An Intersubunit Zinc Binding Site in Rat P2X₂ Receptors*

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P2X receptors are ATP-gated ion channels made up of three similar or identical subunits. It is unknown whether ligand binding is intersubunit or intrasubunit, either for agonists or for allosteric modulators. Zinc binds to rat P2X₂ receptors and acts as an allosteric modulator, potentiating channel opening. To probe the location of this zinc binding site, P2X₂ receptors bearing mutations of the histidines at positions 120 and 213 were expressed in Xenopus oocytes. Studies of H120C and H213C mutants produced five lines of evidence consistent with the hypothesis that these residues in these positions bind zinc. Mixing of subunits containing the H120A or H213A mutation generated receptors that showed zinc potentiation, even though neither of these mutant receptors showed zinc potentiation on its own. Furthermore, expression of trimeric concatamers with His → Ala mutations at some but not all six positions showed that zinc potentiation correlated with the number of intersubunit histidine pairs. These results indicate that zinc potentiation requires an interaction across a subunit interface. Expression of the H120C/H213C double mutant resulted in the formation of ectopic disulfide bonds that could be detected by changes in the physiological properties of the receptors after treatment with reducing and oxidizing agents. Immunoblot analysis of H120C/H213C protein separated under nonreducing conditions demonstrated that the ectopic bonds were between adjacent subunits. Taken together, these data indicate that His₁²₀ and His₁³¹ sit close to each other across the interface between subunits and are likely to be key components of the zinc binding site in P2X₂ receptors.

Colocalization of zinc with P2X₂ receptors in the nervous system suggests a physiological role for this divalent cation in modulating ATP-evoked currents (3, 4). Extracellular zinc potentiates P2X receptor currents in rat sensory and sympathetic neurons as well as in rat PC12 cells (1). The potentiation effect of zinc on P2X₂ receptors results from a decrease in the EC₅₀ for ATP without a concomitant change in the maximum response to ATP (5, 6).

We have previously identified two molecular determinants of zinc potentiation for the rat P2X₂ subunit (6); mutation of either His₁²₀ or His₁³¹ to alanine eliminated potentiation by zinc. Direct participation of these residues in zinc binding is consistent with the established role of histidines in structurally defined zinc binding sites of proteins (7). However, a role for these residues in binding zinc has been called into question by a report that a treatment expected to modify histidines did not alter zinc potentiation (5). In the first part of this paper we demonstrate that His₁²₀ and His₁³¹ are exposed on the extracellular surface and have a number of characteristics expected of residues directly involved in zinc binding. Further experiments that used mixtures of subunits, each bearing one mutation, to demonstrate that zinc potentiation requires an interaction at the interface between adjacent subunits and that these two histidines are indeed at the subunit interface. Taken together, these data implicate His₁²₀ and His₁³¹ as participants in an intersubunit binding site for zinc.

EXPERIMENTAL PROCEDURES

Mutagenesis and Concatamer Construction—Rat P2X₂ cDNA (encoding a 472-amino acid protein) in pcDNA1 was obtained from Dr. D. Julius, University of California, San Francisco. Mutations were generated using the QuickChange Site-Directed Mutagenesis kit (Stratagene). Concatamers were made using methods similar to those described previously (8). In brief, to allow linkage of multiple subunits, the N-terminal sequence was modified from MVRRAR to MVRSIAR to add an MfeI site, and the C-terminal sequence was modified from DPGLAQ to DPKGLAQ to add an EcoRI site. The H120A, H213A, or the H120A/H213A double mutation was then introduced into these modified monomers. The first subunit of each concatamer was prepared by digestion with EcoRI and ligated to the MfeI-EcoRI digest product of the plasmid encoding the desired second subunit. The resulting dimer was then digested with EcoRI and ligated to the MfeI-EcoRI digest product of the plasmid encoding the desired third subunit. Concatamers thus have splice junctions consisting of DPKGLAQ and DPKGLAQ to add an EcoRI site. The H120A, H213A, or the H120A/H213A double mutation was then introduced into these modified monomers. The first subunit of each concatamer was prepared by digestion with EcoRI and ligated to the MfeI-EcoRI digest product of the plasmid encoding the desired second subunit. The resulting dimer was then digested with EcoRI and ligated to the MfeI-EcoRI digest product of the plasmid encoding the desired third subunit. Concatamers thus have splice junctions consisting of DPKGLAQ. The sequences of mutant monomers and splice junctions of the concatamers were confirmed by a combination of restriction analysis and DNA sequencing (University of Michigan DNA Sequencing Core).

Each concatamer also had a T336C mutation in the third subunit. For all four concatamers studied, we verified that 1 mM MTSET (Toronto Research Chemicals, North York, ON, Canada) inhibited the current to an extent similar to that demonstrated previously for other trimeric concatamers (8).

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1 The abbreviations used are: MTSET, (2-trimethylammonium)-ethyl methanethiosulfonate bromide; DEPC, diethylpyrocarbonate; DTT, dithiothreitol.
Expression of P2X2 Receptors—P2X2 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 (9). RNAs encoding wild type and mutant P2X2 receptor monomers and concatamers were synthesized using the mMessage mMachine T7 kit (Ambion, Austin, TX). Each oocyte was injected with 50 nl of RNA (5–10 ng/µl for monomers, 30 ng/µl for concatamers). Two-electrode voltage clamp experiments were performed 2–5 days after RNA injection. All recordings were made at a holding potential of −50 mV. Recording electrodes were pulled from thin walled borosilicate glass and had resistances of 0.5–1 megarms. 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 8 (Axon Instruments, Union City, CA). Subsequent data analysis was done using Clampfit and Excel. The significance of differences between groups was tested using the two-tailed, unpaired t test function of Excel, with significance taken to be p < 0.01.

