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Opposite Effects of Zinc on Human and Rat $P2X_2$ Receptors

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$P2X_2$ receptors from rats show potentiation when a submaximal concentration of ATP is combined with zinc in the range of 10–100 μM. Alignment of the amino acid sequences of human $P2X_2$ ($hP2X_2$) and rat $P2X_2$ ($rP2X_2$) indicated that only one of two histidines essential for zinc potentiation in $rP2X_2$ is present at the homologous position in $hP2X_2$ (H132), with the position homologous to rat H213 instead having an arginine (R225). When expressed in *Xenopus* oocytes, mouse $P2X_{2a}$ and $P2X_{2b}$ receptors showed zinc potentiation indistinguishable from rat $P2X_{2a}$, but $hP2X_{2b}$ receptors were inhibited by zinc. The extent of zinc inhibition of $hP2X_{2b}$ varied with the ATP concentration, with an IC_{50} of 8.4 μM zinc when ATP was applied at 10% of maximal and 87 μM zinc when ATP was applied at 99% of maximal. Site-directed mutagenesis showed that none of the nine histidines in the extracellular domain of $hP2X_{2b}$ were required for zinc inhibition, although inhibition was attenuated in the H204A and H209A mutations. Mutating R225 to a cysteine was sufficient to confer zinc potentiation onto $hP2X_{2b}$, and zinc potentiation was absent in the $hP2X_{2b}$H132A/R225C double mutant. This suggests that zinc potentiation in the mutant $hP2X_{2b}$ uses the same mechanism as zinc potentiation in wild-type $rP2X_{2a}$. Because of the species-specific modulation by zinc, evidence for an *in vivo* role of $P2X_2$ receptors based on studies conducted on genetically modified mice needs to be viewed with caution when extrapolations are made to the function of the human nervous system.

Key words: **mice; ATP; purinergic receptor; zinc; receptor modulation; mouse $P2X_2$**

Introduction

P2X receptors mediate fast neurotransmission by ATP in the nervous system (Burnstock, 2007). The $P2X_2$ subunit is distributed widely throughout the CNS and PNS, as well as in many non-neuronal cell types (Gever et al., 2006). $P2X_2$ subunits of mice have been shown to play a role in pain transmission (Cockayne et al., 2005), mediation of ventilatory responses to hypoxia (Rong et al., 2003), and peristalsis of the small intestine (Ren et al., 2003). Additionally, the $P2X_{2/3}$ heteromeric receptor is likely involved in regulation of urinary bladder reflexes (Cockayne et al., 2005), pain transmission (Cockayne et al., 2005), and taste sensation (Finger et al., 2005).

When expressed in heterologous cell types, rat $P2X_2$ receptors ($rP2X_2$) have a biphasic response to the modulator zinc. In the presence of low ATP, zinc concentrations below 100 μM potentiate current and zinc concentrations higher than 100 μM inhibit current (Nakazawa and Ohno, 1996; Nakazawa et al., 1997; Wildman et al., 1998; Clyne et al., 2002b; Lorca et al., 2005). Both effects of zinc are likely to be through direct interaction with the channel (Clyne et al., 2002b).

Studies of the effects of zinc on native cells likely to be expressing $P2X_2$ receptors have different results depending on the species (Zhong et al., 2001). Experiments on sensory neurons from rats and mice (Li et al., 1993; Dunn et al., 2001) showed potentiation of ATP-elicited current when extracellular zinc was applied. However, experiments on guinea pigs and bullfrogs showed inhibition of current with application of zinc (Li et al., 1997; Zhong et al., 2001; Kanjhan et al., 2003).

It has been shown that two histidines in the extracellular domain of $rP2X_2$ (H120 and H213) are required for zinc potentiation of the receptor and likely directly coordinate zinc (Clyne et al., 2002b; Nagaya et al., 2005). Both histidines are also present in mouse $P2X_2$ and may therefore be responsible for zinc potentiation of ATP-evoked current in native mouse receptors (Dunn et al., 2001). However, neither histidine is present in the analogous positions in guinea pig or *Xenopus tropicalis* $P2X_2$. This might explain the presence of only inhibition by zinc (rather than potentiation at low doses and inhibition at high doses of zinc) in native guinea pig and frog P2X neurons.

In human $P2X_2$ receptors ($hP2X_2$), there is a histidine at the position homologous to rat H120, but $hP2X_2$ has an arginine at the position homologous to rat H213. For this reason, it was important to know whether $hP2X_2$ would potentiate to zinc. We therefore tested wild-type $hP2X_2$ as well as a number of site-directed mutants of rat and human $P2X_2$ for responses to zinc as well as acidic pH (King et al., 1997; Wildman et al., 1997; Clyne et al., 2002b) and negative membrane potential (Zhou and Hume, 1998; Ding and Sachs, 1999), which are also modulators of $rP2X_2$ activity. We also tested the effect of zinc on the $P2X_{2a}$ and $P2X_{2b}$ splice variants of mouse $P2X_2$.

Materials and Methods

The general methods for expressing and characterizing P2X receptors in *Xenopus* oocytes and for inhibiting cysteine mutants with methanethiosulfonate reagents have been described by Nagaya et al. (2005). Addi-

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tional experimental protocols specific to the experiments of the current study are described here.

Expression of $P2X_2$ receptors in Xenopus *oocytes.* All studies on $rP2X_2$ used constructs derived from the cDNA originally isolated by Brake et al. (1994), which was later shown to represent the $rP2X_{2a}$ splice variant. We purchased $hP2X_{2b}$ cDNA in the Gateway entrance vector pENTR 221 (Invitrogen) and subcloned it into the Gateway destination vector pDEST17 using the LR Clonase II enzyme kit (Invitrogen). We obtained cDNAs encoding mouse $P2X_{2A}$ and $P2X_{2B}$ from Taka-aki Koshimizu (Kyoto University, Kyoto, Japan).

We made site-directed mutant receptors as described previously (Tittle et al., 2007). Constructs were expressed by injecting RNA or by injecting plasmids with T7 RNA polymerase recognition sequences upstream of the coding region for $P2X_2$ together with T7 polymerase (Tokmakov et al., 2006). The concentration of RNA or DNA injected for each construct was chosen so that the peak currents in response to saturating ATP would be expected to be −2 to −20 μA at −50 mV. This was typically substantially less nucleic acid than necessary to produce maximal expression. For this reason, the peak amplitude of currents cannot be compared between different experiments.

Preparation of zinc containing solutions. The standard recording solutions used were as described previously (Tittle et al., 2007). For zinc concentrations in the micromolar range, we added the appropriate amount of $ZnCl_2$ from a concentrated stock solution. However, because zinc is often present as a contaminant in the salts used to make physiological solutions, to test the responses of $hP2X_2$ in the nanomolar range, we used mixtures of tricine and zinc to set the zinc concentration (Paoletti et al., 1997). We added 10 mM tricine to our standard recording solution containing 0, 0.26 μM, 2.6 μM, 26 μM, 254 μM, and 5 mM zinc for a final approximate concentration of 0 nM, 1 nM, 10 nM, 100 nM, 1000 nM, and 20 μM, respectively.

