A Putative Extracellular Salt Bridge at the Subunit Interface Contributes to the Ion Channel Function of the ATP-gated P2X2 Receptor

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The recent crystal structure of the ATP-gated P2X4 receptor revealed a static view of its architecture, but the molecular mechanisms underlying the P2X channels activation are still unknown. By using a P2X2 model based on the x-ray structure, we sought salt bridges formed between charged residues located in a region that directly connects putative ATP-binding sites to the ion channel. To reveal their significance for ion channel activation, we made systematic charge exchanges and measured the effects on ATP sensitivity. We found that charge reversals at the interfacial residues Glu63 and Arg274 produced gain-of-function phenotypes that were cancelled upon paired charge swapping. These results suggest that a putative intersubunit salt bridge formed between Glu63 and Arg274 contributes to the ion channel function. Engineered cysteines E63C and R274C formed redox-dependent cross-links in the absence of ATP. By contrast, the presence of ATP reduced the rate of disulfide bond formation, indicating that ATP binding might trigger relative movement of adjacent subunits at the level of Glu63 and Arg274, allowing the transmembrane helices to open the channel.

P2X receptors (P2XRs) are membrane cation channels gated by extracellular ATP. They are widely distributed in excitable and nonexcitable cells of vertebrates (1) and play key roles in synaptic transmission (2), presynaptic modulation (3), taste sensation (4, 5), pain signaling (6, 7), and intestinal motility (8).

P2XRs are allosteric trimeric ion channels formed by the oligomerization of three identical or homologous subunits (9, 10). Each subunit (there are seven identified so far in mammals, termed P2X1 through P2X7) possesses intracellular N and C termini and two transmembrane segments, termed TM1 and TM2, joined by an extracellular ectodomain. The binding of ATP to the ectodomain promotes the rapid opening of the ion channel, referred to as gating. Once the channel is opened, cations transit through the pore down their electrochemical gradients, leading to the transient influx of sodium and calcium into the cell. This in turn leads to depolarization of the cell and downstream calcium signaling. It is thought that gating involves long range conformational changes that are transduced from the ATP-binding sites to the ion channel and even to the cytosolic domain (11). However, the molecular mechanisms underlying the gating process in P2XR are still largely unknown.

Very recently, the crystal structure of zebra fish P2X4R (zP2X4R) has been solved by x-ray crystallography at a resolution of 3.1 Å (12). The structure was solved in the absence of ATP and probably represents the closed state of the ion channel. The location of the ATP-binding sites remains unknown; however, it has been suggested that the nucleotide binds to deep intersubunit grooves, located on the outside of the trimer, 45 Å from the ion channel domain, and surrounded by conserved residues previously shown to be important for ATP function (12). This structure thus represents an outstanding advance in the P2XR field (13) and should facilitate better understanding of the molecular mechanism that couples ATP binding to ion channel opening.

To gain deeper insight into the mechanism of channel opening, we produced by comparative modeling a P2X2R molecular model based on the crystal structure of zP2X4R. This P2X2 model was used, in particular, to identify salt bridges formed by charged and conserved amino acid residues (Fig. 1). Interestingly, these residues were located mainly in the β-sandwich body (12), a central domain connecting the putative ATP-binding site to the ion channel (Fig. 1A). We mutated, one at a time, these residues into the opposite charge to create charge-repelling interactions that may modify the receptor function. Consecutively, we produced charge swap of pairs of residues causing modifications and predicted to be spatially close, to potentially restore the ionic interaction. Using this approach, we show that charge reversals at Glu63 and Arg274 in P2X2R produced gain-of-function effects that were cancelled upon paired charge swapping, suggesting that a critical intersubunit salt bridge formed between these residues contributes to the ion channel function of the receptor at an unexplored subunit interface area. Disulfide bond formation between engineered cysteines confirmed the close proximity of these residues in the
absence of ATP. By contrast, the presence of ATP reduced the rate of disulfide bond formation, suggesting that during gating, Glu63 and Arg273 move away from each other. Based on these results, we propose that this inter-subunit salt bridge contributes to the gating of the ion channel.

**EXPERIMENTAL PROCEDURES**

**Mutagenesis**—pcDNA plasmid containing the cDNA encoding the rat P2X2 subunit tagged at the C terminus with the Myc sequence (EQKLISEEDL) was generously provided by F. Rassendren (CNRS, Montpellier, France). P2X2-2T was designed by mutating Cys348 and Cys430 to threonine. Site-directed mutagenesis was carried out using the QuikChange® II Site-directed mutagenesis kit (Stratagene), and mutation was confirmed by DNA sequencing.

