A Histidine Scan to Probe the Flexibility of the Rat P2X$_2$ Receptor Zinc-binding Site*

Received for publication, February 23, 2007, and in revised form, May 3, 2007. Published, JBC Papers in Press, May 21, 2007, DOI 10.1074/jbc.M701604200

Rachel K. Tittle, Jamila M. Power, and Richard I. Hume

From the Department of Molecular, Cellular, and Developmental Biology, University of Michigan, Ann Arbor, Michigan 48109-1048

The response of P2X$_2$ receptors to submaximal concentrations of ATP is potentiated by low levels of extracellular zinc. Histidines 120 and 213 have previously been shown to be essential in binding zinc across an intersubunit binding site. We tested the flexibility of the zinc-binding site by making mutations that had the effect of shifting the two essential histidines up to 13 residues upstream or downstream from their original positions and then testing the ability of the mutated receptors to respond to zinc. Using this method, we were able to explore potential orientations of the two regions relative to one another. Our data are consistent with a moderately flexible zinc-binding site and inconsistent with parallel and anti-parallel orientations of the regions surrounding histidines 120 and 213.

ATP acts as a signaling molecule in the nervous system, where it elicits a number of effects, including fast excitatory synaptic transmission, through P2X receptors (1). P2X receptors are ligand-gated cation channels that open when exposed to extracellular ATP. Seven P2X receptor subtypes have been cloned in mammals, and they are expressed throughout the body (for review, see Ref. 1). A functional channel is likely made up of three subunits (2) and may be either homo- or heteromeric (1). Each subunit has two transmembrane domains, with intracellular N and C termini and a large extracellular loop (see Fig. 1A). The P2X$_2$ protein is expressed throughout the nervous system of rats (3), and studies from P2X$_2$-deficient mice reveal a role for the subunit in various functions, including peristalsis of the small intestine (4), mediation of ventilatory responses to hypoxia (5), and pain transmission (6). Studies from P2X$_{2/3}$ double knock-out mice reveal a role for heteromeric P2X$_{2/3}$ receptors in pain transmission and the regulation of urinary bladder reflexes (6), as well as in taste sensation (7).

Ionic current through P2X$_2$ receptors is potentiated by low levels (<100 $\mu$M) of extracellular zinc (8, 9). The colocalization of zinc and P2X$_2$ in parts of the nervous system suggests a possible physiological role for this effect (3, 10). Previous studies of recombinant rat P2X$_2$ receptors have identified two key residues involved in zinc potentiation. By individually mutating each extracellular histidine and cysteine to alanine, histidines 120 and 213 were both found to be required for the potentiation of current by zinc (8, 11). A more recent study (12) provided evidence that histidines 120 and 213 are directly involved in zinc binding and that zinc is bound at the interface between adjacent subunits.

We hypothesized that there might be sufficient flexibility across the subunit interface to tolerate some degree of misalignment and still effectively bind zinc. To test this idea, we conducted a histidine scan of the regions surrounding the zinc-binding site (see Fig. 1B). By replacing one of the two essential endogenous histidines with alanine and then substituting histidine for a nearby residue, we could effectively shift each key histidine upstream or downstream of its normal location. Because basic principles of protein structure (13) indicate that the side chains of residues involved in an $\alpha$ helix are expected to point the same direction every 3.6 residues, the side chains in $\beta$ sheets are expected to face the same direction every other residue, and the side chains of adjacent amino acids in a loop can point in the same direction, the location of any histidines that restored zinc potentiation (relative to positions 120 and 213) should allow us to draw inferences regarding the structure of the two distinct regions that comprise this zinc-binding site.

EXPERIMENTAL PROCEDURES

Mutagenesis—Rat P2X$_2$ cDNA (encoding a 472-amino acid protein) in pcDNA1 was obtained from Dr. D. Julius (University of California, San Francisco, CA). The mutations were generated using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). The sequences of mutant subunits were confirmed by DNA sequencing (University of Michigan DNA Sequencing Core). Except where otherwise noted, each mutant is referred to by the original amino acid (one-letter code) followed by the residue number and the substituted amino acid (one-letter code).