Solutions—The external recording solution contained (in mM): 90 NaCl, 1 KCl, 1.3 MgCl2, and 10 HEPES, pH 7.5. Electrodes were filled with an internal solution of 3 M KCl and 400 mM EGTA, pH 7.5. Disodium ATP (Sigma) was prepared as a 100 mM stock in double-distilled water. After the stock concentration of ATP was verified by spectroscopic measurement at 260 nm (BioPhotometer, Brinkmann, New York), Zinc chloride was prepared as a 100 mM stock in double-distilled H2O 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 prior to recording. All ATP recording solutions were used within 48 h.

The histidine-modifying agent diethylpyrocarbonate (DEPC) was diluted to a final concentration of 4 or 7 mM in a phosphate-buffered incubation solution. The 7 mM solution contained (in mM) 94 NaCl, 1.5 NaH2PO4, 6.7 Na2HPO4, 1.0 KCl, 1.7 MgCl2 and was set to pH 7.5. The 4 mM solution contained (in mM) 85 NaCl, 8.75 NaH2PO4, 1.25 Na2HPO4, 2.7 KCl, 1.5 MgCl2 and was set to pH 6.0. Because DEPC degrades more rapidly when exposed to atmospheric conditions, before the solutions were used, we used an experimental protocol that minimized the time between when the solutions were prepared and the oocytes were exposed. A series of oocytes were first characterized for their baseline response to ATP, and each was then placed in a separate well of a 48-well plate. DEPC prepared from a freshly opened bottle was then added rapidly to each well. The time from opening the bottle to exposing the oocytes was typically less than 1 min. After a 10-min incubation in DEPC, the oocytes were washed with incubation solution, returned to the standard HEPES-buffered recording solution, and then retested for their response to ATP.

The sulfhydryl-reactive reagent MTSW was prepared as a 1 mM stock in dimethyl sulfoxide and stored in 10-µl aliquots at −20 °C. For continuous monitoring, MTSW in external recording solution was added to the recording chamber for a 5-min incubation period to produce a final concentration of ~1 mM. However, we more commonly incubated cells in batches, as was done for the DEPC experiments. MTSW was added in the standard HEPES-buffered recording solution. For each construct, pilot experiments were carried out to find the combination of concentration and exposure time which produced a maximal response, and this duration of exposure was used in subsequent experiments. For the cysteine-substituted residues we tested, it required no more than 1 min of exposure to 100 µM MTSW to produce a maximal effect.

The reducing agent dithiothreitol (DTT) was prepared as a 100 mM stock in external solution. DTT was added to the recording chamber for 5–10 min to produce a final concentration of 10 mM. DTT was washed out of the chamber for at least 5 min before recording.

In the zinc protection experiments shown in Fig. 5, oocytes were either treated with a submaximal level of MTSW or preexposed to 100 µM zinc for 1 min and then incubated in 100 µM zinc plus MTSW.

Concentration-Response Analysis—To compare the magnitude of zinc potentiation between groups of cells, it is essential that all cells 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 (5, 6). Furthermore, the EC50 for ATP of different oocytes expressing the same construct can vary significantly (10). In the experiments shown in Figs. 6 and 8, we dealt with these complications by testing each oocyte first with a low concentration of ATP, which we expected would be close to the EC50 based on the average concentration-response relation for each construct, and then with a saturating concentration of ATP, which we hoped would show all constructs used in this study. Only oocytes for which it was verified that the low ATP concentration used was between the EC20 and the EC50 (so that at least a 5-fold increase in current was possible) were included in the data presented in these figures.

Concentration-response relations for ATP were fit to the three-parameter Hill equation using the nonlinear curve fitting program of SigmaPlot 8.0. For displaying average data, the points from each cell 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 S.E. The lines fit to the data indicate the average parameters of the individual fits.

To estimate the concentration-response relation for zinc, it was necessary to correct the data for the inhibition of current by zinc which occurs at high zinc concentrations. This was done using the same methods as in Clyne et al. (6). In brief, the currents in the presence of ATP but no zinc were subtracted from the currents in the presence of ATP plus zinc. The offset data were then scaled up based on the inhibitory zinc concentration-response relation determined from the H120A and H121A mutations. The offset and scaled data were then fit by the same approach as was used to fit ATP concentration-response relations.

Western Blot Analysis—Xenopus oocytes were injected with 50 nl of RNA (10–100 ng/l). After 1–5 days, oocytes were homogenized in buffer H (100 mM NaCl, 20 mM Triz-HCl, pH 7.4, 1% Triton X-100) supplemented with the Complete Mini protease inhibitors (Roche Applied Science). Homogenates were digested for 15 min at 4 °C and then spun for 10 min (20,000 × g) at 4 °C. Laemmli sample buffer (6×) was added at a 1:5 ratio to each sample. Reducing samples contained 10% DTT and nonreducing samples contained 16 mM iodoacetamide (Biorad) to loading buffer. After resolution by SDS-PAGE, proteins were boiled for 5 min. After separation by SDS-PAGE, proteins were blotted to nitrocellulose. Blots were then probed with either a goat polyclonal antibody directed against an extracellular epitope of the human P2X2 receptor (Santa Cruz Biotechnology, Santa Cruz, CA) or a rabbit polyclonal antibody directed against residues 460–472 of the rat P2X2 receptor (Neuromics, Bloomington, MN) and visualized by ECL (Amersham Biosciences).