**Cell Culture and Transfection**—HEK-293 cells were cultured in Dulbecco’s modified Eagle's medium supplemented with 10% fetal bovine serum (Invitrogen), 1× GlutaMax, 100 units/ml penicillin, and 100 μg/ml streptomycin (Invitrogen). Trypsin-treated cells were seeded onto glass coverslips in 35-mm dishes (for patch clamp experiments) or in 100-mm dishes (for biochemical experiments) pretreated with poly-L-Lysine (Sigma) 1 day before transfection and incubated at 37°C with 5% CO2. Transfections were carried out using calcium phosphate precipitation. For patch clamp experiments, cells were transfected with the P2X2 constructs (0.1–0.3 μg) and a green fluorescent protein cDNA construct (0.3 μg) to identify cells that were efficiently transfected. Cells were washed 1 day after transfection with fresh medium and used within 24 h. For biochemical experiments, cells were transfected with the P2X2 constructs (5 μg) and used 1 day after.

**Electrophysiology**—Currents were recorded using the whole-cell configuration of the patch clamp technique only from fluorescent cells. Cells were maintained at a holding potential of −60 mV. Patch pipettes (3–5 megaohms) contained 140 mM KCl, 5 mM MgCl2, 5 mM EGTA, 10 mM HEPES, pH 7.3. External solution contained 140 mM NaCl, 2.8 mM KCl, 2 mM CaCl2, 2 mM MgCl2, 10 mM glucose, 10 mM HEPES, pH 7.3, and was delivered (containing or not containing ATP and/or chemicals) through three parallel tubes placed immediately above the cell. These tubes are displaced horizontally with the aid of a computer-driven system (SF 77A Perfusion fast step, Warner) that were displaced horizontally with the aid of a computer-driven system (SF 77A Perfusion fast step, Warner) that delivers (containing or not containing ATP and/or chemicals) to the gating of the ion channel. These tubes are displaced horizontally with the aid of a computer-driven system (SF 77A Perfusion fast step, Warner) that delivers (containing or not containing ATP and/or chemicals) to the gating of the ion channel.

**Detection of Cross-linked Subunits**—The same protocol as described above was used, except that the non-cleaveable form, cell-impermeant reagent Sulfo-NHS-LC-Biotin was used instead. Solubilization of cells was performed with either 50 μM, 1 mM, or 50 mM iodoacetamide (when indicated) diluted in the lysis buffer. Iodoacetamide was maintained during incubation with neutrophil-agarose beads and was also added in LPS sample buffer. Protein samples were separated by SDS-PAGE as described above, except that no DTT (when indicated) was added in the LPS sample buffer.
equation was fitted (IGOR PRO 5.03) to the data according to Equation 1,

\[
\frac{I}{I_{\text{max}}} = \frac{1}{1 + \left(\frac{[\text{ATP}]}{EC_{50}}\right)^n}
\]

where \(I\) and \(I_{\text{max}}\) are the peak current to a given concentration of ATP and the maximum current, respectively, both normalized to the capacitance of the cell; \(EC_{50}\) is the concentration of ATP giving half response, [ATP] is the concentration of ATP, and \(n\) is the Hill coefficient. The effect of the application of \(H_2O_2\) alone, ATP alone, ATP plus \(H_2O_2\) (as shown in Figs. 5 and 6), or DTT alone on naive cells, was calculated as \(\frac{I_{\text{after}}}{I_{\text{before}}}\) × 100, where \(I_{\text{before}}\) and \(I_{\text{after}}\) are currents recorded before and after treatment, respectively. The effect of reduction (as shown in Fig. 5) after \(H_2O_2\) treatment was then calculated as \(\frac{I_{\text{after}}}{I_{\text{before}}} \times \frac{I_{\text{after}}}{I_{\text{before}}}\) × 100, where \(I_{\text{after}}\) and \(I_{\text{before}}\) are currents recorded before and after application of DTT, respectively. The decrease in ATP responses was plotted versus cumulative time of \(H_2O_2\) exposure and fitted to the single-exponential decay equation,

\[
I_t = I_\infty + A \exp\left(-\frac{t}{\tau}\right)
\]