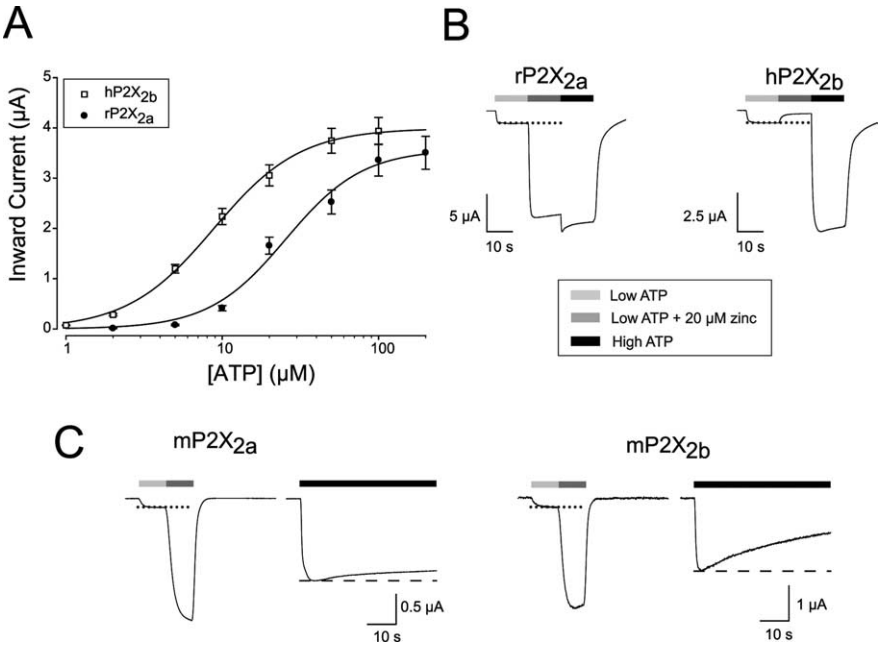

Figure 1. Effect of ATP and zinc on human and rat $P2X_2$ receptors. ***A***, ATP concentration–response relationships for human and rat $P2X_2$. Response of oocytes expressing wild-type $rP2X_{2a}$ or wild-type $hP2X_{2b}$ to increasing concentrations of ATP. Data are the average ± SEM for six oocytes expressing each construct. ***B***, Response of oocytes expressing wild-type $rP2X_{2a}$ receptors and wild-type $hP2X_{2b}$ receptors to ATP in the presence and absence of 20 μM zinc. In each trace, the first bar (light gray) indicates the time that a low concentration of ATP (approximately the EC_{10}) was applied, the second bar (dark gray) indicates the time the same low concentration of ATP but with 20 μM zinc was applied, and the third bar (black) indicates the time that a solution containing a high concentration of ATP that elicited the maximal response was applied. The difference between the amplitude of the current in the presence of zinc and the dotted line extending from the "Low ATP" portion of the trace indicates the amplitude of the zinc modulation, which was quantified as a zinc effect ratio as described in Materials and Methods. For these two traces, the zinc effect ratios were 7.2 and −0.7, respectively. The concentrations of ATP used were as follows: $rP2X_{2a}$: low, 5 μM; high, 500 μM; $hP2X_{2b}$: low, 2.5 μM; high, 500 μM. ***C***, Response of oocytes expressing wild-type $mP2X_{2a}$ receptors and wild-type $mP2X_{2b}$ receptors to ATP in the presence and absence of 20 μM zinc. The bars above the traces and the dotted lines have the same meaning as in ***B***. The dashed lines below the "High ATP" traces provide reference points to help visualize the difference in the extent of desensitization of these two splice variants. In these traces, the "High ATP" application was given after a period of incubation in recording solution alone rather than immediately after the "Low ATP plus 20 μM zinc" application to avoid measurement errors resulting from the different rates of desensitization in $mP2X_{2a}$ and $mP2X_{2b}$. For both constructs, low ATP was 20 μM, and high ATP was 500 μM. Based on the average concentration–response relationships, at this relatively low level of expression, 500 μM was ~88% of the maximal possible response of both $mP2X_{2a}$ and $P2X_{2b}$. Because no difference was found between the a and b splice variants of the mouse $P2X_2$ receptor, in the rest of the figures the constructs are simply labeled $rP2X_2$ or $hP2X_2$.

Quantitative analysis of zinc modulation and pH modulation. In our previous work on zinc potentiation in $rP2X_2$ (Tittle et al., 2007; Friday and Hume, 2008), the effect was quantified by calculating a zinc potentiation ratio. In the current study, in which both potentiation and inhibition were observed, we refer to the result calculated using this same equation as the zinc effect ratio. The equation used was as follows:

$$\text{zinc effect ratio} = \frac{\text{Current to low ATP + zinc}}{\text{Current to low ATP}} - 1.$$

When the low ATP concentration was at the EC_{10}, the maximum possible value for the zinc effect ratio was 9.0, and, for receptors with complete inhibition by zinc, the minimum possible value for the zinc effect ratio was −1.0. A receptor with neither potentiation nor inhibition would give a ratio of 0.0.

Modulation by protons was quantified in a manner similar to zinc modulation by calculating a pH effect ratio using the following equation:

$$\text{pH effect ratio} = \frac{\text{Current to low ATP at pH 6.5}}{\text{Current to low ATP at pH 7.5}} - 1.$$

To ensure that we were studying the response to zinc or pH at a low ATP concentration near the EC_{10} for ATP, we also applied a concentration of ATP to each oocyte, which elicited a maximal response, and compared the response to low ATP alone. We rejected any trials for which the measured EC value was above or below 5 and 15% of maximum. Furthermore, for each mutant, the average EC value across each group of oocytes was between 8 and 12% of maximum.

Significance between experimental and control groups of oocytes was calculated using the two tailed, unpaired *t* test function of Excel (Microsoft), with significance taken to be $p < 0.01$.

Determination of EC_{50} and IC_{50} values. To calculate ATP concentration–response relationships, oocytes were tested with an ascending series of ATP concentrations. Current amplitudes from each oocyte were then fit to the three-parameter Hill equation:

$$\text{Response} = \frac{\text{Maximal response}^* \ [\text{ATP}]^{\text{Hill Coefficient}}}{[\text{ATP}]^{\text{Hill Coefficient}} + \text{EC}_{50}{}^{\text{Hill Coefficient}}},$$

using the curve fitting routine of SigmaPlot 8.0. The data reported in text for the EC_{50} and Hill coefficients are the average of these data ± SEM. For graphing concentration–response relationships, the response of each oocyte was typically normalized to a maximum value of 100% based on the curve fit, and then the average of the normalized amplitudes was plotted ± SEM.

To calculate zinc concentration–response relationships for zinc inhibition, oocytes were tested first with ATP alone, then with an ascending series of zinc solutions mixed with the same concentration of ATP, and

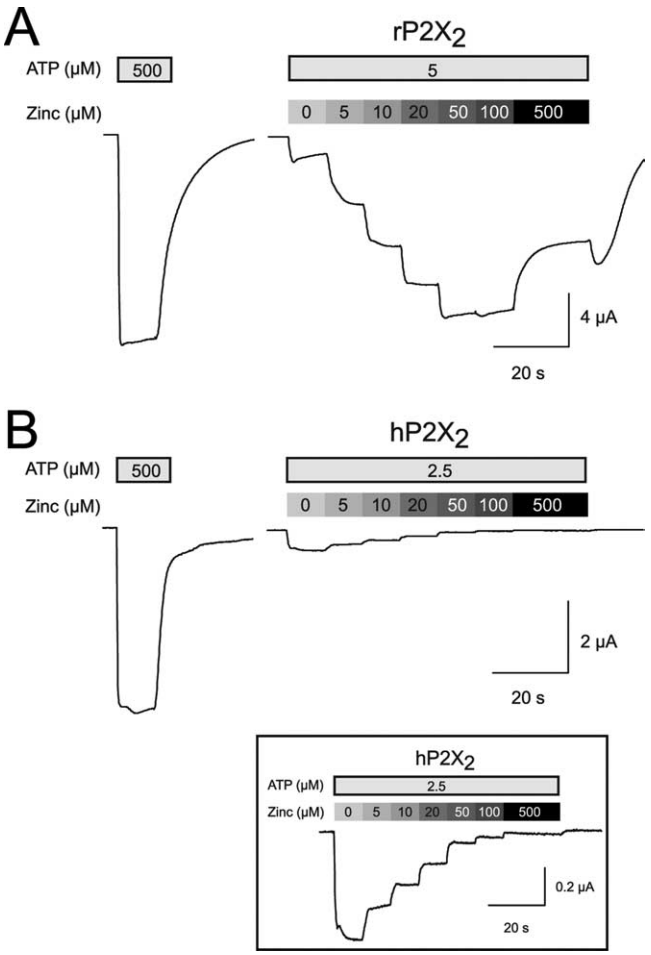

Figure 2. $hP2X_2$ was inhibited by zinc at all concentrations tested. Zinc concentration–response relationships for oocytes expressing wild-type $rP2X_2$ or wild-type $hP2X_2$. ***A***, ***B***, Representative responses to ascending concentrations of zinc in the continuous presence of ATP at approximately the EC_{10}. The response of each oocyte to a maximal concentration of ATP is shown to the left. The boxed trace in ***B*** shows the same zinc concentration–response relationship for wild-type $hP2X_2$ as in the unboxed trace but on a 10-fold larger current scale.

then with maximal ATP (to verify where on the concentration–response relationship the initial application of ATP actually was). In most experiments, the initial ATP concentration was chosen to be near EC_{10}, but, in some experiments, ATP at the EC_{50} or EC_{99} was used. Current amplitudes from each oocyte were then fit to the four-parameter Hill equation:

$$\text{Response} = \text{Response with no zinc} + \frac{(\text{Response with saturating zinc} - \text{Response with no zinc}) * [\text{zinc}]^{\text{Hill Coefficient}}}{[\text{zinc}]^{\text{Hill Coefficient}} + \text{IC}_{50}{}^{\text{Hill Coefficient}}},$$

using the curve fitting routine of SigmaPlot 8.0. For experiments using EC_{99} ATP, we observed desensitization during the protocol. To account for this, each oocyte was also exposed to high ATP alone in the absence of zinc for a duration equal to the length of the original protocol. This control high ATP protocol was applied either before or after the high ATP and ascending zinc protocol, and desensitization observed in the control trace was assumed to be equivalent to that in the zinc concentration–response protocol and added before fitting the curve. For graphing concentration–response relationships, the response of each oocyte was normalized to a maximum value of 100% based on the amplitude of the response to the initial concentration of ATP without zinc, and then the average of the normalized amplitudes was plotted ± SEM.

