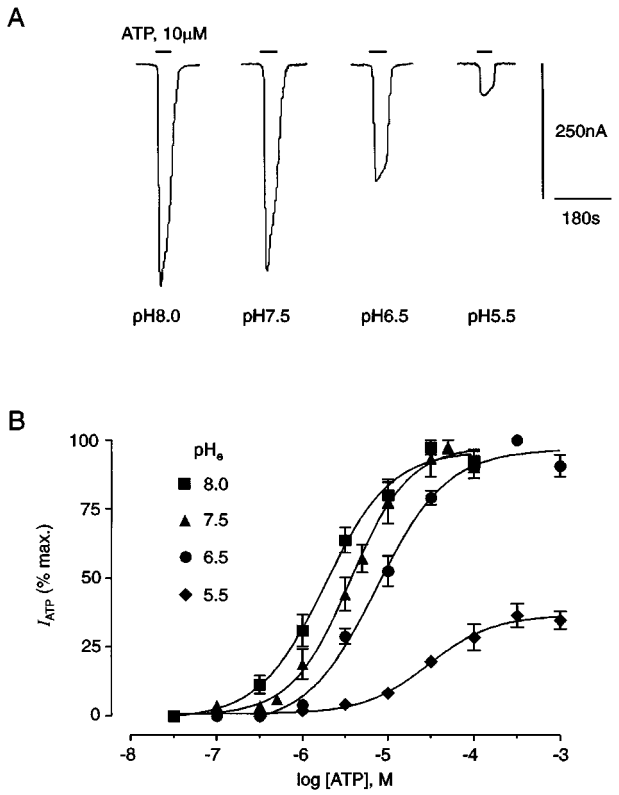

Figure 1 Extracellular pH modulates ATP activity at $rP2X_4$ receptor. (A) Whole-cell currents activated by ATP (10 μM) at four levels of extracellular pH (pH_e) (8.0, 7.5, 6.5, 5.5). All records from the same oocyte ($V_h = -90$ mV). (B) Concentration/response (C/R) curves for ATP (30 nM – 1 mM) at the same four levels of pH_e. Whole-cell currents to ATP (I_{ATP}) were normalized to the maximal ATP-response at pH 7.5. Data points are means ± s.e.mean, $n = 4$.