RESULTS

Evidence that His210 and His213 Are in the Zinc Binding Site of P2X2 Receptors—In zinc-binding proteins with structures that have been solved using x-ray crystallography, histidines are among the most common residues that participate in zinc binding (7). Because the H120A and H121A mutations completely eliminate zinc potentiation of P2X2 receptors (6), an appealing hypothesis is that these two residues are directly involved in zinc binding. However, a study that used DEPC, a compound that would be expected to modify accessible histidines covalently in a way that would make them unable to bind to zinc, reported that DEPC treatment did not attenuate zinc potentiation in wild type P2X2 receptors (5). Because DEPC is reported to have a very short half-life once exposed to air or saline solutions and to react more selectively with histidines at acidic pH (11), we reexamined the effect of DEPC on P2X2 receptors. The significant differences of these new experiments from previous experiments are 2-fold. First, we began the exposure to DEPC within 2 min of opening a fresh bottle. Second, all solutions used to treat oocytes with DEPC had a phosphate buffer to control pH because the 10 mM HEPES used previously as a pH buffer might have been able to bind to the DEPC and make it unable to interact with cellular proteins. After DEPC exposure for 10 min, the oocytes were returned to our standard HEPES-buffered external solution for recording because zinc was precipitated in phosphate-buffered solutions.

We found that when 7 mM DEPC was applied to oocytes...
expressing wild type P2X$_2$ at pH 7.5, there were dramatic decreases in the responses to ATP. On average the currents were less than 5% of control, and many cells that gave large responses prior to DEPC treatment did not respond at all, whether the ATP was applied alone or in the presence of zinc (Fig. 1, A and B). In retrospect, this dramatic effect of DEPC should not have been a surprise, because at pH 7.5 DEPC is known to react with lysine, arginine, cysteine, and tyrosine residues as well as histidines (11), and several lysines and an arginine are believed to be essential elements of the ATP binding site of P2X receptors (12–14).

At more acidic pH levels, the action of DEPC is expected to be somewhat more selective for histidines (11), so we carried out similar experiments on oocytes exposed to 4 mM DEPC at pH 6.0. For oocytes exposed to DEPC at this pH, the maximal ATP-activated currents (elicited by 200 µM ATP) were significantly attenuated in both wild type and the H120A/H213A double mutant (to 33 ± 9% and 45 ± 23% of pretreatment levels, respectively; n = 5 for wild type and 6 for mutant), confirming that much of the inhibitory effect of DEPC was caused by its actions on residues other than His$^{120}$ and His$^{213}$ (Fig. 1, C and D). However, there were sufficient ATP-activated currents remaining to test for zinc potentiation after DEPC treatment at pH 6.0. To maximize the ability to detect zinc potentiation, we used 5 µM ATP in these experiments, which was at approximately the EC$_5$ for the wild type both before and after DEPC treatment (the percent of maximal response before was 4% ± 1% and after was 4% ± 2%). Zinc potentiation (as defined under “Experimental Procedures”) of wild type P2X$_2$ was greatly attenuated after DEPC treatment under these conditions (pre-DEPC potentiation was 16.3 ± 3.1, post-DEPC potentiation was 3.2 ± 1.0, n = 4). In summary, our results demonstrate that under suitable conditions, DEPC attenuates zinc potentiation of P2X$_2$ and are therefore consistent with the possibility that His$^{120}$ and His$^{213}$ are part of the zinc binding site. However, these experiments do not demonstrate that the inhibitory effect we observed required DEPC binding to these particular histidines.

His$^{120}$ and His$^{213}$ Are Accessible on the Cell Surface—For His$^{120}$ and His$^{213}$ to be candidates for participating in zinc binding, they must be accessible on the extracellular surface. As a test of surface accessibility of positions 120 and 213, we made cysteine mutants at each position because cysteines are also competent to coordinate zinc (7) and because many cysteine-reactive reagents are available. We then tested for the ability of the sulfhydryl-reactive reagent MTSET to modify the ATP-activated currents. These two mutants had ATP concentration-response relations relatively similar to wild type (EC$_{50}$ values in µM were 19.6 ± 1.1 for wild type, 24.9 ± 1.9 for H120C, and 32.8 ± 1.6 for H213C; n = 5, 13, and 16, respectively), and wild type and both mutants had peak currents to saturating ATP of ~10 µA.