where \(I_\infty\) and \(A\) are the residual current and maximal amplitude, respectively, \(t\) is the time in seconds, and \(\tau\) is the time constant in seconds. For all experiments, data were analyzed when the variations in the current amplitudes evoked by at least two consecutive ATP applications were lower than 10%. Change in free energy (\(\Delta\Delta G\)) for each mutant (\(mut\)) was computed according to Equation 3,

\[
\Delta\Delta G = RT \ln \frac{EC_{50}^{mut}}{EC_{50}^{WT}}
\]

where \(R = 1.99\) cal/mol/K, \(T = 293\) K, and \(EC_{50}^{mut}\) and \(EC_{50}^{WT}\) are the \(EC_{50}\) for mutant and WT P2X2, respectively. Coupling energy of the interaction between two mutants (\(\Delta\Delta G_{\text{INT}}\)) was computed according to the equation, \(\Delta\Delta G_{\text{INT}} = R\ln \Omega\), where \(\Omega = \frac{EC_{50}^{mut1/mut2}}{EC_{50}^{WT}}\) and where \(EC_{50}^{mut1/mut2}\) is the \(EC_{50}\) for the double mutant. The experimental error of 2\(\sigma\) was calculated for two S.D. from the mean (14). Data were analyzed using one-way ANOVA, followed by a post hoc Dunnett’s test for comparisons of individual mutants against control using STATVIEW 5.0. The level of significance in ATP sensitivity before and after MTS treatment was determined with the appropriate paired Student’s \(t\) test. Results were considered significant for \(p < 0.05\).

Structural Modeling—The sequences were retrieved from the Ligand-Gated Ion Channel data base (15). The sequence alignment was obtained with T-coffee and edited manually (16). Model of the Rattus norvegicus P2X2 receptor was constructed by homology modeling using the structure of Danio rerio P2X4 (12) as a template (Protein Data Bank code 3H9V). Homology modeling was performed with MODELLER 9.7 (17) using default settings; 100 models were prepared, and the best model according to the DOPE energy function was selected. The figures were prepared with PyMOL (DeLano Scientific LLC).

RESULTS

Pairs of Conserved and Spatially Close Charged Residues—We aligned the sequence corresponding to the truncated crystalized form of zP2X4 (\(\Delta zP2X4\)), in which the N and C termini were shortened for crystallization, to that of rat P2X2 (rP2X2). A high sequence identity (48%) was found, providing strong confidence that \(\Delta zP2X4\) is a good template to produce a model by comparative modeling. The rP2X2 molecular model shows a series of extracellular charged amino acid residues that are spatially close (<10 Å between side chains; Fig. 1A) and are well conserved among the P2X family (Fig. 1B). These residues...
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TABLE 1
Functional properties of the investigated mutant receptors