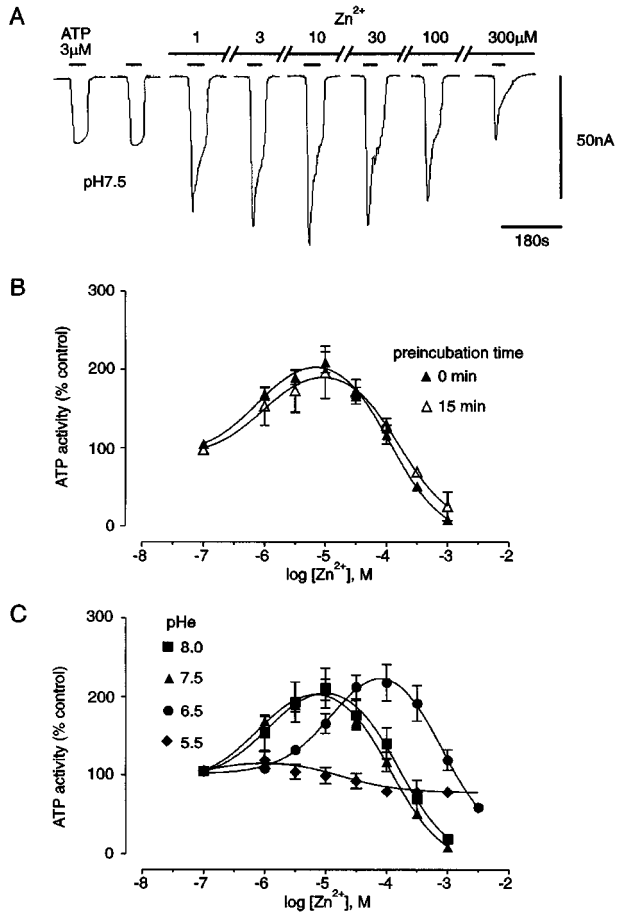

Figure 2 Zn^{2+} modulates ATP activity at $rP2X_4$ receptor. (A) Whole-cell currents to ATP (3 μM) and modulation of agonist activity by Zn^{2+} (1 – 300 μM) added to the superfusate (pH_e, 7.5). All records from the same oocyte ($V_h = -90$ mV). (B) Concentration/response (C/R) curves for Zn^{2+} modulation of whole cell currents at $rP2X_4$ ion-channel activated by three micromolar ATP (at pH 7.5). Zn^{2+} was added to the superfusate and applied either simultaneously with ATP or 15 min prior to, and during, application of ATP. (C) Effect of extracellular pH (pH_e) on the modulatory actions of Zn^{2+} on ATP-responses at $rP2X_4$ receptor. ATP was applied at a concentration equivalent to the EC_{20} value at four levels pH_e (8.0, 7.5, 6.5, 5.5). Data points are means ± s.e.mean, $n = 4$.

1.29 ± 0.2 μM, $n = 4$) (Figure 2A and B). This potentiating effect was not sustained at higher concentrations (30 μM – 1 mM), at which point Zn^{2+} caused an inhibition of ATP-responses (Figure 2A and B). Where Zn^{2+} (0.1 μM – 1 mM) was applied 15 min prior to the addition of ATP, the concentration-dependent potentiating and inhibitory activities of Zn^{2+} remained unaltered (Figure 2B). The potentiating and inhibitory effects of Zn^{2+} were reversed after washout.

Zn^{2+} modulation of ATP-responses was affected by acidifying the extracellular solution. While alkaline conditions (pH 8.0) had no significant effect on Zn^{2+} modulation of ATP-responses, acidification (pH 6.5) displaced the bell-shaped Zn^{2+} curve to the right by 1 log unit without diminishing the extent of Zn^{2+} potentiation (Figure 2C). At pH 5.5, Zn^{2+} failed to potentiate ATP-responses and the inhibitory action of Zn^{2+} was also reduced (Figure 2C).

Effect of Zn^{2+} on concentration dependence of I_{ATP}

The effects of Zn^{2+} on the potency and efficacy of ATP at $rP2X_4$ was studied in detail over a range of pH 7.5 – 5.5. Zn^{2+} was applied at two concentrations at each pH level, the first Zn^{2+} concentration giving maximal potentiation of ATP-responses (pH 7.5, 10 μM; pH 6.5, 100 μM; pH 5.5, 10 μM) and a second concentration causing a significant inhibition of ATP-responses (pH 7.5, 100 μM; pH 6.5, 1000 μM; pH 5.5, 100 μM).

At pH 7.5, the potency of ATP (10 nM – 100 μM) was increased significantly ($P < 0.01$) in the presence of Zn^{2+} (10 μM) (EC_{50} values, 1.0 ± 0.3 μM *vs* 4.1 ± 0.98 μM, $n = 4$). ATP potency was not significantly different at a higher level of Zn^{2+} (100 μM) (EC_{50} value, 1.3 ± 0.3 μM, $n = 4$), but ATP efficacy (i.e., maximal activity) was reduced considerably (59 ± 7% of control) (Figure 3A). Hill co-efficients for ATP curves were similar in the absence and presence of Zn^{2+} (n_H: 0 μM, 1.2 ± 0.1; 10 μM, 1.0 ± 0.1; 100 μM, 1.0 ± 0.1).

Similar effects were seen at pH 6.5. ATP potency was increased significantly ($P < 0.01$) in the presence of Zn^{2+} (100 μM) (EC_{50} values: 1.9 ± 0.5 μM *vs* 8.4 ± 1.2 μM, $n = 4$), whereas ATP potency was not enhanced further by a higher concentration of Zn^{2+} (1000 μM) (EC_{50} 2.1 ± 1.3 μM, $n = 4$) although agonist efficacy was reduced considerably (43 ± 5% of control) (Figure 3B). Hill co-efficients for ATP curves were similar in the absence and presence of Zn^{2+} (n_H: 0 μM, 1.0 ± 0.1; 100 μM, 1.0 ± 0.1; 1000 μM, 0.9 ± 0.1).

At pH 5.5 (and 300 μM UTP present: see Methods), there was no significant change in ATP potency in the presence of Zn^{2+} (10 and 100 μM) (EC_{50} values: 0 μM, 31.7 ± 4.9 μM; 10 μM, 32.6 ± 9.2 μM; 100 μM, 24.9 ± 3.9 μM, $n = 5$). The efficacy of ATP, although reduced considerably at pH 5.5,

was decreased further by Zn^{2+} (peak activity *wrt* maximal ATP activity at pH 7.5: 0 μM, 37$\pm$4%; 10 μM, 27$\pm$4%; 100 μM, 17$\pm$2%) (Figure 3C). Hill co-efficients for ATP curves were similar in the absence and presence of Zn^{2+} (n_H: 0 μM, 1.0$\pm$0.1; 100 μM, 0.8$\pm$0.2; 1 μM, 0.9$\pm$0.1).