When the concentration of ATP was selected so that the response to ATP alone was just detectable (2 µM for wild type H120C and 5 µM for H213C), the maximal potency that could be obtained with 20 µM zinc was significantly larger in oocytes expressing wild type P2X$_2$ than in oocytes expressing the mutants (Fig. 2). After oocytes were incubated with 5 mM MTSET for 1 min (which was more than 50 times the exposure required to produce maximal modification), there was a dramatic decrease in the zinc potentiation of the responses of H120C- and H213C-expressing oocytes but no change in zinc potentiation in the oocytes expressing wild type P2X$_2$ (Fig. 2, A and B). For H120C-expressing oocytes, the responses to low ATP alone were also decreased significantly after MTSET treatment, whereas for H213C-expressing oocytes only the responses in ATP plus zinc were attenuated (Fig. 2B). The effect of MTSET on zinc potentiation of these mutants was quite specific because the EC$_{50}$ for ATP after MTSET treatment was not changed for the wild type or either mutant (p > 0.1, Fig. 3), and there was little change in the peak response to a saturating concentration (500 µM) of ATP (as a percentage of the peak response prior to MTSET, the responses after MTSET were 90.6 ± 9.6, 95.9 ± 4.6, and 91.1 ± 2.4, respectively, for wild type, H120C-, and H213C-expressing oocytes).

In wild type P2X$_2$ receptors, zinc potentiation is an allosteric process that shifts the concentration-response relation for ATP to the left with little if any change in the Hill coefficient or maximum response. The magnitude of the zinc potentiation observed therefore varies greatly depending on the concentration of ATP used, with the largest potentiation obtained at 2 µM, a concentration that produces about 1% of the maximal...
current in the absence of zinc. When the amount of potentiation to 20 μM zinc was plotted as a function of ATP concentration, there was no effect of MTSET on oocytes expressing wild type P2X2 at any concentration of ATP, but both H120C- and H213C-expressing oocytes showed less zinc potentiation after MTSET treatment at all concentrations of ATP that gave zinc potentiation prior to application of MTSET. The effect of MTSET was particularly apparent when these data were plotted on log-log coordinates (Fig. 2C).

A second way to quantify the ATP dependence of zinc potentiation was to measure the change in the EC_{50} to ATP when zinc was present (Fig. 3). The extent of the shift in the EC_{50} was calculated as (EC_{50} without zinc/EC_{50} with zinc). The oocytes expressing wild type receptors showed a substantial left shift to 20 μM zinc, and there was no change in the magnitude of left shift after MTSET treatment (before, 5.6 ± 0.8-fold; after, 5.5 ± 0.3-fold). The two mutants were less sensitive to 20 μM zinc than the wild type receptors, and both were even less sensitive after MTSET treatment. The left shift in the EC_{50} for H120C was 2.4 ± 0.1-fold before MTSET and 1.2 ± 0.1-fold after, whereas for H213C the left shift was 1.8 ± 0.1-fold before MTSET and 1.3 ± 0.1-fold after. However, there was a small residual potentiation to 20 μM zinc after maximal MTSET treatment in both mutants.

In summary, the residues at both positions 120 and 213 are accessible to MTSET, and MTSET modification of cysteines substituted at these positions attenuates zinc potentiation, as would be expected if the native histidines at these positions participate in binding zinc.

H120C and H213C Changed Receptor Properties in Ways Expected for Zinc Binding Site Mutants—If the residues at positions 120 and 213 are in the zinc binding site, it is unlikely that substituting a cysteine for one of the native histidines would produce a site of identical zinc affinity. We therefore determined the zinc concentration-response relations of these mutants before and after treatment with MTSET (Fig. 4). Before MTSET treatment, the oocytes expressing the H120C mutant had zinc concentration-response curves that were left shifted compared with wild type, whereas the oocytes expressing the H213C mutant had zinc concentration-response curves that were right shifted compared with wild type (the respective average EC_{50} values were: wild type, 7.9 μM; H120C, 3.2 μM; H213C, 19.9 μM). There were two factors that contributed to the decline in zinc potentiation after MTSET treatment. First, the EC_{50} for zinc for H120C was shifted more than 25-fold to the right by MTSET treatment, and there was also a modest right shift for H213C (1.8-fold).

The upper traces are normalized to the amplitude of the currents in each oocyte in response to 200 μM ATP alone before and after MTSET treatment. The time scale is identical for all responses to ATP alone before and after MTSET treatment span the same distance (dashed lines) and emphasize the change in the amount of zinc potentiation after MTSET treatment. The time scale is identical for all traces in A and B. C, zinc potentiation (defined as (current in low ATP + 20 μM zinc/current in low ATP)−1) is shown for a series of oocytes studied at different concentrations of ATP. The error bars indicate the S.E.; n = 6 for wild type, 13 for H120C, and 16 for H213C. Additional data were collected for 200 and 500 μM ATP, but these are not shown because there was no significant zinc potentiation at these saturating ATP concentrations before or after MTSET. The dashed horizontal line indicates the maximal zinc potentiation observed in wild type P2X2. The dotted vertical lines indicate the average EC_{50} for each construct.
to the concentration-response relations (not shown) indicated that the maximum response to ATP which could be obtained with a saturating concentration of zinc was considerably lower after MTSET treatment. For H120C studied with 2 μM ATP, the estimated maximal current for a saturating concentration of zinc after MTSET treatment was 29% ± 7% of the current produced before MTSET treatment (n = 8). For H213C studied with 5 μM ATP, the estimated maximal current for a saturating concentration of zinc after MTSET treatment was 25% ± 6% of the current produced before MTSET treatment (n = 3).