Determination of voltage dependence of $P2X_2$ gating. Three paradigms were used to assess the voltage dependence of channel gating. The first approach used voltage steps, the second approach used voltage ramps, and the third approach assessed the concentration–response relationship at five different membrane potentials. In all approaches, the currents in the absence of ATP were subtracted from the currents in the presence of ATP to obtain the ATP activated current in isolation.

In the voltage step and voltage ramp protocols, the flow of ATP was initiated long enough before the onset of the voltage change so that it had reached steady state at the initial holding potential. Furthermore, because both rectification to voltage ramps and the time-dependent relaxations of current to voltage steps varied with the concentration of ATP, for each experiment the ATP concentration is specified.

In the voltage step protocol, membrane potential was stepped from −50 to −100 mV. Because of the large capacitance of the oocytes and the need to turn on the integrator of the Turbotec-03 to maintain clamp of the large ATP-activated currents, the voltage-clamp steps required a few milliseconds to reach their final value. We therefore ignored any time-dependent changes in the first 10 ms of the current response (which were as likely to reflect the settling of the clamp as any change in conductance). We fit a single-exponential function to any time-dependent currents visible after 10 ms.

In the voltage ramp protocol, we applied slowly rising ramps lasting 2 s (typically 0.8 mV/ms beginning at −100 mV) to produce a quasi-steady-state current–voltage relationship. This approach allowed us to rapidly determine the reversal potential (which was defined as the most negative potential at which no inward current was detected) and the amount of inward rectification (which was defined as the ratio of the magnitude of current 50 mV positive and 50 mV negative to the reversal potential). Thus, a strongly inwardly rectifying channel would have a rectification index of near 0, and a cell that did not rectify would have a rectification index of 1.

Results

Zinc inhibition of $hP2X_2$

To study modulation of $P2X_2$ receptors, it is essential that one be at a known point on the ATP concentration–response relationship. We thus began our study of $hP2X_2$ by comparing the ATP concentration–response relationships of $rP2X_2$ and $hP2X_2$ expressed in *Xenopus* oocytes voltage clamped at −50 mV (Fig. 1*A*). Because the EC_{50} of $rP2X_2$ can vary from <10 to >50 μM depending on the level of expression (Clyne et al., 2003; Fujiwara and Kubo, 2004), we injected concentrations of RNA that produced similar peak currents in oocytes expressing $rP2X_{2a}$ and $hP2X_{2b}$ (rat, 3.6 ± 0.3 μA; human, 3.9 ± 0.2 μA; $n = 6$ for each). Under these conditions, the Hill coefficients were nearly the same (rat, 1.8 ± 0.1; human, 1.6 ± 0.1). However, the EC_{50} for $hP2X_{2b}$ was substantially lower than that for $rP2X_{2a}$ (rat, 25.7 ± 1.0 μM; human, 9.0 ± 0.2 μM).

Zinc produces a biphasic modulatory response of ATP-elicited current in $rP2X_2$ receptors (Wildman et al., 1998; Clyne et al., 2002b). In a previous study, we determined that 20 μM zinc was the highest concentration of zinc that produced potentiation of current without apparent inhibition of current (Clyne et al., 2002b). This concentration of zinc is also in line with current estimates of physiological zinc concentrations at synapses after vesicular release (Frederickson et al., 2005). Therefore, in our initial tests of $hP2X_2$, we applied a low concentration of ATP near the expected EC_{10}, followed by the same concentration of ATP together with 20 μM zinc, followed by a high concentration of ATP alone to verify that the low concentration of ATP tested on that oocyte was close to the EC_{10}. We found that 20 μM zinc, which elicited robust potentiation of current in $rP2X_{2a}$ (zinc effect ratio, 7.5 ± 0.2; $n = 6$), inhibited current in $hP2X_{2b}$ (zinc effect ratio, −0.7 ± 0.01; $n = 12$) (Fig. 1*B*).

Human cDNAs encoding $P2X_2$ were cloned in 1999 from pancreas and pituitary RNA (Lynch et al., 1999). This initial cloning reported that four different splice variants of $hP2X_2$ mRNA ($P2X_{2a}$–$P2X_{2d}$) are expressed and subsequent analysis has re-

vealed two additional splice variants ($P2X_{2b}$, $P2X_{2i}$), with only the "a" and "b" isoforms producing functional channels. The $hP2X_{2b}$ protein is 67 amino acids shorter than $hP2X_{2a}$ because of alternative splicing that removes 201 bp, with the deleted region corresponding to the middle of the intracellular C-terminal domain. Unlike $rP2X_{2a}$ and $rP2X_{2b}$, which have very different desensitization kinetics (Brandle et al., 1997; Simon et al., 1997), $hP2X_{2a}$ and $hP2X_{2b}$ are virtually indistinguishable in desensitization rate (Lynch et al., 1999). The two human receptors also respond similarly to ATP agonists and antagonists (Lynch et al., 1999). We had ready access only to the rat $P2X_{2a}$ isoform and the human $P2X_{2b}$ isoform, but we were kindly given plasmids encoding both isoforms of mouse $P2X_2$ (Koshimizu et al., 2006). This allowed us to test whether the difference between the C-terminal intracellular tails of the a and b isoforms had any effect on zinc modulation. As reported previously (Koshimizu et al., 2006), these isoforms differed in the extent of desensitization to high ATP (Fig. 1*C*). After 60 s of 500 μM ATP, the currents from oocytes expressing mouse $P2X_{2a}$ ($mP2X_{2a}$) were still nearly as large as the peak response (86 ± 2%; $n = 6$), whereas the currents from oocytes expressing $mP2X_{2b}$ had fallen to less than half of their peak amplitude (48 ± 2%; $n = 4$). Both the $mP2X_{2a}$ and $mP2X_{2b}$ isoforms showed robust potentiation to 20 μM zinc (Fig. 1*C*) when ATP was applied near the EC_{10} (for $mP2X_{2a}$, the average concentration of low ATP tested was 8.4% of maximal ± 0.7%, N = 10 and for $mP2X_{2b}$ the average concentration of low ATP tested was 8.1% of maximal ± 1.0%; $n = 8$). The average zinc effect ratios were 12.7 ± 1.0 ($n = 10$) for $mP2X_{2a}$ and 11.0 ± 0.6 ($n = 8$) for $mP2X_{2b}$, which did not differ from each other, nor from $rP2X_{2a}$ studied under similar conditions. These results were not surprising, because we were adding zinc to the outside of the cell and the extracellular domains of the $mP2X_{2a}$ and $mP2X_{2b}$ are identical. Furthermore, the extracellular domain of $rP2X_2$ differs from $mP2X_2$ only at six amino acids, none of which are near the sites identified in the rat as essential for zinc potentiation (see Fig. 10). Because of this evidence that zinc responsiveness was independent of whether the a or b splice variant was present, for the rest of this paper we will refer to $rP2X_{2a}$ and $hP2X_{2b}$ simply as $rP2X_2$ and $hP2X_2$.

Exposure of human and rat $P2X_2$ receptors to a range of zinc concentrations (Fig. 2) confirmed that the lack of zinc potentiation in $hP2X_2$ was not a consequence of the specific zinc concentration selected for the initial studies. In addition to the concentrations illustrated, we also tested much lower concentrations of zinc on $hP2X_2$ in the presence of tricine (see Materials and Methods), because some neurotransmitter-gated ion channels respond to zinc in the nanomolar range (Laube et al., 1995; Paoletti et al., 1997). We found no evidence of a potentiating effect of zinc on $hP2X_2$ at any concentration of zinc tested.

We compared the properties of wild-type $hP2X_2$ with mutant versions of $rP2X_2$ that lacked the ability to respond to zinc with potentiation. It had been shown previously (Clyne et al., 2002b) that the $rP2X_2$ mutant receptor H213A has no potentiating response to zinc, and we replicated this result (zinc effect ratio of −0.1 ± 0.03; $n = 6$). However, the inhibitory effect of 20 μM zinc on the $rP2X_2H213A$ mutant was extremely small when compared with that of $hP2X_2$ because the IC_{50} of this $rP2X_2$ mutant is over 100 μM (Clyne et al., 2002b). To determine whether the arginine in $hP2X_2$ at the analogous position to H213 of the rat receptor was responsible for the enhanced zinc inhibition, we made the $rP2X_2$ mutant receptor H213R and tested its response to 20 μM zinc. The response of $rP2X_2H213R$ to zinc (zinc effect ratio, −0.1 ± 0.01; $n = 6$) was not significantly different from that of $rP2X_2H213A$ (Fig. 3*A*). The IC_{50} for zinc at EC_{10} concentrations of ATP for rH213R was 125.4 μM zinc ($n = 5$), whereas the IC_{50} for zinc at EC_{10} concentrations of ATP for $hP2X_2$ was 8.4 μM zinc ($n = 6$) (Fig. 3*B*).