Expression of P2X$_2$ Receptors—P2X$_2$ receptors were expressed in defolliculated stage V-VI Xenopus laevis oocytes. Oocytes were harvested using procedures approved by the University of Michigan Committee on the Use and Care of Vertebrate Animals and have been described in detail previously (14). RNAs encoding wild type and mutant P2X$_2$ receptors were synthesized using the mMessage mMachine T7 kit (Ambion, Austin, TX). Each oocyte was injected with 50 nI of RNA at 5–10 ng/$\mu$l.

Electrophysiological Recordings—Two-electrode voltage clamp experiments were performed 2–5 days after RNA injection. All of the recordings were made at a holding potential of $\pm$50 mV. Recording electrodes were pulled from thin-walled
borsilicate glass on a model P-87 Flaming Brown puller (Sutter Instrument Company, Novato, CA) and had resistances of 0.5–1 MΩ. The currents were recorded with a Turbo TEC-03 voltage clamp amplifier (npi electronic GmBH, Tamm, Germany). Data acquisition was performed using a Digidata 1322A interface controlled by pCLAMP 9 (Molecular Devices, Union City, CA).

Solutions—The external recording solution contained 90 mM NaCl, 1 mM KCl, 1.3 mM MgCl₂, and 10 mM HEPES, pH 7.5. Electrodes were filled with an internal solution of 3 M KCl. Dissodium ATP (Sigma-Aldrich) was prepared as a 100 mM stock in double-distilled H₂O and stored at −20°C. For recording, ATP solutions were made by diluting the stock in external recording solution. To compensate for the chelation of Mg²⁺ by ATP, we added MgCl₂ to our solutions such that all solutions contained 1 mM free Mg²⁺, as determined by the program Bound and Determined (15). Zinc chloride was prepared as a 10 mM stock in external recording solution that was acidified with 0.01 M HCl to prevent precipitation. The pH of ATP solutions with and without zinc was adjusted to 7.5 with NaOH prior to recording.

In some experiments, apyrase was added to solutions to remove any endogenous ATP released from the oocytes. We used grade 1 apyrase (Sigma-Aldrich) that was prepared as 100 units/ml aliquots in double-distilled H₂O and stored at −20°C. The apyrase recording solutions were diluted to a final concentration of 1 unit/ml apyrase in external recording solution and were used within 20 min of preparation.

Quantification of Zinc Potentiation and Selection of Zinc and ATP Concentrations—The response of P₂X₂ receptors to zinc is biphasic; at low levels, zinc causes potentiation, but at high levels it results in inhibition of current (8, 9). In a previous study, we used the nonpotentiating mutants H120A and I226A to demonstrate that these two effects of zinc are separable. At a zinc concentration of 20 μM or below the inhibition is negligible, whereas at all higher zinc concentrations both processes are occurring (8). We therefore used 20 μM zinc in all experiments testing only zinc potentiation, even though 50 and 100 μM zinc produce greater potentiation (see Fig. 5).

We defined potentiation to zinc as [(current in low ATP + 20 μM zinc)/current in low ATP] − 1. Thus, a cell that showed no increase in current in response to zinc had a zinc potentiation ratio equal to 0. To compare the magnitude of zinc potentiation between groups of oocytes, it is essential that all of the oocytes be studied at similar points on the ATP concentration-response relation, because as the concentration of ATP increases, potentiation decreases so that there is no zinc potentiation when a saturating concentration of ATP is present (8, 9). Furthermore, the EC₅₀ for ATP of different oocytes expressing the same construct can vary significantly (16). In all experiments except those shown in Fig. 5, we dealt with these complications by testing each oocyte first with a low concentration of ATP that we expected would be close to the EC₅₀ based on the average concentration-response relation for each construct and then with a high concentration of ATP, which was a maximal concentration (again based on the average concentration-response relation). Only oocytes for which it was verified that the low ATP concentration used was between the EC₅₀ and the EC₂₀ (so that at least a 5-fold increase in current was possible) were included in the data presented.

For the experiments presented in Fig. 5, we used a simpler approach to determine which oocytes should be excluded. For each construct we already knew from experiments presented in Figs. 2 and 3 the amount of potentiation that would be obtained in response to 20 μM zinc when the ATP level was correctly set. Therefore, in the zinc concentration-response experiments, any oocyte that gave zinc potentiation to 20 μM zinc more than one standard deviation above or below the known average value was considered to have had an EC₅₀ for ATP outside of the acceptable range and was excluded from the average data.