If a cysteine residue is participating in a zinc binding site, then when zinc is bound to it, the cysteine should be inaccessible to modification by MTSET. The binding of MTSET is irreversible, whereas the binding of zinc is reversible, so the steady-state level of inhibition produced by MTSET is expected to be the same regardless of whether zinc is present. To test for competition we therefore identified a concentration of MTSET that produced significant but not maximal inhibition (1 min of 0.2 μM for H120C and 2 min of 1 μM for H213C), so that protection would be evident as a lessening of MTSET inhibition. We found that 100 μM zinc significantly protected the H120C and H213C mutants from inhibition by MTSET (Fig. 5). As a control for the specificity of this treatment, we tested whether I328C, a residue near the mouth of the pore that is known to be sensitive to MTSET (15, 16), was protected from...
MTSET modification (1 μM for 2 min) by 100 μM zinc. Zinc produced no protection in I328C; in fact on average there was greater inhibition of I328C when 100 μM zinc was present during the MTSET incubation (Fig. 5G).

Intersubunit Interactions Are Required for Zinc Potentiation—If His120 and His213 are components of the zinc binding site, the two histidines within a single subunit could form an intrasubunit binding site, as is the case for the N-methyl-D-aspartate receptor (17). Alternatively, the histidines between adjacent subunits could form an intersubunit binding site, as is the case for γ-aminobutyric acid type A receptors (18) and glycine receptors (19). A recent study using cross-linking to study the extracellular loop of P2X2 receptors (20) established that the N-terminal end of one loop is in close proximity to the C-terminal end of the loop of an adjacent subunit, as might be expected if His120 and His213 form an intersubunit zinc binding site.

As an initial test of whether His120 and His213 participate in an intersubunit interaction in P2X2 receptors, the H120A and H213A mutants were coexpressed in *Xenopus* oocytes at a 1:1 ratio. In cells expressing equal amounts of the two subunits, 75% of the receptors are predicted to be a mixture of subunits (two H120A and one H213A or one H120A and two H213A), whereas the other 25% will be homotrimeric H120A or H213A receptors (Fig. 6A). If zinc binding occurs within a single subunit, then zinc potentiation should be absent because every subunit has one of the mutations. However, if zinc binding occurs between subunits then 75% of the receptors should have one functional binding site and might be capable of potentiation. Indeed, zinc significantly increased ATP-evoked currents in oocytes coexpressing the two mutant subunits, but not in oocytes expressing either mutant on its own (Fig. 6B). To develop a quantitative index of the amount of zinc potentiation, the currents in response to a low concentration of ATP with and without 20 μM zinc and to 200 μM ATP without zinc were measured (for details on how the low ATP concentration was selected, see “Experimental Procedures”). For cells expressing wild type P2X2, H120A, H213A, or coexpressing H120A and H213A, the average potentiation to high ATP was near 9, indicating that each construct was tested with a low ATP concentration close to the EC10 (Fig. 6C). Because zinc potentiation does not increase the current beyond the maximum response to a saturating concentration of ATP (5, 6) the maxi-
mal possible potentiation by zinc of the response to an EC₁₀ concentration of ATP would also be 9, and wild type P2X₂ showed zinc potentiation close to the maximum possible (7.8). The zinc potentiation observed in oocytes coexpressing the mixture of these two mutant subunits was significantly smaller (1.5) than wild type P2X₂ receptors (Fig. 6).

To characterize further the intersubunit dependence of zinc potentiation, we made a series of concatameric receptors by splicing three coding units together. The trimers were constructed from P2X₂ monomers that were slightly modified at their N- and C-terminal ends to facilitate concatenation (see “Experimental Procedures”). To determine whether P2X₂ concatamers are expressed as full-length trimers, proteins from oocytes expressing wild type P2X₂, or trimer HH-HH-HH, AA-AA-HH, HA-AH-HH, or HA-AA-AH were subjected to SDS-PAGE and immunoblot analysis (Fig. 7). A single protein band of ~190 kDa was present for all four concatameric receptors, indicating that they were processed into full-length trimers. Thus, in contrast to results reported for P2X₁ concatamers (21), P2X₂ concatamers did not appear to aggregate or be expressed.
 maximal currents tending to have lower EC50 values. The response curve varied from cell to cell with oocytes with higher trimers remain intact.

Using P2X1 concatamers. First, the C-terminal intracellular tail is quite a bit longer in P2X2, which may allow the adjacent subunits to adopt more easily the folding conformation necessary for assembly. Second, the P2X1 concatamers contained polyglutamine linkers, which may have promoted aggregation, whereas our P2X2 concatamers were linked by modestly modified versions of the endogenous N and C termini. Similar to previously described P2X2 trimeric concatamers (8), all of our trimeric constructs were functional (Fig. 8), although the concatameric constructs consistently desensitized more rapidly than the wild type monomers. As was the case for wild type P2X2, the parameters of the concentration-response curve varied from cell to cell with oocytes with higher maximal currents tending to have lower EC50 values. The average parameters of the concentration-response curves for all four trimers were within the range of values that can be observed in wild type P2X2 receptors expressed in oocytes (H120C/H213C, the single mutant H120C, the single mutant H213C, or the double mutant H120C/H213C) appeared as a single band of about 63 kDa. Under nonreducing conditions the major band for wild type P2X2, the single mutant H120C, the single mutant H213C, or the double mutant H120C/H213C was expressed in oocytes and only a very low level of zinc potentiation was observed (Fig. 9A). However, when these oocytes were treated with the reducing agent DTT, the current evoked by 10 μM ATP increased more than 20-fold, and robust zinc potentiation was restored (Fig. 9B). In contrast, no change in response to DTT was observed in wild type oocytes (Fig. 9C). The increase in responsiveness to ATP and zinc after DTT treatment of H120C/H213C-expressing oocytes lasted for 24 h, so presumably the cell surface is not a sufficiently oxidizing environment to reform the disulfide bonds once they are broken. However, a 5-min treatment with the oxidizing agent H2O2 was sufficient to return the receptors to the low sensitivity state, and a second application of DTT could return the receptors to the high sensitivity state. It should be noted that at sufficiently high concentrations, the H120C/H213C double mutant responded to ATP even when it was in the oxidized state and that DTT treatment dramatically left shifted the ATP concentration-response relation (Fig. 9D).