Effect of H^+ and Zn^{2+} on suramin blockade

The P2 receptor antagonist, suramin (50 μM), failed to inhibit ATP-responses at $rP2X_4$ receptors at pH 7.5. Instead, suramin caused a modest potentiation of ATP-activated inward currents (Figure 4A). The extent of this potentiation (149$\pm$11%, n=3) was not significantly altered at pH 6.5 (148$\pm$4%, n=3) and pH 5.5 (143$\pm$13%, n=3). In the presence of Zn^{2+} (10 μM) which, of itself, potentiated ATP-responses, suramin (50 μM) failed either to potentiate further or inhibit ATP-activated currents (Figure 4B). This apparent Zn^{2+} antagonism of suramin activity was observed at pH 7.5 and 6.5, but not at 5.5. At this lowest pH level, Zn^{2+} failed to potentiate ATP-responses and also failed to reduce suramin potentiation of ATP-responses. These results suggest that potentiating actions of Zn^{2+} and suramin are not additive at pH 7.5 and pH 6.5. Also, suramin potentiation may involve a mechanism different from Zn^{2+} potentiation, since only the former can occur at pH 5.5.

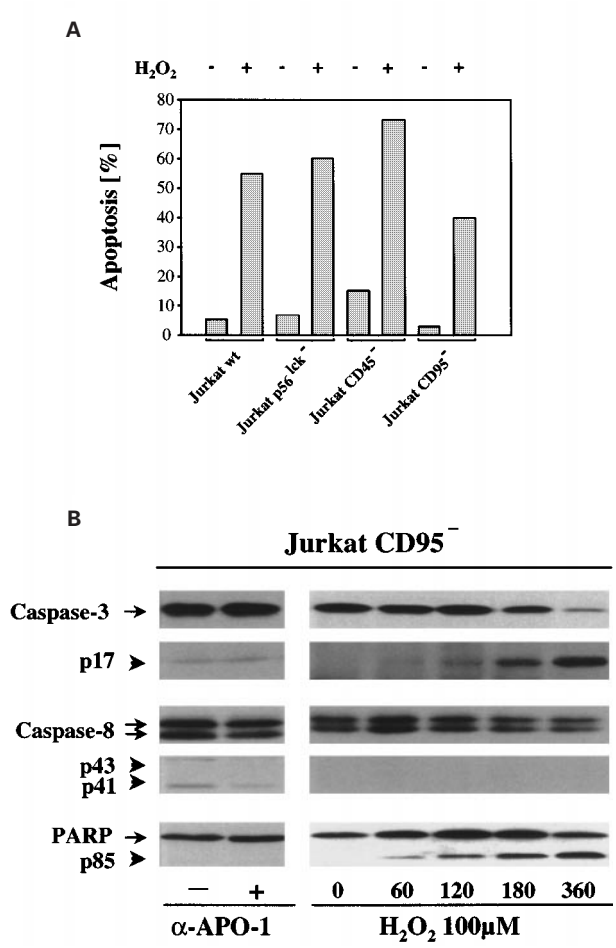

Figure 3 Interaction of H^+ and Zn^{2+} on ATP activity at $rP2X_4$ receptor. Concentration/response (C/R) curves for ATP at three levels of extracellular pH (in A, pH 7.5; in B, pH 6.5; in C, pH 5.5), in the absence then presence of concentrations of Zn^{2+} ions that caused potentiation and inhibition of ATP-responses. Zn^{2+} potentiation was caused by an increase in ATP potency, displacing C/R curves to the left. Zn^{2+} inhibition was due to a decrease in ATP efficacy, without altering agonist potency. Data points are means$\pm$s.e.mean, n=4.