We also tested the concentration–response relationship for zinc of $hP2X_2$ at ATP concentrations equal to the EC_{50} and EC_{99} for individual oocytes (Fig. 3*C*). With greater concentrations of ATP, the IC_{50} for zinc increased (zinc IC_{50} at EC_{50} ATP, 21.9 μM zinc, $n = 6$; zinc IC_{50} at EC_{99} ATP, 86.6 μM zinc; $n = 6$). Although at the EC_{10} for ATP the current was eventually predicted to decrease to near 0 as the concentration of zinc was increased (minimum current prediction, 3.6% of maximum), the predicted minimum current in the presence of EC_{50} ATP was 5.4% of maximum, and the predicted minimum current in the presence of EC_{99} ATP was 29.3% of maximum. The shift in the response to zinc as the ATP concentration was increased indicated that the mechanism of inhibition is likely to be allosteric rather than by a noncompetitive mechanism, such as channel blocking. A second

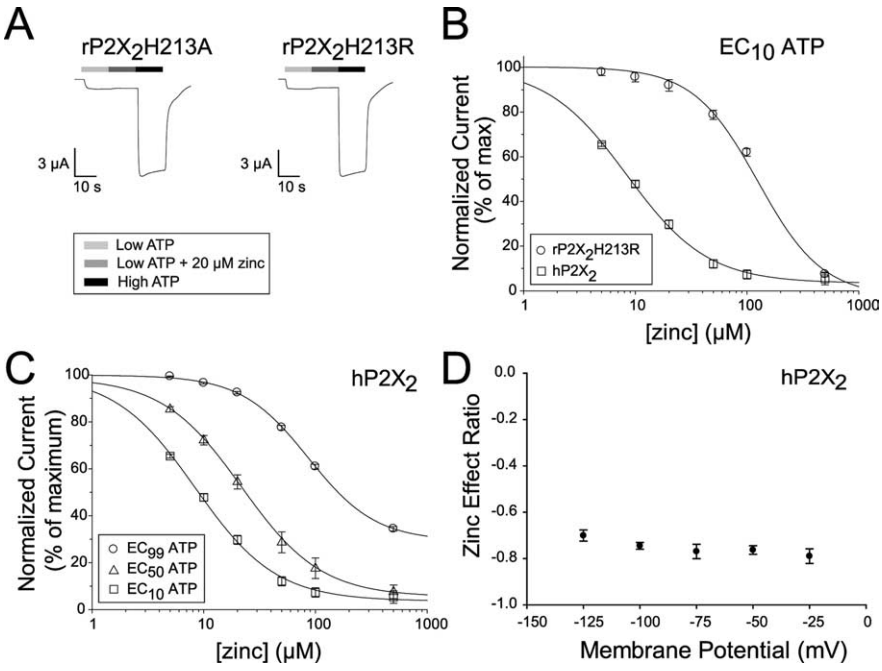

Figure 3. Concentration dependence of zinc inhibition in $rP2X_2$ and $hP2X_2$. ***A***, Response of oocytes expressing $rP2X_2H213A$ and $rP2X_2H213R$. The concentrations of ATP used were as follows: $rP2X_2H213A$: low, 10 μM; high, 1000 μM; $rP2X_2H213R$: low, 6 μM; high, 500 μM. ***B***, The zinc concentration–response relationship for oocytes expressing $rP2X_2H213R$ and wild-type $hP2X_2$ receptors based on experiments like those shown in Figure 2. The ATP concentration used was approximately the EC_{10} for each oocyte tested. For $rP2X_2H213R$, the ATP concentration used was 4.5 μM, whereas for wild-type $hP2X_2$ the ATP concentration used was 2.5 μM. ***C***, The zinc concentration–response relationship for oocytes expressing wild-type $hP2X_2$ at different concentrations of ATP. The ATP concentration used was approximately the EC_{10}, EC_{50}, or EC_{99} for each oocyte tested. The average concentrations of ATP applied to oocytes were as follows: EC_{10}, 2.5 μM; EC_{50}, 7.6 μM; EC_{99}, 500 μM. ***D***, Lack of effect of membrane potential on zinc inhibition of $hP2X_2$. Data are the average ± SEM for five oocytes tested with 20 μM zinc at the EC_{10} for ATP.

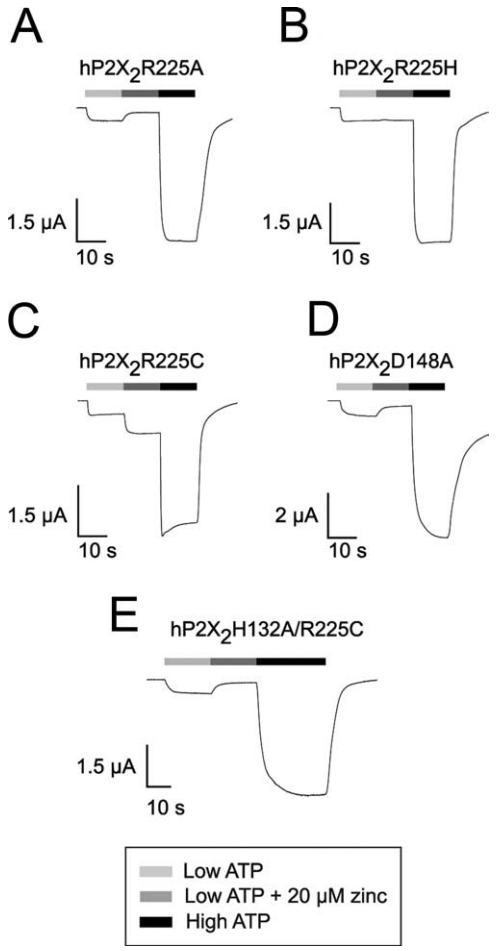

Figure 4. Responses of $hP2X_2$ mutants at sites required for zinc potentiation in $rP2X_2$. The low concentrations of ATP used were as follows: $hP2X_2$R225A, 5 μM; $hP2X_2$R225H, 4 μM; $hP2X_2$R225C, 5.2 μM; $hP2X_2$D148A, 1.5 μM; $hP2X_2$H132A/R225C, 10 μM. In all panels, the concentration of high ATP was 500 μM.

line of evidence that the inhibitory action of zinc was not attributable to channel block was that the inhibition was independent of membrane potential (Fig. 3*D*).

Role of candidate amino acids in zinc responses of $hP2X_2$

In $rP2X_2$, histidines at both 120 and 213 are necessary for zinc potentiation (Clyne et al., 2002b). Might the residue at either of these positions in $hP2X_2$ play a role in zinc inhibition? When R225 (the residue at the equivalent position to rat H213) was mutated to alanine to produce the mutant $hP2X_2$R225A (Fig. 4*A*), zinc inhibition was no different from wild-type $hP2X_2$ (zinc effect ratio, -0.6 ± 0.01; $n = 6$). However, when R225 was mutated to histidine, which mimics the residue present in the $rP2X_2$, the $hP2X_2$R225H mutant showed significantly less inhibition than in wild-type $hP2X_2$ (zinc effect ratio, -0.2 ± 0.02; $n = 6$) (Fig. 4*B*). Although a zinc effect ratio below 0 is not considered potentiation as we have defined it, this result suggested that this receptor might have zinc binding to an $rP2X_2$-like potentiating zinc site that is being overwhelmed by the inhibitory action of zinc at another site. We therefore made the mutant human receptor $hP2X_2$R225C because cysteines are also common amino acids for zinc coordination (Auld, 2001). For this mutant, there was clear zinc potentiation (zinc effect ratio, 1.3 ± 0.01; $n = 6$) (Fig. 4*C*). Another mutant we tested for zinc potentiation was $hP2X_2$D148A (Fig. 4*D*). The homologous aspartate in $rP2X_2$ (D136) has been suggested to be a candidate for coordination of zinc in the potentiating binding site (Friday and Hume, 2008). However, its mutation to alanine did not affect the zinc effect ratio in $hP2X_2$ (zinc effect ratio, -0.7 ± 0.03; $n = 5$). We also tested the double-mutant $hP2X_2$H132A/R225C. The rationale for this experiment was that, if replacing R225 with a cysteine created a potentiating zinc binding site like that of $rP2X_2$, then mutating H132 to alanine should abolish the potentiation (because the homologous residue, rat H120, is essential for zinc binding at the potentiating site). Indeed, this mutant showed no sign of potentiation, with a zinc effect ratio like wild-type $hP2X_2$ (zinc effect ratio, -0.7 ± 0.1; $n = 4$) (Fig. 4*E*).