The physiological results with DTT treatment demonstrated that when cysteines are at positions 120 and 213 they are in close enough proximity to form a disulfide bond but are equally consistent with the two residues interacting at a subunit interface or within a single subunit. Western blot analysis showed that the interaction is between subunits (Fig. 10). The illustrated data are from one of five experiments that gave similar results. Under reducing conditions, proteins from oocytes expressing wild type P2X2, the single mutant H120C, the single mutant H213C, or the double mutant H120C/H213C appeared as a single band of about 63 kDa. Under nonreducing conditions the major band for wild type P2X2, H120C-, or H213C-expressing oocytes was slightly smaller (most likely because of the five endogenous intrasubunit disulfide bonds). In addition, a minor band consistent with subunit dimers was observed in some experiments (although not in the experiment shown in Fig. 10), but a band of the size expected for subunit trimers was never seen for these three types of subunits.

In truncated monomeric and dimeric forms. We suspect that two factors might account for the differences between our results on P2X2 concatamers and the results of Nicke et al. (21) using P2X1 concatamers. First, the C-terminal intracellular tail is quite a bit longer in P2X2, which may allow the adjacent subunits to adopt more easily the folding conformation necessary for assembly. Second, the P2X1 concatamers contained polyglutamine linkers, which may have promoted aggregation, whereas our P2X2 concatamers were linked by modestly modified versions of the endogenous N and C termini.

To test for potentiation to 20 μM zinc, a concentration of ATP that produced approximately an EC10 response was selected (Fig. 8C). Zinc potentiation in the concatamer with all six histidines intact (trimer HH-HH-HH) did not differ significantly from zinc potentiation when three monomers assembled independently (Fig. 8D). As predicted by the hypothesis that His120 and His213 from adjacent subunits participate in a zinc binding site, they must be within a few angstroms of each other (23). This prediction was tested with both physiological and biochemical experiments. These experiments built upon the observation that both H120C- and H213C-expressing oocytes are capable of potentiating to zinc. We therefore predicted that if both cysteine mutations were present, these residues might be close enough to form an ectopic disulfide bond. When the H120C/H213C double mutant was expressed in oocytes and tested with 10 μM ATP, the ATP-evoked currents were tiny, and only a very low level of zinc potentiation was observed (Fig. 9A). However, when these oocytes were treated with the reducing agent DTT, the current evoked by 10 μM ATP increased more than 20-fold, and robust zinc potentiation was restored (Fig. 9B). In contrast, no change in response to DTT was observed in wild type oocytes (Fig. 9C). The increase in responsiveness to ATP and zinc after DTT treatment of H120C/H213C-expressing oocytes lasted for 24 h, so presumably the cell surface is not a sufficiently oxidizing environment to reform the disulfide bonds once they are broken. However, a 5-min treatment with the oxidizing agent H2O2 was sufficient to return the receptors to the low sensitivity state, and a second application of DTT could return the receptors to the high sensitivity state. It should be noted that at sufficiently high concentrations, the H120C/H213C double mutant responded to ATP even when it was in the oxidized state and that DTT treatment dramatically left shifted the ATP concentration-response relation (Fig. 9D).

The physiological results with DTT treatment demonstrated that when cysteines are at positions 120 and 213 they are in close enough proximity to form a disulfide bond but are equally consistent with the two residues interacting at a subunit interface or within a single subunit. Western blot analysis showed that the interaction is between subunits (Fig. 10). The illustrated data are from one of five experiments that gave similar results. Under reducing conditions, proteins from oocytes expressing wild type P2X2, the single mutant H120C, the single mutant H213C, or the double mutant H120C/H213C appeared as a single band of about 63 kDa. Under nonreducing conditions the major band for wild type P2X2, H120C-, or H213C-expressing oocytes was slightly smaller (most likely because of the five endogenous intrasubunit disulfide bonds). In addition, a minor band consistent with subunit dimers was observed in some experiments (although not in the experiment shown in Fig. 10), but a band of the size expected for subunit trimers was never seen for these three types of subunits. In contrast, the protein detected in material from oocytes expressing the H120C/H213C double mutant expressed two prominent bands near the size expected for a cross-linked trimer when run under nonreducing conditions, but not when run under reducing conditions. This is a direct demonstration that this pair of cysteines can form intersubunit disulfide bonds. The two bands might represent differences in glycosylation, but also might represent complexes held together by two versus three such bonds. There was also a band at the monomer size, but no band at the size of a dimer, suggesting that once the receptor assembled to a state in which disulfide bond formation is possible, cysteines at no fewer than two of the three subunit interfaces formed bonds. The monomer may arise mainly from intracellular subunits that have not yet assembled. A second confirmation that H120C and H213C can form a highly specific cross-link between adjacent subunits was obtained by studying material from oocytes coexpressing the two single mutants H120C and H213C. As illustrated in Fig. 6, in an experiment that mixes subunits with two different mutations, no more than one subunit interface of a trimeric receptor can have both a mutation at His120 and a...
mutation at His\textsuperscript{213} present. It therefore is predicted that the receptor proteins from such oocytes studied under nonreducing conditions would show monomer and dimer, but no trimer, and that is exactly the result that was obtained. These results make clear that His\textsuperscript{120} and His\textsuperscript{213} lie close to each other at the interface between two adjacent subunits.