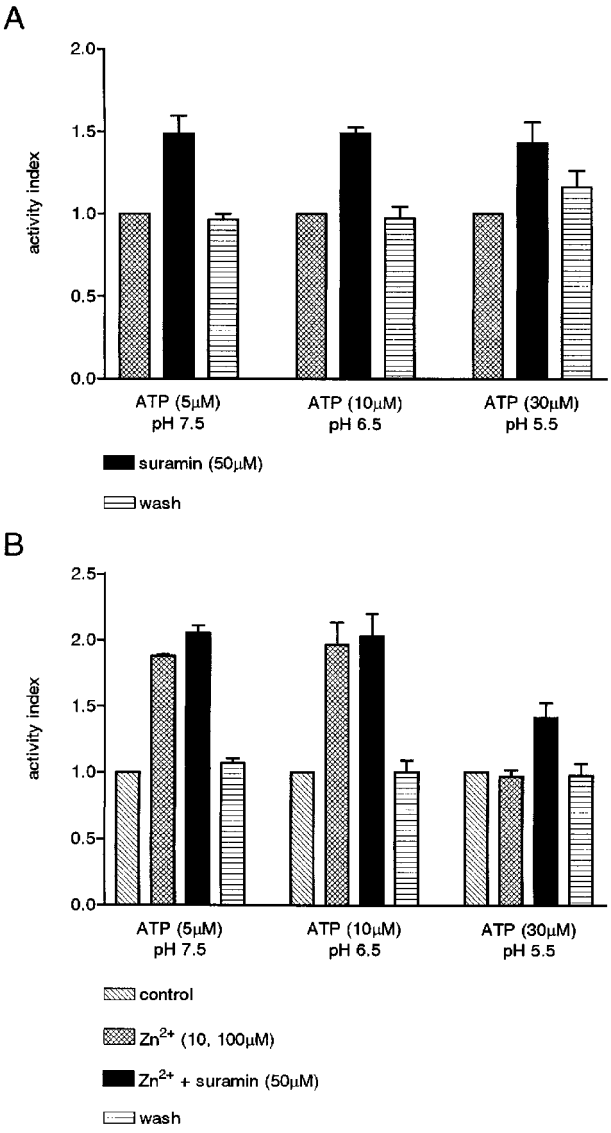

Figure 4 Suramin activity at $rP2X_4$ receptor. (A) Histograms of ATP activity in the absence and presence of suramin (50 μM), and 20 min after washout of suramin. The agonist was applied at the respective EC_{50} value at pH 7.5, 6.5 and 5.5, and control responses were taken as 1 (activity index=1). (B) Histograms of ATP activity in the absence and presence of Zn^{2+} ions, further addition of suramin (50 μM), and 20 min after washout of suramin and Zn^{2+} ions. Zn^{2+} was applied at 10 μM (pH 7.5 and 5.5) and 100 μM (pH 6.5), these concentrations causing maximal potentiation of ATP-responses at the above pH levels.

Discussion

In the present study, ATP-activated inward currents at the $rP2X_4$ receptor were found to be sensitive to changes in extracellular pH. Acidification of the bathing medium progressively shifted the ATP C/R curve to the right and decreased agonist potency by as much as 8 fold, whereas alkaline changes shifted the C/R curve marginally to the left. Similar phenomena were observed for $rP2X_4$ receptors expressed in either HEK293 cells (Stoop *et al*., 1997) or *Xenopus* oocytes (Clarke *et al*., 1998). We have extended these observations by calculating EC_{50} values and showing that H^+ (at pH 5.5) also reduces the efficacy of ATP. At pH 5.5, H^+ appears to decrease the number of $rP2X_4$ channels available for agonist activation, in a manner comparable to a non-competitive antagonist, although the effects of H^+ were reversed by washout. Can levels as low as pH 5.5 be reached *in vivo*? Localized acidosis has been reported following bone fracture (pH 4.7), during ischaemia (pH 5.7), inflammation (pH 5.4), during epileptic seizures and injuries related to CNS degenerative changes (DeSalles *et al*., 1987; Chesler, 1990; Steen *et al*., 1992; Ransom & Philbin, 1992). It is also evident that acidic shifts occur transiently during CNS neurotransmission (Yanovsky *et al*., 1995).