| Mutants         | EC_{50} \mu M | n_{st} | I_{max} \text{pA/picofarad} | n | ΔΔG kcal/mol | Ω | ΔΔG_{INT} kcal/mol |
|-----------------|--------------|--------|-----------------------------|---|--------------|---|-------------------|
| WT P2X2         | 28.7 ± 3.2   | 1.9 ± 0.2 | 361 ± 45                   | 6 | 0            | 0 | 0                 |
| K53D            | 37.0 ± 2.9   | 2.1 ± 0.3 | 7.4 ± 4.0 \text{a}         | 4 | 0.14 ± 0.04  | 0 | 0                 |
| D57K            | 16.9 ± 2.5 \text{a} | 1.7 ± 0.05 | 168 ± 77                   | 5 | -0.33 ± 0.08 | 0 | 0                 |
| E59K            | 14.8 ± 1.1 \text{a} | 2.1 ± 0.1  | 8.5 ± 3.5 \text{a}        | 4 | -0.39 ± 0.04 | 0 | 0                 |
| E63R            | 10.5 ± 1.5   | 1.6 ± 0.1 | 277 ± 82                   | 9 | -0.61 ± 0.09 | 0 | 0                 |
| E63K            | 4.0 ± 0.5 \text{a} | 1.9 ± 0.1  | 240 ± 68                   | 8 | -1.18 ± 0.08 | 0 | 0                 |
| E63A            | 9.9 ± 0.8 \text{a} | 1.4 ± 0.1  | 39 ± 8 \text{a}           | 7 | -0.63 ± 0.05 | 0 | 0                 |
| K195D           | 26.0 ± 7.3   | 2.0 ± 0.05 | 42 ± 19 \text{a}           | 4 | -0.11 ± 0.17 | 0 | 0                 |
| K195E           | 27.9 ± 3.2   | 2.3 ± 0.1 | 95 ± 54 \text{a}          | 5 | -0.03 ± 0.07 | 0 | 0                 |
| K197D           | 28.5 ± 3.0   | 2.2 ± 0.4 | 278 ± 84                   | 5 | -0.01 ± 0.07 | 0 | 0                 |
| D259K           | NR           | NR       | NR                         | 5 | -0.01 ± 0.07 | 0 | 0                 |
| D261K           | NR           | NR       | NR                         | 5 | -0.01 ± 0.07 | 0 | 0                 |
| R274E           | 14.0 ± 2.1 \text{a} | 2.0 ± 0.1  | 246 ± 49                   | 8 | -0.45 ± 0.08 | 0 | 0                 |
| R274A           | 6.3 ± 0.8 \text{b} | 1.8 ± 0.2  | 189 ± 77                   | 6 | -0.91 ± 0.08 | 0 | 0                 |
| K324D           | NR           | NR       | NR                         | 5 | -0.91 ± 0.08 | 0 | 0                 |
| K53D/D259K      | NR           | NR       | NR                         | 4 | -0.91 ± 0.08 | 0 | 0                 |
| K53D/D261K      | NR           | NR       | NR                         | 4 | -0.91 ± 0.08 | 0 | 0                 |
| D57K/K195D      | 48.9 ± 4.0 \text{a} | 1.9 ± 0.2  | 143 ± 65                   | 6 | 0.30 ± 0.05  | 3.2 ± 0.3 | 0.67 ± 0.05 \text{b} |
| D57K/R274E      | 12.6 ± 1.7 \text{a} | 0.9 ± 0.1 \text{a} | 7.0 ± 4.0 \text{a}    | 4 | -0.49 ± 0.08 | 1.5 ± 0.2 | 0.24 ± 0.08      |
| D57K/K324D      | NR           | NR       | NR                         | 4 | -0.49 ± 0.08 | 1.5 ± 0.2 | 0.24 ± 0.08      |
| E59K/K195E      | 35.6 ± 4.4   | 2.4 ± 0.2 | 115 ± 34 \text{a}         | 7 | 0.10 ± 0.08  | 2.5 ± 0.3 | 0.50 ± 0.08 \text{b} |
| E63R/R274E      | 19.8 ± 0.9   | 1.7 ± 0.1 | 284 ± 63                   | 6 | -0.22 ± 0.03 | 3.7 ± 0.2 | 0.77 ± 0.03 \text{b} |
| E63K/R274E      | 30.3 ± 4.9   | 1.6 ± 0.1 | 327 ± 133                  | 5 | 0.01 ± 0.11  | 15.6 ± 2.5 | 1.58 ± 0.10 \text{b} |
| E63A/R274A      | 13.4 ± 1.3 \text{a} | 1.4 ± 0.1  | 191 ± 46                   | 8 | -0.46 ± 0.05 | 6.2 ± 0.6 | 1.05 ± 0.05 \text{b} |
| D259K/K324D     | NR           | NR       | NR                         | 4 | -0.46 ± 0.05 | 6.2 ± 0.6 | 1.05 ± 0.05 \text{b} |
| D261K/K324D     | NR           | NR       | NR                         | 4 | -0.46 ± 0.05 | 6.2 ± 0.6 | 1.05 ± 0.05 \text{b} |

a Values are significantly different from WT P2X2, p < 0.05 (one-way ANOVA).
b Values are significantly different from D57K/R274E, p < 0.05 (one-way ANOVA).

belong to five different segments (namely post-M1 loop, β8-β9 loop, β11-β12 loop, strand β12, and pre-M2 loop) separated in the primary sequence but possibly interacting with each other in the three-dimensional space of the receptor (Fig. 1B). Interestingly, these segments directly connect the putative ATP-binding site to the ion channel, and consequently we hypothesized that interactions between these residues through salt bridges could be involved in the gating process. To test this hypothesis, we disrupted these potential salt bridges by individually reversing the charges and investigated the functional effects thereof.

Effects of Charge Reversals on ATP Activation—We made 12 individual charge reversals in P2X2R and transiently expressed the corresponding mutants in HEK-293 cells. Cell surface expression and functionality of each mutant were determined by biotinylation of plasma membrane-targeted receptors and by patch clamp electrophysiology, respectively (see “Experimental Procedures”).