Data Analysis—Data were analyzed using Clampfit and Microsoft Excel. The significance of differences between groups was tested using the two-tailed, paired t test function of Excel, with significance taken to be p < 0.01. Concentration-response relations for ATP were fit to the three parameter Hill equation using the nonlinear curve fitting program of SigmaPlot 8.0 (Systat Software Inc., San Jose, CA). For displaying average data, the points from each oocyte were normalized to between 0 and 100% based on the maximum value of the fitted curve. The scaled data were then averaged and plotted with error bars indicating the standard error of the mean. The lines fit to the data indicate the average parameters of the individual fits.

RESULTS

We made a total of 22 double mutants in which one essential histidine remained in its original position and the other was near its original position (Fig. 1B). For the H120A scan, these included all residues between serine 116 and threonine 123. For the H213A scan, all residues between aspartate 209 and isoleucine 226 were mutated, except for cysteines 214 and 224, which form an essential disulfide bond (11). The rationale for extending this scan so far from position 213 in the C-terminal direction was that the disulfide bond might bring residues near 224 close to position 213. By comparing the level of zinc potentiation in these mutants with that of wild type P₂X₂, we were able to assay the extent to which the altered receptors could respond to zinc.

Responses to ATP—Two mutations in this study resulted in nonfunctional P₂X₂ receptors: H213A/P225H and H213A/I226H. This result is consistent with the finding that P225A and I226A were nonresponsive to ATP (17). All other mutations made in the present study resulted in functional receptors, and the ATP concentration-response relationship was determined (Table 1).

Many of the histidine scan mutants had EC₅₀ values that were significantly different from wild type P₂X₂. Seven mutant receptors had an EC₅₀ that was shifted more than 2-fold from that of wild type P₂X₂. Of these, six shifted to the right, and in the most extreme case (L211H/H213A), the EC₅₀ was right-shifted more than 10-fold. One mutant (H213A/D221H) was shifted to the left, with an EC₅₀ of 6 μM compared with 22 μM for wild type.

Responses to Zinc—Fig. 2 shows representative traces obtained when the zinc potentiation protocol was tested on oocytes...
Histidine Scan of P2X2 Zinc-binding Site

FIGURE 1. Model of the rat P2X2 receptor. A, each subunit has two transmembrane domains and 10 conserved extracellular cysteines (gray circles), all of which are thought to be involved in disulfide bonds. Two of the three subunits that make up a receptor are shown to illustrate the intersubunit binding of zinc by residues His120 and His213 (open circles). B, summary of experimental strategy. In wild type P2X2 receptors, His120 and His213 (top panel, large black type) are essential for zinc potentiation and directly bind zinc. In the double mutants tested, one of these two histidines was mutated to an alanine (outlined type), whereas a nearby residue was mutated to histidine (large black type). Oocytes expressing each construct were then tested for responsiveness to zinc. Three cysteine residues (gray type) were not mutated because they form disulfide bonds essential for normal responses to ATP. All other residues from Ser116 to Thr123 and Asp209 to Ile226 were substituted with histidines.

TABLE 1
The EC50 values of all mutant receptors characterized in this study
All of the data are ± S.E.

| Construct | EC50  | n a |
|-----------|-------|-----|
| P2X2      | 22 ± 1| 7   |
| Single mutants |       |     |
| H120A     | 18 ± 2| 6   |
| K212A     | 27 ± 2| 6   |
| K212H     | 53 ± 1 b| 6 |
| H213A     | 29 ± 1 b| 6 |
| Double mutants |       |     |
| S116H/H120A | 29 ± 2| 10 |
| M117H/H120A | 15 ± 1 b| 8 |
| K118H/H120A | 90 ± 13 b| 5 |
| V119H/H120A | 35 ± 3 b| 10 |
| H120A/S121H | 33 ± 3 | 8 |
| H120A/S122H | 70 ± 20 | 6 |
| H120A/T123H | 21 ± 1 | 6 |
| D209H/H213A | 15 ± 0 b| 10 |
| Y210H/H213A | 34 ± 6 | 6 |
| L211H/H213A | 380 ± 56 b| 9 |
| K212H/H213A | 187 ± 13 b| 7 |
| H213A/T215H | 13 ± 1 b| 15 |
| H213A/F216H | 46 ± 4 b| 6 |
| H213A/D217H | 14 ± 1 b| 5 |
| H213A/Q218H | 27 ± 1 b| 6 |
| H213A/D219H | 26 ± 1 | 6 |
| H213A/S220H | 34 ± 2 b| 6 |
| H213A/D221H | 6 ± 0 b| 6 |
| H213A/P222H | 26 ± 2 | 6 |
| H213A/Y223H | 89 ± 12 b| 6 |
| Quadruple mutants |       |     |
| V119H/H120A/L211H/H213A (H119/H211) | 2,549 ± 118 b| 6 |
| H120A/S121H/L211H/H213A (H121/H211) | 503 ± 126 b| 6 |
| V119H/H120A/K212H/H213A (H119/H212) | 578 ± 58 b| 6 |
| H120A/S121H/K212H/H213A (H121/H212) | 452 ± 15 b| 6 |