The above findings at $rP2X_4$ receptors differed radically from the effects of H^+ at $rP2X_2$ receptors (King *et al*., 1996, 1997), where acidic changes to the bathing solution enhanced ATP potency without affecting the efficacy of the agonist. It was further noted that $rP2X_2$ receptors were more sensitive than $rP2X_4$ receptors to small changes in extracellular pH, as confirmed by Stoop *et al*., (1997). At both $rP2X_4$ and $rP2X_2$ receptors, however, the change in amplitude of ATP-activated currents was immediate when changing extracellular pH, did not alter with time and was reversed immediately on washout. The speed with which H^+ exerts its action on these two P2X subtypes suggests H^+ ions act at extracellular site, but there is little structural information to implicate specific (and strategic) amino acid residues. It appears that histidine residues in the extracellular loop of the $rP2X_2$ subunits can be discounted (Stoop *et al*., 1997; King *et al*., 1997).

The software programme, Bound and Determined (BAD), calculates the fractional ratios of ATP species for a given amount of ATP (Brookes & Storey, 1992). We have used this programme beforehand, when studying H^+ modulation of ATP activity at $P2X_2$ receptors, to try to determine the ATP species most likely to activate the $rP2X_2$ subunit (King *et al*., 1996). We have repeated such analysis for $rP2X_4$, comparing EC_{50} values at the four pH_e levels tested against the calculated fractional ratios of ATP species present (data not shown). The fractional amount of free ATP (ATP^{4-}) remained constant (about 30%) for the respective EC_{50} values for ATP over the range of pH 8.0–6.5, but fell sharply (to 8%) at pH 5.5. Thus, it is unlikely that ATP^{4-} alone stimulated the $rP2X_4$ receptor. Of the other ATP species present, the fractional amounts of HATP and BaHATP increased while NaATP, KATP and BaATP decreased with progressive acidification of the superfusate. Such changes in the relative amounts of ATP species could not explain the observed changes in ATP potency over the range of pH 8.0–5.5. As concluded in an earlier paper on $rP2X_2$ receptors (King *et al*., 1996), it is more likely that receptor protonation rather than agonist protonation accounts for the change in ATP potency at $rP2X_4$ receptors.

Other investigators have already shown that extracellular Zn^{2+} can potentiate ATP-responses at rat and human $P2X_4$ receptors (Séguéla *et al*., 1996; Soto *et al*., 1996; Garcia-Guzman *et al*., 1997; Nakazawa & Ohno 1997). Here, we demonstrated the concentration-dependence of this effect and also confirmed that actions of Zn^{2+} were reversed on washout. Additionally, we found that high concentrations of Zn^{2+} can exert an inhibitory effect on ATP activity. A bell-shaped C/R relationship also has been observed for the actions of Zn^{2+} on ATP-activated currents in rat sympathetic neurons (Cloues *et al*., 1993), at which $rP2X_2$ and $rP2X_4$ transcripts have been localized (Collo *et al*., 1996). From studying the effects of both potentiating and inhibitory concentrations of Zn^{2+} on the C/R curve for ATP, it appears that inhibition by Zn^{2+} was due to decrease in agonist efficacy and not a decrease in ATP potency. Thus, high concentrations of Zn^{2+} can reduce the number of $rP2X_4$ receptors available for agonist activation in a manner comparable to a non-competitive antagonist. Since Zn^{2+} further reduced the efficacy of ATP at pH 5.5, it appears that the ability of Zn^{2+} and H^+ to reduce the number of available $rP2X_4$ receptors were additive. The locus for the inhibitory actions of Zn^{2+} and H^+ has not been determined. However, these inhibitory actions raise an interesting issue. Since Zn^{2+} and H^+ are found in synaptic vesicles (Johnson & Scarpa, 1976; Assaf & Chung, 1984) and probably are released along with ATP during central neurotransmission, these modulators at the right concentrations might exert a physiological antagonism of $rP2X_4$ receptors which, otherwise, are insensitive to known P2 receptor antagonists.

Extracellular Zn^{2+} acted differently at $rP2X_4$ and $rP2X_2$ receptors. Both the potentiating and inhibitory actions of Zn^{2+} at $rP2X_4$ were dependent on concentration and independent of time, while the potentiating effect of Zn^{2+} at $rP2X_2$ is dependent on time and, irrespective of concentration, eventually replaced by inhibition (Wildman *et al*., 1998). Additionally, the maximal Zn^{2+} potentiation of ATP-responses is markedly less pronounced at $rP2X_4$ (2 fold) than $rP2X_2$ receptors (15 fold) at pH 7.5 (see Figure 5), and the respective EC_{50} values for Zn^{2+} are somewhat dissimilar ($rP2X_4$, 1.29 ± 0.2 μM; $rP2X_2$, 6.1 ± 1.2 μM). Furthermore, the time-dependent inhibitory actions of Zn^{2+} at $rP2X_2$ involve a reduction in ATP potency and efficacy, whereas inhibition at