The most common residues at zinc binding sites are histidines and cysteines (Auld, 2001). Because it seems likely that all of the extracellular cysteines are linked in a network of disulfide bonds (Clyne et al., 2002a; Ennion and Evans, 2002), we set out to explore the possible role of the nine extracellular histidines of $hP2X_2$ by mutating them one at a time to alanines. None were essential for zinc inhibition (Fig. 5*A*), but two mutants ($hP2X_2$H204A and $hP2X_2$H209A) had zinc effect ratios that indicated that 20 μM zinc produced significantly less inhibition in these mutants than in wild-type $hP2X_2$ (Fig. 5*B*). Compared with the value of ~8 μM zinc for wild-type $hP2X_2$, the zinc concentration–response curve in the presence of EC_{10} ATP was shifted to the right for both mutants (IC_{50} of 20.3 μM zinc for $hP2X_2$H204A; IC_{50} of 30.3 μM zinc for $hP2X_2$H209A; $n = 6$ for each). The double-mutant $hP2X_2$H204A/H209A also showed a decrease in zinc inhibition, but the magnitude of the change was no greater than for the more affected of the two single mutants (zinc effect ratio, -0.43 ± 0.03; $n = 5$)

To test whether H204 and/or H209 are required for normal zinc inhibition because they contribute to zinc binding (rather than altering receptor structure in a way that indirectly affects zinc modulation), we made cysteine mutants at each of these positions. The rationale for these experiments was that a cysteine can often substitute for a histidine in binding zinc but that, if this is the case, when a bulky thiol reactive reagent is then bound to the cysteine, zinc should no longer be able to bind. Zinc inhibition of both $hP2X_2$H204C and $hP2X_2$H209C was attenuated by 2-(trimethylammonio)ethyl-methanethiosufonate (MTSET) (3 min of 100 μM, which was a saturating application), but substantial zinc inhibition remained after treatment (for $hP2X_2$H204C pretreatment, zinc effect ratio of -0.79 ± 0.03, $n = 8$; posttreatment zinc effect ratio of -0.40 ± 0.04, $n = 8$; for $hP2X_2$H209C, pretreatment zinc effect ratio of -0.84 ± 0.03, $n = 8$; posttreatment zinc effect ratio of -0.53 ± 0.03, $n = 8$). A simpler way to describe the change in zinc inhibition, which we will use for the rest of this paper, was to calculate the average change in the zinc effect ratio. MTSET produced a positive shift in of +0.39 for $hP2X_2$H204C and +0.31 for $P2X_2$H209C, but no change was seen for wild-type $hP2X_2$ (mean shift of -0.03 ± 0.02; $n = 6$). In contrast, when oocytes were treated with recording solution containing 1% DMSO (the final concentration present when MTSET was added), the average changes in the zinc effect ratio were very close to 0 (wild-type $hP2X_2$, -0.01 ± 0.01, $n = 8$; $hP2X_2$H204C, 0.03 ± 0.01, $n = 7$; $hP2X_2$H209C, 0.05 ± 0.04, $n = 7$), indicating no effect of the vehicle alone on the zinc effect ratio.

For both $hP2X_2$H204C and $hP2X_2$H209C, in addition to the change in the zinc effect ratio produced by MTSET treatment, there was a substantial change in the amplitude of the currents evoked by a maximum concentration of ATP after MTSET treatment (Fig. 6). For wild-type $hP2X_2$-expressing oocytes treated only with the DMSO vehicle, the maximum currents were some-

what smaller when a second application of high ATP was made 5 min after the initial application (85 ± 1.8%; $n = 8$), and the effect of MTSET treatment was no greater than this (83 ± 4.0%; $n = 7$). When $hP2X_2H204C$ was treated with MTSET, the amplitude 5 min after the initial trial (54 ± 3.6%; $n = 10$) was substantially smaller than after DMSO alone (83 ± 3.4%; $n = 7$). The same protocol gave a very different result for $hP2X_2H209C$. The amplitude of the currents evoked by saturating ATP were dramatically increased after MTSET treatment (to 443 ± 34%; $n = 15$), although there was no change in the Hill coefficient of the concentration–response relationship and only a small change in the EC_{50} for ATP (8.7 ± 0.3 μM before MTSET and 6.8 ± 0.3 μM after MTSET; $n = 6$).

To test whether the effects of MTSET were attributable to its positive charge, we also determined the effects of the negatively charged reagent MTSES. MTSES treatment also attenuated the zinc inhibition of both $hP2X_2H204C$ and $hP2X_2H209C$ (respectively, the average changes in zinc effect ratio were +0.19 ± 0.03, $n = 6$ and +0.31 ± 0.03, $n = 8$) but not wild-type $hP2X_2$ (−0.03 ± 0.02; $n = 6$). MTSES also produced a large increase in the current to saturating ATP from oocytes expressing $hP2X_2H209C$ (to 370 ± 44%; $n = 11$) and a significant decrease in the current to saturating ATP from oocytes expressing $hP2X_2H204C$ (to 49 ± 2.4%; $n = 7$).

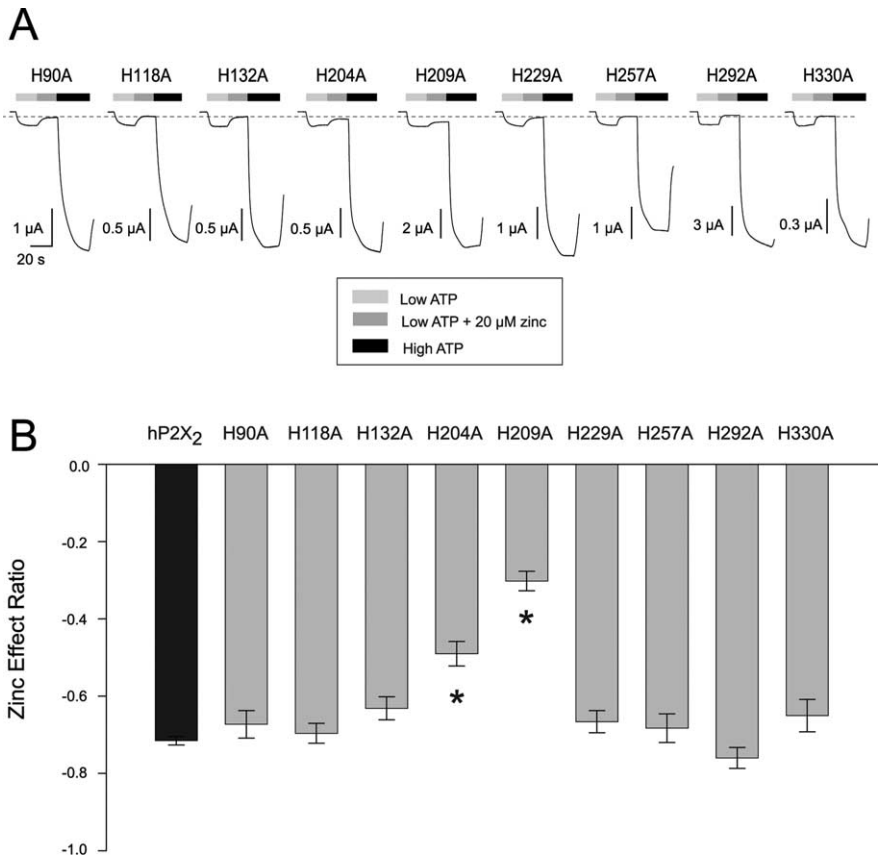

Figure 5. None of the nine histidines in the extracellular domain of $hP2X_2$ were required for zinc inhibition. ***A***, Responses of oocytes expressing $hP2X_2$ receptor mutants in which a single histidine of the extracellular domain was mutated to alanine. All traces have been scaled so that the currents to low ATP are the same size. The dashed line indicates the typical amplitude of the currents observed when wild-type $hP2X_2$ at its EC_{10} for ATP was treated with 20 μM zinc. The low concentrations of ATP used were as follows: $hP2X_2H90A$, 4 μM; $hP2X_2H118A$, 5 μM; $hP2X_2H132A$, 3.5 μM; $hP2X_2H204A$, 2.4 μM; $hP2X_2H209A$, 3.5 μM; $hP2X_2H229A$, 4 μM; $hP2X_2H257A$, 4 μM; $hP2X_2H292A$, 1.5 μM; $hP2X_2H330A$, 2.4 μM. In all panels, the concentration of high ATP was 500 μM. ***B***, Bar graph of average zinc effect ratios for wild-type $hP2X_2$ and all histidine to alanine mutant receptors listed in ***A***. The number of oocytes tested for each mutant ranged from 4 to 12. Asterisks indicate mutants that were significantly different from wild type ($p < 0.01$).

Effect of other modulators on $hP2X_2$

A second modulator that has been studied on $rP2X_2$ is acidic pH (King et al., 1997; Wildman et al., 1997; Clyne et al., 2002b). Altering the pH of a low ATP solution from 7.5 to 6.5 elicits potentiation of current through $rP2X_2$ (Clyne et al., 2002b). We tested the effects of acidic pH on ATP-elicited current in $hP2X_2$. We found that, although there was some potentiation of EC_{10} ATP-elicited current when pH was shifted from 7.5 to 6.5, the potentiation was much less in $hP2X_2$ than in $rP2X_2$ (pH effect ratio for $rP2X_2$, 7.6 ± 0.3, $n = 7$; for $hP2X_2$, 2.5 ± 0.1, $n = 5$) (Fig. 7). It has been shown that the $rP2X_2$ mutant H319A displays greatly reduced potentiation of current in acidic pH, indicating that this histidine residue might be responsible for coordinating protons (Clyne et al., 2002b). We made the homologous mutant in $hP2X_2$ (H330A) and found that this mutant receptor displayed no potentiation when exposed to EC_{10} ATP in 6.5 pH solution (pH effect ratio, 0.0 ± 0.1; $n = 5$).