**DISCUSSION**

**The Role of His\textsuperscript{120} and His\textsuperscript{213} in Binding Zinc**—In our previous work, we demonstrated that either the H120A or H213A mutation could eliminate zinc potentiation. Because histidines are present in the zinc binding sites of many structurally characterized proteins, they seemed to be good candidates in the P2\textsubscript{X}\textsubscript{2} receptor as well. However, a previous report had suggested that zinc modulation of P2\textsubscript{X}\textsubscript{2} currents was not sensitive to DEPC, which is known to modify histidines covalently. This raised the possibility that His\textsuperscript{120} and His\textsuperscript{213} might be buried and not have a direct role in binding zinc. We showed here that the previously reported DEPC results were misleading. It is possible that the problem with the earlier experiments was that there was HEPES in the incubation buffer, which interacted avidly with the DEPC, leaving little available to interact with the receptor. In the experiments reported here, a phosphate buffer was used.

Five lines of evidence based on studies of H120C and H213C

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**Fig. 8. Concatameric constructs suggest an intersubunit zinc binding site.** A, predicted number of intrasubunit and intersubunit binding sites in four different concatameric receptor constructs. The format of this figure is similar to Fig. 6A, except that the concatameric constructs have only one N terminus and one C terminus. B, responses of Xenopus oocytes expressing the indicated subunits to a low concentration of ATP, the same low concentration of ATP plus 20 \textmu M zinc, and then a high concentration of ATP that produced a maximal response in all of these constructs (200 \textmu M). The concentration of ATP was selected to produce a response to ATP in the absence of zinc which was ~10\% of the maximal response in the same cell (see C). The low ATP concentration was 7.5 \textmu M for trimer HH-HH-HH, 5 \textmu M for HA-AH-HH, 10 \textmu M for P2\textsubscript{X}\textsubscript{2}, and 12.5 \textmu M for AA-AA-HH and HA-AA-AH. C, potentiation to high ATP was calculated as in Fig. 6. These data are taken from the same oocytes used to test zinc potentiation in D. Each bar is the mean \pm S.E. for 7–10 oocytes. There was no significant difference among the five constructs tested. D, zinc potentiation was calculated as in Fig. 6. A single asterisk indicates that the mean was significantly different from oocytes expressing P2\textsubscript{X}\textsubscript{2}, and from oocytes expressing trimers HH-HH-HH, HA-AH-HH and HA-AA-AH. A double asterisk indicates that the mean was significantly different from oocytes expressing P2\textsubscript{X}\textsubscript{2}, and from oocytes expressing trimers HH-HH-HH, HA-AA-HH, and AA-AA-HH. A triple asterisk indicates that the mean was significantly different from oocytes expressing P2\textsubscript{X}\textsubscript{2}, and from oocytes expressing trimers HH-HH-HH, HA-AA-AH, and AA-AA-HH.
mutants are consistent with the hypothesis that the residues in these positions bind zinc. First, the H120C and H213C receptors are both potentiated by zinc. This is the expected result if these histidines are in the zinc binding site because it is well established from studies of zinc finger transcription factors that cysteines and histidines are interchangeable at zinc binding sites. Second, the EC50 values of H120C and H213C for zinc are significantly shifted from that of wild type, as might be expected for binding site residues, because of the size difference between histidine and cysteine. Third, zinc potentiation is greatly attenuated when either H120C or H213C is treated with MTSET. This is direct evidence that positions 120 and 213 are accessible on the surface as is required if they are to participate in binding extracellular zinc. Fourth, H120C and H213C are partially protected from MTSET when zinc is present. Fifth, the residual zinc potentiation remaining for H120C after maximal exposure to MTSET has a significantly right shifted EC50.

It should be noted that although the H120C and H213C mutations had many similar properties, they were not identical. A likely explanation is that zinc binding sites typically have three or four ligating residues (7, 23) and that the disruption of the zinc binding site is slightly different depending on whether His120 or His213 is mutated. It was also somewhat of a surprise that maximal treatment with MTSET did not completely eliminate zinc potentiation because the H120A and H213A mutants, which might seem to be a less drastic alteration, did eliminate all potentiation. One possible explanation for the failure to eliminate completely zinc potentiation with saturating levels of MTSET is that it is not possible to bind...
MTSET to all three cysteines present in homotrimeric H120C or H213C receptors, and it is zinc binding to the unmodified sites that produces the residual MTSET sensitivity. However, it is also possible that receptors modified by MTSET on all three cysteines can still respond to zinc but in a way different from unmodified receptors.