In whole-cell recordings, ATP evoked robust current densities (I_{max} > 100 pA/picofarad) for five of the mutants comparable with those obtained from the WT P2X2, except for K53D, E59K, K195D, and K195E, for which significant reductions of the current densities were recorded (Table 1). No response to ATP (up to 1 mM) was observed for D259K, D261K, and K324D. Cell surface labeling experiments showed normal expression for E63K, E63R, K197D, and R274E but reduced expression for the other mutants (Fig. 2A). For all mutants, however, total expression (internal plus cell surface) was not altered (Fig. 2B). Overall, cell surface labeling results correlated well with those of the current densities, suggesting that charge reversal at positions 53, 59, 195, 259, 261, and 324 affects normal receptor trafficking. An exception was found for D57K, of which normal current density was recorded, but this mutant

FIGURE 2. Cell surface and total expression of charge reversal and charge swap mutants expressed in HEK-293 cells. A, top, Western blot analysis probed with anti-c-Myc antibody of biotinylated WT and mutant P2X2 receptors, which represent cell surface-targeted receptors. Bottom, summary of the cell surface expression data. The dashed line indicates the level of 2 μM error, which corresponds to ±18%. B, top, corresponding Western blot of total expression probed with anti-c-Myc antibody. Bottom, summary of the total expression data. Data are from 3–7 independent transfections. The dashed line indicates the level of 2 μM error, which corresponds to ±35%. For both A and B, SDS-polyacrylamide gels were run in the presence of DTT. Also indicated is the position of the apparent molecular mass marker (in kDa). WT P2X2 expression was set to 100%.
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**TABLE 2**

Proximity of the investigated charged residues predicted from the model of rP2X2 receptor

| Residues             | Close to* | Location of the interaction |
|----------------------|-----------|-----------------------------|
| Lys53               | Asp57     | Intrasubunit |
| Asp57               | Lys195 and Lys324 | Intrasubunit |
| Glu59               | Lys195     | Intrasubunit |
| Glu63               | Arg274     | Intersubunit |
| Lys195              | Asp63 and Glu59 | Intrasubunit |
| Lys197              | None       | |
| Asp259              | Lys63 and Lys274 | Intrasubunit |
| Asp261              | Lys63 and Lys274 | Intrasubunit |
| Arg274              | Glu63      | Intersubunit |
| Lys274              | Asp57, and Asp259, and Asp261 | Intrasubunit |

* Oppositely charged side chains separated by less than 10 Å.

Charge Swap of Glu63 and Arg274 Restores WT-like ATP Sensitivity—We next tested the effect of the swap of the charges on ATP sensitivity. We considered that if acidic and basic residues were interacting in the WT P2X2R, then this exchange of charges might restore WT-like function. First, we selected only the positions that resulted in significant modifications of the ATP EC50 (Asp57, Glu59, Glu63, and Arg274). The mutants displaying no ATP response and showing dramatic effects on cell surface expression were also included (Asp259, Asp261, and Lys324). Second, we restricted the number of all possible charge swap combinations by selecting oppositely charged side chains that are separated by less than 10 Å in our rP2X2R model (Table 2). By combining these two criteria, we produced the following double mutants: K53D/D259K, K53D/D261K, D57K/K195D, D57K/K324D, E59K/K195E, E63K/R274E, E63R/R274E, D259K/K324D, and D261K/K324D.

Charge swapping for K53D/D259K, K53D/D261K, D57K/K324D, D259K/K324D, and D261K/K324D did not restore either ATP function or cell surface expression (Fig. 2A and Table 1). However, the double mutants D57K/K195D and E59K/K195E were functional and displayed higher EC50 values than those of gain-of-function mutants D57K and E59K (Table 1), suggesting that Asp57 and Glu59 might interact with Lys195.

Remarkably, when the charges were swapped between Glu63 and Arg274, the receptor responded to ATP in a concentration-dependent manner that was very similar to that of the WT P2X2R (Fig. 3A). ATP EC50 values for the double mutants E63K/R274E and E63R/R274E were very similar to that of the WT (Fig. 3B and Table 1), indicating that charge swap completely restored WT-like ATP function. This suggests that Glu63 and Arg274 are spatially close to each other and functionally coupled to contribute to the ATP activation.