A third modulator of $rP2X_2$ is membrane potential (Zhou and Hume, 1998). When the membrane potential was stepped from −50 to −100 mV in the continuous presence of a low concentration of ATP (Fig. 8*A*), the current jumped instantly to a larger level, reflecting both an increase in driving force and an apparent increase in the unitary conductance of the channels (Zhou and Hume, 1998; Ding and Sachs, 1999). However, in addition to these changes, there was an increase in the inward current that occurred gradually, with a time constant of many tens of milliseconds that reflected the opening of additional channels. Conversely, when the membrane potential was jumped back to −50 mV, there was a slow component that reflected the closing of some channels. In contrast, in $hP2X_2$, no voltage-dependent relaxations were present over the temporal scale accessible with two-electrode clamping of oocytes (Fig. 8*B*). However, there was a voltage dependence to the response of $hP2X_2$, because there was clearly strong inward rectification in the current–voltage relationship in response to ramp depolarizations in the presence of low concentrations of ATP (Fig. 8*C*). As is the case for $rP2X_2$, the rectification in $hP2X_2$ was less when a saturating concentration of ATP was used (Fig. 8*D*). Another manifestation of the voltage-dependent modulation of $hP2X_2$ was that the concentration–response relationship varied systematically with membrane potential (Fig. 9*A*). At more negative membrane potentials, the EC_{50} decreased and the Hill coefficient increased (Fig. 9*B*, *C*).

Possible evolutionary origin of zinc potentiation in $P2X_2$ receptors

The fact that zinc potentiation occurs in $hP2X_2$ when cysteine was substituted for the arginine at the position equivalent to rat H213 and that this zinc potentiation could be eliminated when the residue equivalent to rat H120 was mutated to alanine in the $hP2X_2H132A/R225C$ double-mutant suggests that an $rP2X_2$-like

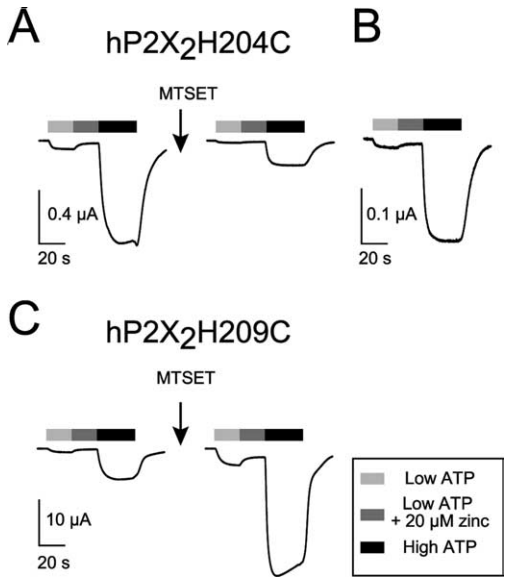

Figure 6. Effect of MTSET on $hP2X_2$ mutants H204C and H209C. ***A***, Effect of MTSET treatment (3 min at 100 μM) on the response of an oocyte expressing $hP2X_2H204C$. The low ATP was 3 μM and the high ATP was 500 μM. ***B***, The response of the same oocyte in ***A*** after MTSET shown on a different amplitude scale to allow the extent of zinc inhibition to be more easily seen. ***C***, Effect of MTSET treatment (3 min at 100 μM) on the response of an oocyte expressing $hP2X_2H209C$. The low ATP was 3 μM and the high ATP was 500 μM.

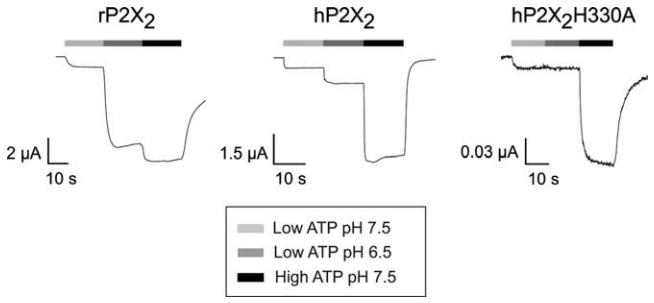

Figure 7. Potentiation to acidic pH in $rP2X_2$ and $hP2X_2$. Response of oocytes expressing wild-type $rP2X_2$, wild-type $hP2X_2$, and $hP2X_2$ mutant receptor H330A. In each trace, the first bar (light gray) indicates the time that a low concentration of ATP (approximately the EC_{10}) at pH 7.5 was applied, the second bar (dark gray) indicates the time the same low concentration of ATP at pH 6.5 was applied, and the third bar (black) indicates the time that a solution containing a high concentration of ATP in pH 7.5 (which elicited the maximal response) was applied. The concentrations of ATP used were as follows: wild-type $rP2X_2$, low, 12 μM; high, 500 μM; wild-type $hP2X_2$, low, 3 μM; high, 100 μM; $hP2X_2$ H330A, low, 2 μM; high, 500 μM.

potentiating zinc binding site might have existed in the receptor that was ancestral to both $rP2X_2$ and $hP2X_2$, and that the two proteins have retained a structural similarity through the region despite the ~60 million years since the divergence from the last common ancestor of rats and humans. To explore this issue, we aligned the sequences of $P2X_2$ sequences from mammals along with sequences from birds and amphibians (Fig. 10*A*). A histidine at the position analogous to rat H213 is present in the majority of mammals, but arginines are also common. Based on a statistical algorithm widely used in evolutionary biology to predict the most likely character state at the root of a phylogenetic tree (Zhang and Nei, 1997), the posterior probability that the mammalian ancestral residue at this position was histidine is 1.00 (i.e., any other ancestor is highly statistically unlikely). This means that mutation to arginine occurred independently several times in different mammalian lineages, including the one that produced humans. Although both rat and human $P2X_2$ have a histidine residue at the position homologous to $rP2X_2$ 120, a histidine at this position is not universal among other mammals. Indeed, based on the same statistical algorithm, the most likely residue present in this position in the ancestor of all mammals is proline with a posterior probability of 0.996. It therefore was of interest to test the H213P mutation in $rP2X_2$. The $rP2X_2R120P$ mutant expressed well in oocytes, with maximum currents in excess of -50 μA at -50 mV after injection of saturating amounts of RNA and had a concentration–response relationship (EC_{50} of 53 $\pm$ 3.6 μM; Hill coefficient, 1.69 $\pm$ 0.05; $n = 11$) (Fig. 10*B*) slightly right shifted from wild-type $rP2X_2$. We found that this mutant receptor showed no significant change in amplitude when 20 μM zinc was added to a concentration of ATP at approximately the EC_{10} (zinc effect ratio, 0.06 $\pm$ 0.02, $n = 14$ compared with 0.03 $\pm$ 0.02, $n = 5$ when ATP without zinc was added from a second tube) (Fig. 10*C*). Furthermore, lowering the ATP concentration to the EC_3 (which dramatically increases the zinc effect ratio in wild-type $rP2X_2$) or tripling the zinc concentration to 60 μM while the ATP concentration was kept near the EC_{10} showed no zinc potentiation and indeed revealed only the zinc inhibition characteristic of the low potency inhibitory site of the rat receptor (zinc effect ratio, -0.09 ± 0.05, $n = 4$ for EC_3 ATP with 20 μM zinc; zinc effect ratio, -0.09 ± 0.01, $n = 5$ for EC_{10} ATP with 60 μM zinc). Thus, a plausible scenario is that the $P2X_2$ receptor present in the earliest mammals was at a tipping point, with only a few mutations needed to create strong zinc potentiation as in rats and mice, or to completely lack zinc potentiation as in humans.

As far as the amino acids at these key positions in other members of the P2X receptor family, the receptor most closely related to $P2X_2$ is $P2X_3$ (North, 2002). The point of divergence between these genes predates all known fish (which have both a $P2X_2$ and a $P2X_3$). None of the available fish $P2X_2$ sequences has a proline at the position equivalent to rat H120 or a histidine at the position equivalent to rat H213 (these residues first appear in $P2X_2$ receptors of amphibians and marsupials, respectively). Indeed, there is such variety of amino acids at these positions in the fish $P2X_2$ and $P2X_3$ receptors that it is not possible to infer the amino acids at these positions in the last common ancestor to these two genes, let alone to infer the ancestral sequence of the other five more distantly related mammalian P2X receptors or the even more distantly related P2X receptors of invertebrates and single-celled organisms.