a n refers to the number of oocytes tested.

b Receptors with an EC50 that significantly differed from wild type receptors at p < 0.01.

expressing wild type P2X2, H120A, H213A, and the four histidine scan mutants that involved the smallest shifts to either side of the normal position of the histidine. Average data for all of the double mutants studied are presented in Fig. 3. The average zinc potentiation ratio for wild type P2X2 was 6.4 ± 0.37. As reported previously, mutation of His120 or His213 to alanine produces receptors that are almost completely unaffected by the application of zinc (8), with an average zinc potentiation ratio of −0.1 ± 0.01 for H120A and 0.2 ± 0.06 for H213A. In the scan around His213, histidine substitution at 11 of the 13 positions tested resulted in zinc potentiation ratios that were not greater than the H213A single mutant. One double mutant, L211H/H213A, showed modest but statistically significant potentiation (zinc potentiation ratio of 0.50 ± 0.05), whereas a second (K212H/H213A) had an average zinc potentiation ratio of 13.9 ± 0.6, which was significantly greater than wild type P2X2. Similarly, in the scan around His120, substitution at only a single position (H120A/S121H) resulted in a zinc potentiation ratio greater than 0.5 (3.5 ± 0.09). However, in this region, four of the five other positions tested gave zinc potentiation that was small but significantly greater than that exhibited by the H120A single mutant. Thus, robust zinc potentiation was present only when a histidine was shifted no more than one residue downstream or upstream of its endogenous location.

Further Analysis of the K212H/H213A Mutant—The level of zinc potentiation in the mutant K212H/H213A was greater than wild type P2X2. Furthermore, the combination of zinc and low ATP exceeded the maximum current attained by saturating levels of ATP, which is not the case for wild type P2X2. There were a number of possible explanations for this phenomenon. We first tested whether this effect was due to the loss of the lysine at position 212 rather than to the new position of the histidine. By studying the properties of two single mutants: K212A and K212H (Fig. 4). Because lysine 212 is conserved across all seven P2X receptors, it might be expected to play a critical role. The ATP concentration-response relation and zinc potentiation ratio for K212A were not significantly different from wild type P2X2 (EC50 of 27 μM and zinc potentiation ratio of 7.7 ± 0.3). This was a similar result to that reported by Ennion et al. (18), who found that mutating the equivalent lysine in P2X2 to alanine or arginine had almost no effect on ATP potency. K212H was slightly right-shifted in its response to ATP when compared with wild type P2X2 (EC50 of 53 μM), but its zinc potentiation ratio, at 7.8, was not significantly different from wild type. Thus, removing the lysine at 212 or adding an additional histidine at 212 is insufficient to produce the
phenotype exhibited by the K212H/H213A double mutant. We conclude that it is the movement of the histidine from position 213 to position 212 and not the loss of the lysine at position 212 that accounts for the properties of this double mutant.

It was possible that K212H/H213A might have a shifted potency for its potentiating or inhibiting response to zinc compared with wild type. To test this possibility, we compared the zinc concentration-response relations for wild type P2X2 and the K212H/H213A mutant (Fig. 5, A, B, and E). This was accomplished by adding higher and higher concentrations of zinc to the [EC_{10}] ATP. Although the fold potentiation for K212H/H213A was higher than that for wild type P2X2 at every concentration of zinc tested, the concentration-response relation for both potentiation and inhibition was similar.