Non-additive Effects of Salt Bridge Mutations—Mutant cycle analysis is routinely used to compute the coupling energy between amino acid residues intimately involved in protein-protein interactions on the basis of the free energy change associated with a perturbation (here a mutation) (14) and was previously used to investigate other ligand-gated ion channels (19). If the effects of the mutations are independent, the change in free energy (ΔG) for the double mutant is expected to be the sum of those for the two single mutations, whereas if the mutated residues are energetically coupled, then the change in free energy for the double mutant differs from the sum of the two single mutants. We adopted this concept to investigate the significance of the intermolecular interactions between the residues.

showed little surface expression. Finally, the fact that we detected little surface expression and no ATP-evoked current for mutants D259K, D261K, and K324D prevents us from further evaluating the significance of the charge reversal at these positions upon ATP activation.

Dose-response curves were constructed for functional mutants, and the effective concentration of ATP giving half-response (EC50) as well as the Hill coefficient (nH) were determined (Table 1). For the WT P2X2R, these parameters were in the same range as previously reported (18) (Fig. 3B). ATP EC50 and nH values for all functional mutants were not significantly different from that of the WT P2X2R, except for D57K, E59K, E63R, E63K, and R274E mutants, for which significantly lower EC50 values were recorded (Table 1). The concentration-response curves were accordingly shifted left (see Fig. 3B for E63K and R274E). These data indicate that charge reversal at amino acid residues located in the post-M1 loop and strand β12 on P2X2R produces an increase of ATP sensitivity resulting in gain-of-function phenotypes.
charged residues. Coupling energy (ΔΔG_{INT}) turned out to be small but significant for the pairs D57K/K195D and E59K/K195E, confirming that interactions are present, but the consequence on ATP function is relatively weak (Table 1). By contrast, a significantly higher value of ΔΔG_{INT} was computed for E63K/R274E, although less for E63R/R274E (Fig. 3, C and D, and Table 1), suggesting a strong interaction between Glu63 and Arg274. As a control, we compared these values with that obtained with D57K/R274E, an additional double mutant in which the distance between the side chains of Asp57 and Arg274 was predicted from the model to be longer than 10 Å (Fig. 1A). As expected, ΔΔG_{INT} value for this double mutant was not significant, close to the experimental error (Fig. 3D and Table 1), indicating that the interaction observed between Glu63 and Arg274 is specific for this pair.

It has been previously reported that analysis of double mutant cycles is most reliable for alanine mutations because interactions are mostly abolished without new interactions being formed (20). We thus neutralized Glu63 and Arg274 independently and in combination by introducing alanine mutations. The single mutations E63A and R274A increased ATP sensitivity compared with that of the WT, and, as expected for interacting residues, the ΔΔG for the double mutant E63A/R274A was not the additive sum of the ΔΔG calculated from the single mutants (Table 1, and a significant ΔΔG_{INT} value was computed (Fig. 3D). These data thus confirmed that Glu63 and Arg274 are energetically coupled. Notably, the double alanine mutant did not restore WT-like EC_{50} as successfully as did charge swapping mutants, suggesting that the electrostatic interaction between Glu63 and Arg274 is necessary for normal receptor gating.

**Cysteine Substitutions and Modifications with Charged MTS Reagents**—To confirm the electrostatic nature of the interaction between Glu63 and Arg274 residues, we examined the effects of inserting in real-time positive and negative charges brought by sulphydryl-reactive reagents at these positions substituted by cysteine residues. As detailed in the supplemental material, single cysteine mutants were introduced in the double mutant C348T/C430T (here called P2X2-2T; supplemental Fig. S1). ATP robustly activated the P2X2-2T with an EC_{50} value that was similar to that of the WT P2X2R (Tables 1 and 3), in line with a previously published work (21) reporting that the triple mutant P2X2-3T (C9T/C348T/C430T) remained functional. Both single mutants E63C and R274C were functional, and cell surface expression appeared normal for E63C. However, similar to the D57K mutant, surface expression was very low for R274C (total expression appeared normal; see supplemental Fig. S2), but current density was not significantly different from that of P2X2-2T (Table 3).

We then examined the effects of modifying E63C with the negatively charged sulphydryl-reactive reagent MTSES. MTSES applied alone on cells expressing the mutant for 60 s modified the ATP concentration-response curve (Fig. 4, B and D) when compared with that obtained before treatment (Table 3), resulting in a significant increase of the ATP EC_{50} value and slight decrease of n_{H} with no significant change of I_{max}, whereas the same treatment had no significant effect on P2X2-2T-expressing cells (Fig. 4, A and D). Interestingly, the ATP EC_{50} value after MTSES application on cells expressing E63C was indistinguishable from that of WT (Table 3), suggesting that the negatively charged tethered sulfonate moiety accommodates well to the glutamate side chain cavity.