Discussion

Rat $P2X_2$ receptors exposed to low ATP show dramatic potentiation when exposed to zinc concentrations of 10–100 μM and show inhibition to higher zinc concentrations (Nakazawa and Ohno, 1996; Nakazawa et al., 1997; Wildman et al., 1998; Clyne et al., 2002b). The mouse $P2X_2$ receptor has an amino acid sequence identical to $rP2X_2$ in the region homologous to both $rP2X_2H120$ and $rP2X_2H213$, and mouse neurons known to express $P2X_2$ receptors show potentiation to zinc (Dunn et al., 2001). It therefore was not a surprise that we found that mouse $P2X_2$ receptors were also potentiated by 20 μM zinc. In contrast, we found that $hP2X_2$ receptors do not potentiate when exposed to any concentration of zinc and instead are profoundly inhibited by the same range of zinc concentrations that potentiate $rP2X_2$ and $mP2X_2$. The inhibitory effect of zinc on $hP2X_2$ is ~10-fold more potent than the zinc inhibition of $rP2X_2$ that is uncovered when residues essential for zinc potentiation are eliminated by mutagenesis.

Human $P2X_2$ receptors are also substantially less sensitive to changes in pH than rat receptors.

The inhibitory zinc binding sites of $rP2X_2$ and $hP2X_2$

The higher potency of zinc inhibition in human compared with rat $P2X_2$ could have two explanations. First, similar parts of the extracellular domains of $rP2X_2$ and $hP2X_2$ might comprise a common inhibitory zinc binding site with nearly identical structure, with differences in the local amino acid sequence accounting for the difference in potency. Second, zinc might bind to $hP2X_2$ at a high-affinity inhibitory zinc binding site that is not present in $rP2X_2$. We attempted to explore this issue by mutating candidate residues in both $rP2X_2$ (Clyne et al., 2002a,b; Friday and Hume, 2008) and $hP2X_2$ (this study), using the initial criterion that, if a residue is part of the inhibitory zinc binding site, mutation to alanine would result in a receptor deficient in zinc inhibition.

In $rP2X_2$, neither the extracellular histidines (Clyne et al., 2002b) nor negatively charged glutamates and aspartates (Friday and Hume, 2008) are required for low-affinity zinc inhibition. Furthermore, if one accepts the circumstantial evidence that all 10 extracellular cysteines form disulfide bonds and are unavailable for zinc binding (Clyne et al., 2002a; Ennion and Evans, 2002), then all of the top candidates based on other known zinc binding proteins appear to have been eliminated.

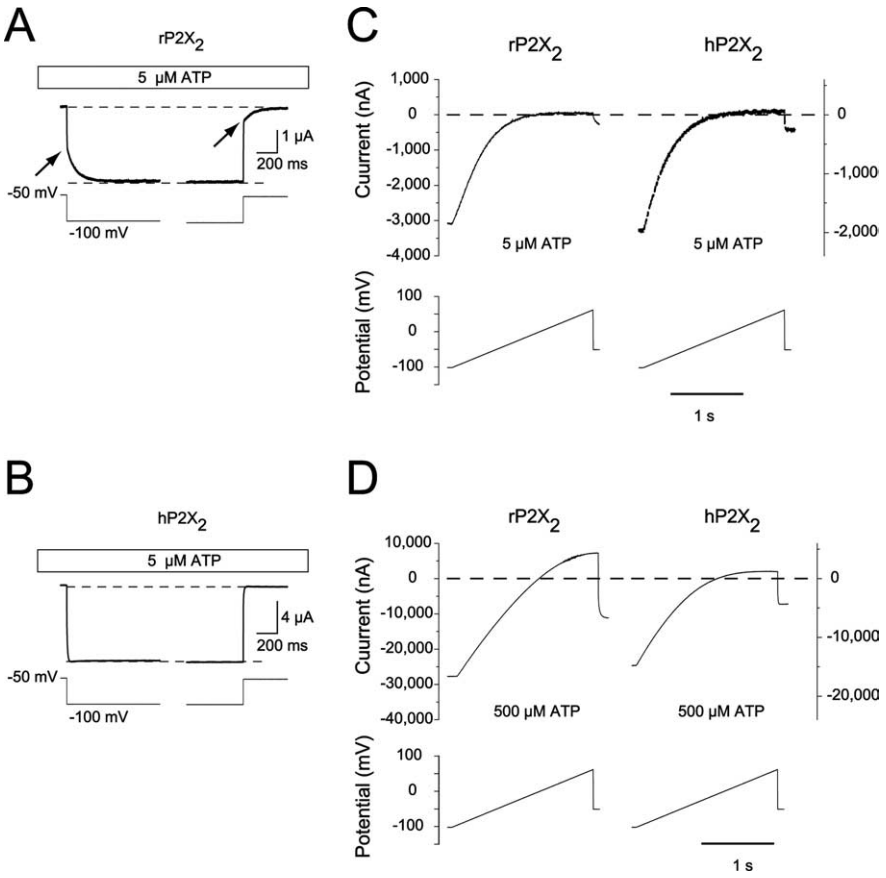

Figure 8. Voltage-dependent modulation of $hP2X_2$. ***A***, In $rP2X_2$, there was a slow increase in ATP-dependent current after a step from −50 to −100 mV and a slow decrease in the current when the potential was stepped back to −50 mV (arrows). ***B***, No such slow relaxations of current were visible when the same protocol was applied to $hP2X_2$. For these particular oocytes, 5 μM ATP represented approximately the EC_{10} concentration for both the rat and human receptors. ***C***, Responses to a ramp depolarization from −100 to +60 mV when a low concentration of ATP was present. Both $rP2X_2$ and $hP2X_2$ currents showed strong inward rectification. ***D***, The same cells as in ***C*** tested with 500 μM ATP, which produced a maximal response. Both $rP2X_2$ and $hP2X_2$ currents showed inward rectification that was less pronounced than when low ATP was used. These traces also demonstrate that, in these particular cells studied at −50 mV, 5 μM ATP was approximately the EC_{10} for both human and rat $P2X_2$.

In $hP2X_2$ we showed in the current study that mutation of two of the nine extracellular histidines to alanines led to a significant attenuation of zinc inhibition. In contrast, when cysteines were placed at these sites, zinc inhibition was very similar to wild-type $hP2X_2$. Furthermore, when the $hP2X_2H204C$ or $hP2X_2H209C$ mutants were challenged with MTSET, the zinc inhibition was attenuated. Taken on their own, the ability of the cysteine mutants to sustain full zinc inhibition and for MTSET treatment of these mutants to partially attenuate zinc inhibition are consistent with the hypothesis that these two histidines participate in an inhibitory zinc binding site of $hP2X_2$ but that modifying either single residue is insufficient to completely destroy the binding site. It is therefore of potential interest that H204 is common to rat and human $P2X_2$ receptors (the equivalent residue in the rat is H192), whereas H209 is only found in $hP2X_2$ (the equivalent residue in the rat is K197). However, several lines of evidence suggest another explanation for these results. First, the modification of $hP2X_2H204C$ and $hP2X_2H209C$ by MTSET produced large changes in the amplitude of the responses to ATP alone (a 50% decrease for $hP2X_2H204C$ and a fivefold increase for $hP2X_2H209C$), indicating that alterations in this region clearly had effects on processes other than zinc inhibition. Second, MTSES was nearly as effective as MTSET in producing changes to the zinc effect ratio of $hP2X_2H204C$ and $hP2X_2H209C$ receptors, although if the effect was at the zinc binding site, the charge of the adduct would have been expected to make a difference because zinc is positively charged. Finally, the double-mutant $hP2X_2H204A/H209A$ was no more deficient in zinc inhibition than either single mutant, although if both $hP2X_2H204$ and $hP2X_2H209$ contribute to the zinc binding site, this mutant would have been expected to be much more severely affected. From these results, we conclude that it is most likely that modification of the $hP2X_2$ receptor at positions H204 and H209 altered zinc inhibition indirectly and that the residues responsible for binding zinc to produce inhibition are yet to be identified.

There are at least two caveats to the conclusion that all the most obvious candidates for involvement in the inhibitory zinc binding sites have been tested and excluded. First, in the studies of inhibition of $rP2X_2$, the higher-affinity zinc potentiation site was left intact, so if removal of each residue contributing to the inhibitory zinc site has only a small effect, it might have been masked. Second, the evidence that all cysteines are in disulfide bonds is indirect and has recently been questioned for $P2X_4$ (Coddou et al., 2007). It would therefore be of considerable interest to apply more direct biochemical methods to determine conclusively whether all 10 cysteines are in disulfide bonds and, if not, to carefully test their potential role in binding zinc at the inhibitory sites of $rP2X_2$ and $hP2X_2$.