Recent reports suggest that airway epithelial cells respond to extracellular zinc in the absence of ATP by a process that involves P2X4, P2X6, or coassembly of the two subunits (19, 20). We therefore tested the responses of wild type P2X2 and the K212H/H213A mutant to ascending concentrations of zinc (2, 5, 20, 50, 100, and 500 μM) to see whether zinc alone could elicit an inward current. For oocytes with holding currents of 35 nA or less at 50 mV, the responses to zinc were small outward currents that did not differ significantly from uninjected oocytes. For oocytes with larger holding currents at 50 mV, we sometimes observed small inward currents in response to zinc. However, the amplitudes of the zinc responses were positively correlated with the amplitudes of the holding currents so it seemed plausible that these oocytes were leaking ATP and that the inward currents were due to zinc acting on the small proportion of receptors that had ATP bound rather than due to an effect of zinc alone. We therefore tested the effect of zinc alone in the presence of 1 unit/ml of apyrase to destroy endog-
enous ATP. With apyrase, the average response to 20 μM zinc was a very small outward current in oocytes expressing wild type P2X2 (+3 ± 3 nA), K212H/H213A (+3 ± 2 nA) and in un.injected oocytes (+6 ± 1 nA, n = 6 each), regardless of the holding current (Fig. 5, C and D). There was no significant difference between the three groups of oocytes. We therefore conclude that zinc alone cannot open the channels of P2X2 receptors expressed in Xenopus oocytes.

The Effect of Simultaneously Moving Both Histidines—If the region near His120 and the region near His213 ran in a parallel orientation (as suggested in Fig. 1B), then simultaneously moving both histidines in the C-terminal direction or in the N-terminal direction might be expected to create a receptor that more closely mimics the normal distance between these histidines than either double mutant alone and thus might restore normal zinc potentiation. Conversely, if these two regions ran in an anti-parallel orientation, the normal distance would be restored when one histidine was moved in the N-terminal direction and the other in the C-terminal direction. To test these ideas, we made quadruple mutants in which both critical histidines were moved from their endogenous positions (Fig. 6). Because Cys214 is an essential cysteine, we only explored combinations in which the second histidine was moved in the N-terminal direction, either to position 211 or 212, whereas the first histidine was moved from 120 in both directions. For clarity of presentation, we will refer to these quadruple mutants by the positions of their histidines. Thus, V119H/H120A/K212H/H213A, a quadruple mutant, will be referred to as H119/H212. Using this nomenclature, wild type P2X2 is H120/H213, and the most highly zinc potentiating double mutant is H120/H212.

As noted previously, the ATP concentration-response relations of H120/H211 and H120/H212 double mutant receptors were right-shifted compared with wild type P2X2 (EC50 values of 380 and 187 μM, respectively). The four quadruple mutants that included His211 and His212 were much more severely right-shifted, with three of the four EC50 values near 500 μM and the fourth exceeding 2 mM (Table 1).

To test the predictions of the parallel and anti-parallel models, we compared zinc potentiation data from wild type, double mutant, and quadruple mutant receptors (Table 2). The double mutant H120/H211 showed very modest potentiation to zinc, suggesting that a better positioned first histidine should have been able to substantially augment zinc potentiation when paired with His211. The parallel model predicts that His119 will be closer to His211 than His121, whereas the anti-parallel model predicts that His121 will be closer to His211, so if one of these models is a good description of this region, then one quadruple mutant receptor should have zinc potentiation enhanced and the other suppressed. However, neither H119/H211 nor H121/H211 receptors showed significant zinc potentiation. Conversely, if orientation were either parallel or anti-parallel, then either His119 or His121 would be expected to be too distant to form a zinc site with His212. However, both H119/H212 and H121/H212 supported substantial zinc potentiation. In summary, regardless of the position of the other histidine, the ability to potentiate to zinc followed the order His212 > His213 > His211 and His120 > His121 > His119. We conclude from these experiments that it is likely that the regions containing His120 and His213 are neither parallel nor anti-parallel to each other.