The Zinc Binding Site Is at a Subunit Interface—Four lines of evidence indicate that the zinc binding site is at the interface between subunits. First, mixing P2X2 subunits that contain the zinc-insensitive H120A mutation and subunits that contain the zinc-insensitive H213A mutation produces receptors that are zinc-sensitive. Thus, an interaction between subunits is required for zinc potentiation. Second, in trimeric concatamers in which three subunits are linked together, the concatamer with histidines at both 120 and 213 in a single repeat but no paired histidines at a subunit interface (AA-AA-HH) did not show any zinc potentiation. In contrast, a concatamer with paired histidines at one subunit interface (HA-AH-HH) showed much less potentiation than the wild type. A concatamer with paired histidines at two subunit interfaces (HA-AH-HH) gave significantly more zinc potentiation than the concatamer that allowed for interaction at only one subunit interface. These data support the inference that the zinc binding site is at a subunit interface and show that occupancy of more than one site by zinc gives greater potentiation. Third, the H120C/H213C double mutant gave virtually no current in response to 10 μM ATP, a concentration that effectively activated wild type receptors and both single mutants. However, after exposure to the reducing agent DTT, currents from the double mutant were greatly enhanced, indicating that an ectopic disulfide bond formed when cysteines were present at both positions 120 and 213. Receptors previously enhanced by DTT treatment could be returned to the low response state by reoxidation with H2O2, indicating that these residues are within a few angstroms of each other on the cell surface in functioning receptors. Fourth, when proteins from oocytes expressing wild type P2X2 or the single mutants H120C and H213C were studied, the detected protein appeared as monomers under reducing and nonreducing conditions. In contrast, the protein detected in material from oocytes expressing the H120C/H213C double mutant had prominent bands at the size expected for a cross-linked trimer when run under nonreducing conditions, but not when run under reducing conditions. This is a direct demonstration that this pair of cysteines can form an intersubunit disulfide bond. Additional confirmation that H120C and H213C can form a highly specific cross-link between adjacent subunits was obtained by Western blot studies of proteins from oocytes coexpressing the two single mutants H120C and H213C. These results demonstrate that His120 and His213 of P2X2 lie close to each other at the interface between two adjacent subunits. In summary, multiple lines of evidence support the conclusion that zinc binds to P2X2 receptors at the interface between subunits and that the binding site involves histidines 120 and 213.

Implications for the Location of the ATP Binding Site—In both glutamate receptors and Cys-loop receptors, allosteric modulators bind at a site physically far from the agonist binding site (17–19, 24), so identification of the location of the zinc site involved in potentiating P2X2 receptors gives no direct information about where ATP binds to the receptor. Indeed, because His120 and His213 are not conserved in other P2X receptors, it seems unlikely that residues at these positions play an essential role in ATP binding. However, in the two well characterized families of ligand-gated channels, there is a striking correlation between the type of interface at which agonist binding sites and allosteric modulator binding sites are found. In the ionotropic glutamate receptors, both the glutamate binding site (25, 26) and the allosteric zinc binding site (17) are intrasubunit, whereas in the γ-aminobutyric acid type A and glycine receptors, both the agonist binding sites (27, 28) and the allosteric zinc binding sites (18, 19, 24) are intersubunit. If our assignment of His120 and His213 to a direct role in zinc binding is correct, these correlations suggest that the ATP binding site of P2X receptors might be intersubunit as well. Is this consistent with other information available regarding the location of the ATP binding site?

For both glutamate receptors (26) and nicotinic receptors (29), crystallization of a protein that has the essential features of the ligand binding domain has been achieved, and the structure of this ligand binding domain has been solved. This has not yet been achieved for P2X receptors, so the available information on the structure of P2X receptors comes from mutagenesis studies and modeling studies. Mutagenesis studies have implicated residues spread over most of the extracellular domain of P2X receptors as potentially involved in ATP binding (30). Jiang et al. (12) came up with two independent lines of evidence to support the idea that the region in the vicinity of
Lys<sup>69</sup> of P2X<sub>2</sub> is involved in ATP binding. Ennion et al. (13) presented evidence that the equivalent region of P2X<sub>2</sub> is part of the ATP binding site, and there is also additional evidence consistent with direct roles of Phe<sup>185</sup>, Phe<sup>291</sup>, Arg<sup>292</sup>, and Lys<sup>309</sup> of P2X<sub>1</sub> in ATP binding (13, 31). Modeling studies have placed a focus on residues in the C-terminal half of the extracellular domain as potentially being involved in ATP binding. By searching for possible folding motifs, Freist et al. (32) suggested that a portion of the extracellular domain of P2X receptors might fold into a six-stranded antiparallel β-pleated sheet structure in a manner similar to class II aminoacyl-tRNA synthetases. This idea has recently been extended significantly by experimental work on P2X<sub>1</sub> (14). Yan et al. examined a series of mutations of amino acids that would be predicted to be near the ATP binding site of P2X receptors, like the binding site of glutamate receptors. This idea has recently been extended significantly by experimental work on P2X<sub>4</sub> (14). Yan et al. examined a series of mutations of amino acids that would be predicted to be near the ATP binding site of P2X receptors, like the binding site of glutamate receptors.

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