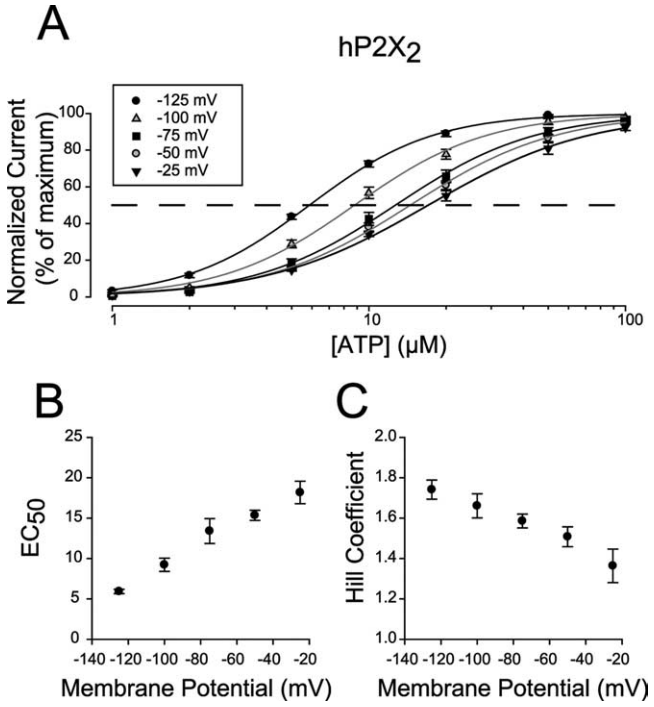

Figure 9. Effect of membrane potential on the concentration–response relationship of $hP2X_2$. ***A***, Average concentration–response relationship for oocytes clamped at the indicated membrane potentials. The amplitude of responses at each potential were normalized to the amplitude of the maximum response obtained at that potential before the data for different oocytes were averaged. The dashed line indicates the half-maximal response. ***B***, Average EC_{50} for ATP at each membrane potential. ***C***, Average Hill coefficient at each membrane potential. $n = 5$ for all three panels.

Potential physiological role of zinc modulation of $P2X_2$ receptors

Because zinc has opposite effects on human and rat $P2X_2$ receptors *in vitro*, it likely has opposite effects on cells expressing $P2X_2$ receptors in living rats and humans. Zinc is sequestered into the axonal boutons of some neurons and is released after synaptic stimulation (Frederickson et al., 2005). Estimates of the resulting concentration of free zinc present in the synapse are between 10 and 30 μM (Frederickson et al., 2005). The concentration of ATP in the synapse after vesicular release has been estimated at between 5 and 500 μM (Pankratov et al., 2006). If we take 100 μM ATP as a typical value, then the effect of zinc on any rat or human $P2X_2$ receptors directly postsynaptic to a nerve terminal releasing ATP would be very different. In the rat, if the zinc concentration reached 20 μM (the concentration that produces optimal potentiation for low ATP), one would expect it to have little modulatory effect on $rP2X_2$ because, at saturating ATP, zinc binding does not increase current beyond that elicited by high ATP alone (Clyne et al., 2002b). In contrast, if the zinc reached 20 μM at human synapses using $hP2X_2$, the zinc present would attenuate the ATP response to ~80% of its value in the absence of zinc. For perisynaptic $P2X_2$ receptors, in which the concentration of ATP is expected to be lower than at the synapse itself, zinc would likely have more dramatic and opposite effects on $rP2X_2$ and $hP2X_2$. For instance, if the ATP concentration were 2 μM (approximately the EC_2 for $rP2X_2$ and the EC_8 for $hP2X_2$), then 20 μM zinc would increase the current through $rP2X_2$ by >20-fold and attenuate current through $hP2X_2$ to ~20% of its value in the absence of zinc. Among the parts of the nervous system that contain both zinc-containing neurons and $P2X_2$ receptors are the hippocampus, cerebral cortex, and spinal cord (Smart et al., 1994; Kanjhan et al., 1999; Frederickson et al., 2005). When zinc is applied to cultured rat spinal neurons, it elicits an increase in the frequency of IPSCs through potentiation of presynaptic $P2X_2$ responses (Laube, 2002). Our results suggest that this phenomenon would not occur in humans.

Transgenic, knock-out, and knock-in mouse models are frequently used to assess the role of specific molecules in normal physiology and to model human diseases. Because human and mouse $P2X_2$ receptors respond oppositely to zinc, hypothesized actions of $P2X_2$ receptors that are based on experimental evidence from mice or rats must be carefully scrutinized for their relevance to the physiology of the human nervous system.

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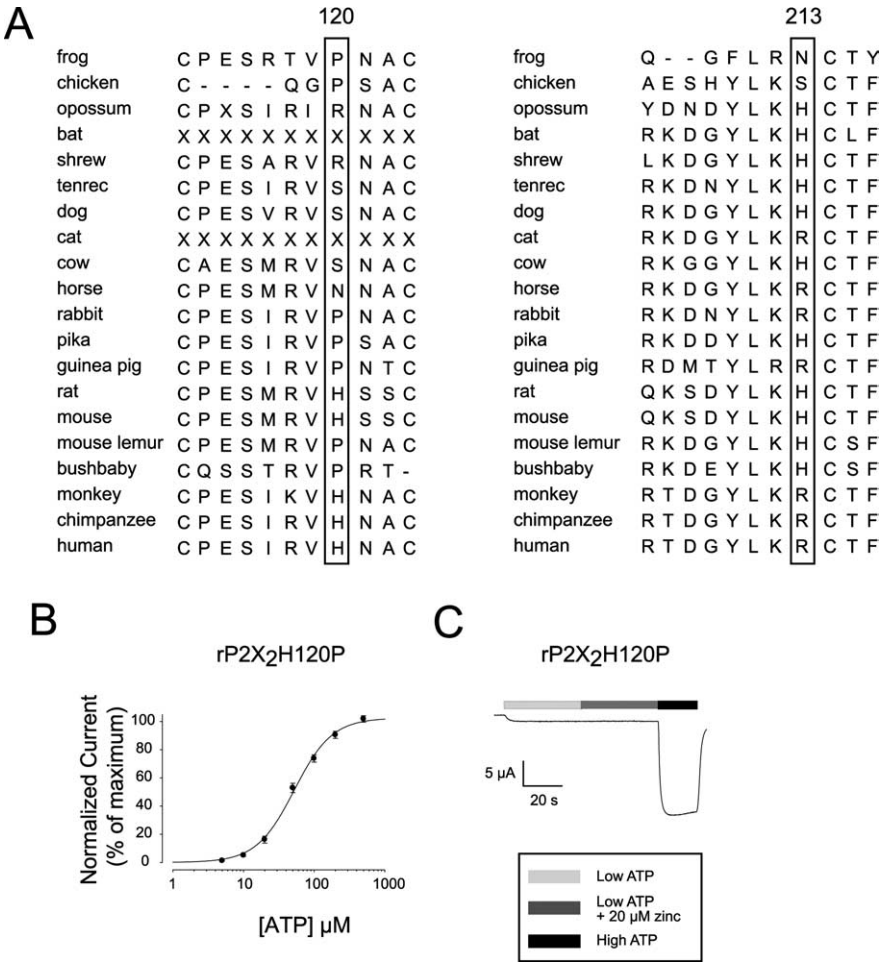

Figure 10. ***A***, Comparison of the amino acid sequence of $P2X_2$ receptors from amphibian, avian, and mammalian species. The order of species represents their approximate evolutionary distance from humans, with the most distant species at the top (http://www.ncbi.nlm.nih.gov/Taxonomy). The boxes indicate the position of the residues in the same position as H120 of $rP2X_2$ (left) and H213 of $rP2X_2$ (right). X indicates sequence not available, and − indicates no residue at this position when the full sequences were aligned. All sequences except for the guinea pig came from Ensemble release 49 (http://www.ensembl.org). The species and accession numbers were as follows: frog, *Xenopus tropicalis*, ENSXETG00000016152; chicken, *Gallus gallus*, ENSGALG00000007551; opossum, *Monodelphis domestica*, ENSMODG00000015154; bat, *Myotis lucifugus*, ENSMLUG00000016490; shrew, *Sorex araneus*, ENSSARG00000008417; tenrec, *Echinops telfairi*, ENSFCAG00000002495; dog, *Canis familiaris*, ENSCAFG00000006270; cat, *Felis catus*, ENSFCAG00000002495; cow, *Bos taurus*, ENSBTAG00000007496; horse, *Equus caballus*, ENSECAG00000016406; rabbit, *Oryctolagus cuniculus*, ENSOCUG00000017737; pika, *Ochotona princeps*, ENSOPRG00000003206; rat, *Rattus norvegicus*, ENSRNOG00000037456; mouse, *Mus musculus*, ENSMUSG00000029503; mouse lemur, *Microcebus murinus*, ENSMICG00000003948; bushbaby, *Otolemur garnettii*, ENSOGAG00000006635; monkey, *Macaca mulatta*, ENSMMUG00000015459; chimpanzee, *Pan troglodytes*, ENSPTRG00000005653; human, *Homo sapiens*, ENSG00000187848. The source for the guinea pig sequence was 070397–1 (uniprot.org). ***B***, Concentration–response relationship of $rP2X_2$H120P. ***C***, Lack of response of $rP2X_2$H120P to 20 μM zinc. Low ATP, 17.5 μM; high ATP, 500 μM.

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