We applied the same protocol to cells expressing R274C, except that the positively charged MTSET reagent was used instead. MTSET did not significantly change the ATP concentration-response curve of P2X2-2T when compared with that before treatment (Fig. 4E and Table 3). However, a significant rightward shift of the concentration-response curve was observed for R274C with a slight decrease of I_{max} and larger decrease of n_{H} (Fig. 4, C and E, and Table 3), suggesting that the trimethylammonium group was covalently attached to the cysteine. Overall, these results might indicate that MTSES and MTSET interacted respectively with the positively and negatively charged residues Arg274 and Glu63 to restore an electrostatic interaction.

**Disulfide Bond Forms between E63C and R274C**—The coupling energy observed for charge swapping between Glu63 and Arg274 suggested that the charged residues are separated by 5 Å or less (14). We thus carried out cysteine cross-linking experiments to confirm the spatial proximity between these residues by introducing in combination E63C and R274C in the P2X2-2T background. This background is appropriate for studying disulfide bond formation induced by the oxidizing reagent hydrogen peroxide (H_{2}O_{2}) because a recent work has shown that mutating Cys430 to alanine produced a receptor that was insensitive to H_{2}O_{2} (22). The double mutant E63C/R274C was functional but showed very weak cell surface expression (supplemental Fig. S2), preventing, as for single cysteine mutants, the monitoring of disulfide bond formation on Western blot. However, the double mutant displayed sufficient currents, although the I_{max} value was significantly reduced (Table 3), for monitoring possible redox-dependent modifications by patch clamp electrophysiology. The ATP EC_{50} value for E63C/R274C lies significantly in between those of single cysteine mutants and P2X2-2T (Table 3). In addition, the Hill coefficient value significantly fell to unity when compared with that of P2X2-2T (Table 3).

We directly probed the extent of spontaneous cross-linking by measuring the effect of H_{2}O_{2} on currents evoked by three repeated applications of ATP spaced by 10–30 s at a low con-
centration of ATP (~EC\textsubscript{20} = 40) for the single cysteine mutants and P2X2-2T and at a higher concentration for E63C/R274C (~EC\textsubscript{70}) because of low current density (Table 3) and then by recording on the same cell the effect of the reducing reagent DTT, known to reduce specifically disulfide bonds. H\textsubscript{2}O\textsubscript{2} (0.3%, 120 s) and DTT (10 mM, 120 s) had no significant effect on ATP-evoked currents from P2X2-2T and single cysteine mutants (Fig. 5, A and B). However, H\textsubscript{2}O\textsubscript{2} significantly reduced ATP-evoked currents for the double mutant receptor E63C/R274C, and subsequent application of DTT significantly potentiated currents (Fig. 5, A and B), providing strong evidence that a disulfide bond is formed between E63C and R274C. Interestingly, the amplitude of the potentiated current after subsequent applications of H\textsubscript{2}O\textsubscript{2} and DTT was larger than that of initial current (compare \(I_{\text{after}}\) for H\textsubscript{2}O\textsubscript{2}/DTT and \(I_{\text{before}}\) in Fig. 5A), suggesting that some disulfide bonds were spontaneously formed. This was further confirmed by the fact that potentiation (224 \pm 66%, \(n = 7\)) of the current was observed after application of DTT to naive cells. Following reduction by DTT, currents progressively returned within 2–5 min to initial current \(I_{\text{before}}\) (data not shown). Overall, these results (i) demonstrate that E63C and R274C are relatively proximal in the closed state of the receptor, (ii) show that disulfide bond formation affects ATP activation of the receptor, and (iii) probably indicate the existence of two different populations of channels in cells expressing E63C/R274C, one with spontaneous cross-linked cysteines and a second with reduced cysteine.