DISCUSSION

From our previous work (12), we knew that the interaction of His120 and His213 with zinc is across the interface between adjacent subunits. Because the interface of two subunits was involved, one possibility was that there might be sufficient flexibility in one subunit or the other to allow the positions of the critical histidines to be moved and still retain zinc binding. However, we also knew that all 10 extracellular cysteines in P2X receptors are probably involved in disulfide bonds (11, 21).
Histidine Scan of P2X₂ Zinc-binding Site

Because His¹²⁰ is only four residues from Cys¹²⁴ and His²¹³ is next to Cys²¹⁴, the presence of disulfide bonds so close to these histidines suggested that the structure around them might be highly ordered. In this case, movement of the histidines to other locations might be expected to render the receptor incapable of responding to zinc. To test these ideas, we conducted a histidine scan of the rat P2X₂ zinc-binding site by replacing one of the endogenous required histidines with alanine and replacing a nearby residue with histidine. By assaying the resulting double and quadruple mutants for their responsiveness to zinc, we were able to assess the tolerance of the zinc-binding site to shifted positions of the essential histidines. It should be noted that our zinc potentiation assay does not measure zinc binding directly, so there are multiple potential explanations for mutations that do not potentiate to zinc. However, when potentiation was present, the magnitude of the effect provided interesting new information about the structure of these receptors.

Our results suggest a zinc-binding site that is intermediate between the highly flexible and the highly ordered models and suggest that the regions around His¹²⁰ and His²¹³ take on neither an α helix nor a β sheet secondary structure. The latter interpretation of the data is based on the observation that the side chains of residues involved in an α helix are expected to point the same direction every 3.6 residues, and the side chains in β sheets are expected to face the same direction every other residue (13). However, the pattern of residues that could support zinc potentiation did not fit neither of these models. As far as the region around position 120, moving the histidine to any of five nearby positions gave detectable zinc potentiation. Thus, any of six positions somewhat symmetrically spread in either direction around position 120 can come close enough to His²¹³ to form a zinc-binding site, although all but 120 and 121 do this very poorly. The spread around position 213 was unidirectional. When we inserted a histidine at positions beyond 213, the location tested was able to produce receptors that could respond to zinc. This suggests that the entire loop formed by the disulfide bridge between Cys²¹⁴ and Cys²²² is pointed away from the zinc binding interface. In contrast, placing a histidine at either of the two positions just before the location of the endogenous histidine produced receptors that showed slight (position 211) or substantial (position 212) zinc potentiation. A possible structure for each of these regions therefore is a loop in which the residues directly before and after the endogenous histidines point in a similar direction.

By moving both histidine residues at once, we were able to make predictions about relative orientation of the two regions of the zinc-binding site. If the region near 120 and the region near 213 were in loops in the same plane (as shown in Figs. 1A and 6A), then the peptide backbones of the two regions would be either relatively parallel or anti-parallel to each other. Because our results with quadruple mutants do not support either model, it seems likely that these two regions are in different planes. One potential model is that they approach each other only at a point normally defined by 120 and 121 on one side and by 212 and 213 on the other but that some side to side movement is possible in either subunit.

There is a functional dissociation between the ability of receptors to respond to zinc and their ability to respond to ATP. Wild type receptors (EC₅₀ for ATP of 22 μM), K212H/H213A receptors (EC₅₀ for ATP of 187 μM), and H121/H212 receptors (EC₅₀ for ATP of 452 μM) all responded robustly to zinc. Indeed, the K212H/H213A receptors potentiated to zinc better than the wild type P2X₂. Conversely, the H120A and H213A mutants responded to ATP like wild type receptors but were zinc-insensitive. The response to ATP and the response to zinc seem to be functionally separate processes. Are the binding sites also structurally distinct?

One approach to understanding ATP binding has been through site-directed mutagenesis of residues conserved in all P2X receptors, followed by additional tests to distinguish binding from gating (18, 22–25). This approach has implicated several amino acids as directly binding ATP (26). A recent study
Histidine Scan of P2X$_2$ Zinc-binding Site

A)

\[
\begin{array}{cccc}
\text{Construct} & \text{EC}_{10} \text{ for ATP} & \text{Zinc potentiation at EC}_{10} \text{ ATP} & n^* \\
\hline
\text{H119/H211}^a & 500 & -0.3 \pm 0.03 & 5 \\
\text{H119/H212}^a & 78 & 0.8 \pm 0.16 & 6 \\
\text{H119/H213} & 12 & 0.3 \pm 0.10 & 6 \\
\text{H120/H211} & 100 & 0.5 \pm 0.12 & 6 \\
\text{H120/H212} & 37 & 13.9 \pm 1.52 & 6 \\
\text{H120/H213 (wild type P2X$_2$)} & 3 & 6.8 \pm 0.90 & 6 \\
\text{H121/H211}^b & 62 & 0.0 \pm 0.05 & 5 \\
\text{H121/H212}^b & 71 & 4.8 \pm 0.76 & 9 \\
\text{H121/H213} & 15 & 3.5 \pm 0.23 & 6 \\
\end{array}
\]

$^a$ refers to the number of oocytes tested.