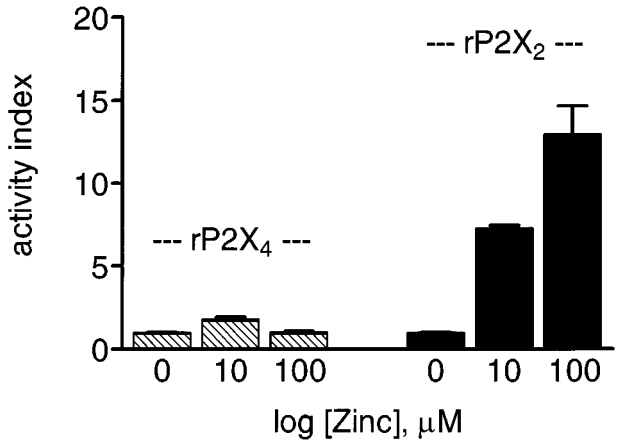

Figure 5 Comparison of Zn^{2+} modulation on ATP-responses at $rP2X_4$ and $rP2X_2$ receptors. Potentiating effects of Zn^{2+} (10 and 100 μM) on the ATP activity at $rP2X_4$ receptor and $P2X_2$ receptor, at pH 7.5. ATP was applied at a concentration just above threshold to give $\sim$5% of the maximal response at each P2X subtype, and these control responses were taken as 1 (activity index = 1). Zn^{2+} had a more profound effect on ATP-responses at $rP2X_2$ receptors at which Zn^{2+} maximally potentiated ATP-responses were increased 15 fold. In contrast, Zn^{2+} maximally potentiated ATP-responses at $rP2X_4$ receptors by 2 fold only.

$rP2X_4$ involves a reduction in ATP efficacy alone. These distinguishing features for Zn^{2+} modulation at $rP2X_2$ and $rP2X_4$ receptors might be useful criteria to determine the presence of either $P2X_2$ or $P2X_4$ subunits in native P2X receptors in neurons in the CNS and periphery. In the same vein, H^+ potentiation of ATP-responses at native P2X receptors is now viewed as signatory for the presence of $P2X_2$ subunits (Stoop *et al*., 1997).

In agreement with earlier reports (Bo *et al*., 1995; Buell *et al*., 1996; Collo *et al*., 1996; Séguéla *et al*., 1996; Soto *et al*., 1996; Wang *et al*., 1996), suramin failed to inhibit ATP-responses at $rP2X_4$ receptors. We attempted to uncover an inhibitory action by suramin by altering pH or adding Zn^{2+}, or using both, on the basis that the blocking activity of suramin is greatly enhanced at $rP2X_2$ at pH 5.5 (King *et al*., 1997) and in the presence of Zn^{2+} (Wildman *et al*., 1998). However, there was no evidence for an inhibitory action by suramin at $rP2X_4$ under such modified conditions. In point of fact, H^+ and Zn^{2+} appeared to be better inhibitors of ATP-responses at $rP2X_4$ than any of the known P2 receptor antagonists.

In conclusion, extracellular pH and Zn^{2+} affect only agonist activity and not antagonist activity at $rP2X_4$ receptors. H^+ and Zn^{2+} exert opposing actions by decreasing and increasing agonist potency, yet both share a common feature of also lowering the efficacy of ATP. These actions are in sharp contrast to the effects of H^+ and Zn^{2+} on agonist and antagonist activity at $rP2X_2$ receptors. Although both $rP2X_4$ and $rP2X_2$ transcripts are found throughout central and peripheral nervous system, their differing activity profiles with H^+ and Zn^{2+} suggest that P2X signalling can be altered in an opposite manner by these modulators. It has been reported for the enteric nervous system that P2X receptors show either a $P2X_2$-like phenotype (Zhou & Galligan, 1996) or a $P2X_4$-like phenotype (Barajas-Lopez *et al*., 1996), although neither H^+ nor Zn^{2+} have been tested on ATP-responses in the ENS. If similar phenotypic subsets of endogenous P2X receptors occur elsewhere throughout the CNS and PNS, the modulatory properties of H^+ and Zn^{2+} may have significant and selective actions on P2X signalling at discrete nuclei and ganglia, and provide the means to amplify or inhibit such signalling.

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