$^b$ Receptors in which both histidines were moved.

showed that two of these residues, Lys$_{68}$ and Phe$_{291}$, are in very close proximity across the subunit interface (25) in a P2X$_{1/2}$ chimera. If these residues are directly involved in binding ATP, this indicates that ATP as well as zinc binds between subunits in P2X receptors. Another recent study also suggests an intersubunit ATP-binding site (27).

A second approach to understanding ATP binding has been to make models of the ATP-binding site based on homology to other proteins with known structures. One model for the ATP-binding site is based on the crystal structure of synapsin II (28). The synapsin II model includes roles for many of the residues identified as critical in mutagenesis scans. Another interesting model for ATP binding to P2X receptors (29) is based on similarities between residues in the extracellular domain of P2X receptors and the catalytic domains of the class II aminoacyl-tRNA synthetases, which also bind ATP. This model has been extended to P2X$_4$ receptors (30, 31) and tested by making site-directed mutants of several residues predicted to be critical in ATP binding. Potential limitations of this model are that several of the key residues are not conserved across the P2X receptor family and that the region modeled includes only residues from Lys$_{320}$ to Lys$_{326}$ (which represent about the second half of the extracellular domain), even though a role for Lys$_{69}$ in binding ATP has been supported by a number of studies (18, 22, 23, 27). Although this model may be incomplete, it makes interesting predictions about the structure of P2X$_2$ in the vicinity of His$_{213}$. The position of this histidine is predicted to be in a loop between strands $\beta_1$ and $\beta_2$ of a six-strand $\beta$-pleated sheet. Although none of the residues mutated in our histidine scan are directly implicated in binding the ATP molecule, the next residue in the C-terminal direction after our scan, Phe$_{227}$ (P2X$_2$ numbering), was implicated in binding to the adenine ring. If this is correct, His$_{213}$ would also be close to the adenine ring of a bound ATP because of the disulfide bond between Cys$_{214}$ and Cys$_{224}$. Although this model does not include a prediction for the His$_{120}$ region of the scan, we know that when histidines 120 and 213 are both mutated to cysteine, they are close enough to form an intersubunit disulfide bond (12). Therefore, if the model by Yan et al. (30) is a good approximation of the residues that bind ATP, it would mean that the region surrounding His$_{120}$ is also very close to the bound ATP. This model suggests a possible reason that no residues other than His$_{120}$ and His$_{213}$ have thus far been identified as binding to zinc, although most zinc-binding sites are tetrahedral (32). This result could be explained if the coordination of zinc within P2X$_2$ receptors involves an interaction with ATP, which has a significant affinity for divalent cations, including zinc (33).

Acknowledgments—We thank members of the Hume laboratory for critical reading of the manuscript.

REFERENCES

1. North, R. A. (2002) Physiol. Rev. 82, 1013–1067
2. Nicke, A., Baumert, H. G., Rettinger, J., Eichele, A., Lambrecht, G., Mutschler, E., and Schmalzing, G. (1998) EMBO J. 17, 3016–3028
3. Kanjhan, R., Housley, G. D., Burton, L. D., Christie, D. L., Kippenberger, A., Thorne, P. R., Luo, L., and Ryan, A. F. (1999) J. Comp. Neurol. 407,
Histidine Scan of P2X$_2$ Zinc-binding Site

11–32

4. Ren, J., Bian, X., DeVries, M., Schnegsberg, B., Cockayne, D. A., Ford, A. P., and Galligan, J. J. (2003) *J. Physiol.* **552**, 809–821

5. Rong, W., Gourine, A. V., Cockayne, D. A., Xiang, Z., Ford, A. P., Spyer, K. M., and Burnstock, G. (2003) *J. Neurosci.* **23**, 11315–11321

6. Cockayne, D. A., Dunn, P. M., Zhong, Y., Rong, W., Hamilton, S. G., Knight, G. E., Ruan, H. Z., Ma, B., Yip, P., Nunn, P., McMahon, S. B., Burnstock, G., and Ford, A. P. (2005) *J. Physiol.* **567**, 621–639

7. Finger, T. E., Danilova, V., Barrows, J., Bartel, D. L., Vigers, A. J., Stone, L., Hellekant, G., and Kinnamon, S. C. (2005) *Science* **310**, 1495–1499

8. Clyne, J. D., LaPointe, L. D., and Hume, R. I. (2002) *J. Physiol.* **539**, 347–359

9. Wildman, S. S., King, B. F., and Burnstock, G. (1998) *Br. J. Pharmacol.* **123**, 1214–1220

10. Smart, T. G., Xie, X., and Krishek, B. J. (1994) *Prog. Neurobiol.* **42**, 393–441

11. Clyne, J. D., Wang, L. F., and Hume, R. I. (2002) *J. Neurosci.* **22**, 3873–3880

12. Nagaya, N., Tittle, R. K., Saar, N., Dellal, S. S., and Hume, R. I. (2005) *J. Biol. Chem.* **280**, 25982–25993

13. Branden, C., and Tooze, J. (1998) *Introduction to Protein Structure*, 2nd Ed., pp. 13–87, Garland Publishing, New York

14. Zhou, Z., and Hume, R. I. (1998) *J. Physiol.* **507**, 353–364

15. Brookes, S. P., and Storey, K. B. (1992) *Anal. Biochem.* **201**, 119–126

16. Clyne, J. D., Brown, T. C., and Hume, R. I. (2003) *Neuropharmacology* **44**, 403–412

17. Nakazawa, K., Ojima, H., Ishii-Nozawa, R., Takeuchi, K., and Ohno, Y. (2004) *Eur. J. Pharmacol.* **483**, 29–35

18. Ennion, S., Hagan, S., and Evans, R. J. (2000) *J. Biol. Chem.* **275**, 35656

19. Zsembery, A., Fortenberry, J. A., Liang, L., Bebok, Z., Tucker, T. A., Boyce, A. T., Braunstein, G. M., Welty, E., Bell, P. D., Sorscher, E. I., Clancy, J. P., and Schwiebert, E. M. (2004) *J. Biol. Chem.* **279**, 10720–10729

20. Liang, L., Zsembery, A., and Schwiebert, E. M. (2005) *Am. J. Physiol.* **289**, C388–C396

21. Ennion, S. J., and Evans, R. J. (2002) *Mol. Pharmacol.* **61**, 303–311

22. Jiang, L. H., Rassendren, F., Surprenant, A., and North, R. A. (2000) *J. Biol. Chem.* **275**, 34190–34196

23. Roberts, J. A., and Evans, R. J. (2004) *J. Biol. Chem.* **279**, 9043–9055

24. Roberts, J. A., and Evans, R. J. (2007) *J. Neurosci.* **27**, 4072–4082

25. Marquez-Klaka, B., Rettinger, J., Bhargava, Y., Eisele, T., and Nicke, A. (2007) *J. Neurosci.* **27**, 1456–1466

26. Vial, C., Roberts, J. A., and Evans, R. J. (2004) *Trends Pharmacol. Sci.* **25**, 487–493

27. Wilkinson, W. J., Jiang, L. H., Surprenant, A., and North, R. A. (2006) *Mol. Pharmacol.* **70**, 1159–1163

28. Roberts, J. A., Vial, C., Digby, H. R., Agboh, K. C., Wen, H., Atterbury-Thomas, A., and Evans, R. J. (2006) *Pflugers Arch.* **452**, 486–500

29. Freist, W., Verhey, J. F., Stuhmer, W., and Gauss, D. H. (1998) *FEBS Lett.* **434**, 61–65

30. Yan, Z., Liang, L., Tomic, M., Obsil, T., and Stojilkovic, S. S. (2005) *Mol. Pharmacol.* **67**, 1078–1088

31. Yan, Z., Liang, L., Obsil, T., and Stojilkovic, S. S. (2006) *J. Biol. Chem.* **281**, 32649–32659

32. Auld, D. S. (2001) *Biometals* **14**, 271–313

33. Sigel, H., and Griers, R. (2005) *Chem. Soc. Rev.* **34**, 875–900