Presence of ATP Reduces the Rate of Engineered Disulfide Bond Formation—We next determined whether H\textsubscript{2}O\textsubscript{2} could promote disulfide bond formation between E63C and R274C in the presence of ATP. To achieve this goal, we would have to apply H\textsubscript{2}O\textsubscript{2} plus ATP for 120 s, because so far we had applied H\textsubscript{2}O\textsubscript{2} for 120 s (Fig. 5). Because sustained ATP application potentially induces strong desensitization and/or rundown of the currents, we decided to determine first if shorter applications of H\textsubscript{2}O\textsubscript{2} alone still sufficiently reduce ATP-evoked currents. We thus performed a kinetic analysis of inhibition by H\textsubscript{2}O\textsubscript{2} alone on cells expressing E63C/R274C by recording at least two consecutive 30 s ATP-evoked test responses separated by a 10–60-s application of 0.3% H\textsubscript{2}O\textsubscript{2} (Fig. 6, A and B). We found that the rate of H\textsubscript{2}O\textsubscript{2}-induced inhibition was fast (\(\tau = 13.0 \pm 1.8\) s) and that the inhibition level reached a plateau that corresponded to 50% of the initial ATP-evoked currents (Fig. 6B). We thus decided to use a 20-s co-application of 0.3% H\textsubscript{2}O\textsubscript{2} plus 100 \(\mu\)M ATP. We chose 100 \(\mu\)M ATP for co-application because this concentration is known to be the maximal dose evoking the highest open channel probability (23). Co-application of ATP plus H\textsubscript{2}O\textsubscript{2} did not significantly reduce subsequent ATP responses (Fig. 6C, bottom) when compared with currents recorded after application of ATP alone for 20 s (Fig. 6C, A and B).
6C, top), contrasting with the significant inhibition recorded after application of \( \text{H}_2\text{O}_2 \) alone (Fig. 6D). These results strongly suggest that in the presence of ATP, no substantial disulfide bond formation induced by \( \text{H}_2\text{O}_2 \) occurred within 20 s.

**DISCUSSION**

Using a charge reversal scanning approach, followed by charge swapping and chemical modification of cysteine-substituted mutants, we have identified a critical intersubunit salt bridge formed between Glu\(^{63} \) and Arg\(^{274} \) in P2X2R that contributes to the ion channel function of the receptor. Engineered disulfide bond trapping experiments further confirmed the close proximity of the residues substituted by cysteines. We found that oxidation with \( \text{H}_2\text{O}_2 \) decreased ATP-evoked currents in the E63C/R274C double mutant, whereas reduction with DTT potentiated currents, showing that disulfide bond formation affects activation by ATP. In proteins of known crystal structure, the average separation between disulfide-bonded cysteine \( \alpha \)-carbons is 5.6 Å (24). To form a disulfide bond, cysteines must approach this distance for a productive sulfhydryl collision; however, geometrical factors, including the average distance separating the two cysteines, the relative side chain orientations, and dynamic factors, such as thermal mobility, might affect sulfhydryl collision frequency and consequently disulfide bond formation. It has been shown in the \( \text{d} \)-galactose chemosensory receptor (25) that when a cysteine pair exceeds the distance between the sulfhydryl collision spheres, a minimum translational motion, ranging from 4.5 to 15.2 Å, is required for a productive sulfhydryl collision. We observed that only a fraction of disulfide bonds was spontaneously formed and that \( \text{H}_2\text{O}_2 \) increased rates of disulfide bond formation in the absence of ATP, suggesting that the separation between \( \alpha \)-carbons of residues 63 and 274 must exceed 5.6 Å in the closed state of the receptor. This is in line with the atomic model of rP2X2R built on the recent crystal structure of zfP2X4R, which shows an average distance of \( \sim 10 \) Å between the \( \alpha \)-carbons (Fig. 7B). This implies that there are some thermal fluctuations of the protein backbone in the apo form of the receptor, at least at this subunit interface region.

Side chains of Glu\(^{63} \) and Arg\(^{274} \), which are larger than those of cysteines, are in close contact according to our molecular model (average of \( \sim 3.6 \) Å from the three interfaces), suggesting a possible electrostatic interaction between these residues, such as a salt bridge (Fig. 7B). Our results show that mutations disrupting this interaction produce gain-of-function phenotypes. Two possible mechanisms can account for these data. First, this interaction might contribute directly to the ATP binding affinity, and one may envision that disrupting the salt bridge could remodel the shape and/or the electrostatic potential of the ATP-binding pocket in such a way that ATP binds with a higher affinity to its site. A second hypothesis is that the electrostatic interaction is located far away from the ATP-binding site and

**FIGURE 5.** Engineered disulfide cross-linking experiments support interaction between positions 63 and 274. **A**, top, a 120-s application of 0.3% \( \text{H}_2\text{O}_2 \) (first arrow) reduces responses evoked by 30 \( \mu \text{M} \) ATP in the E63C/R274C double mutant introduced in the P2X2-2T background. A subsequent 120-s application of 10 mM DTT (second arrow) on the same cell potentiates responses. \( I_{\text{before}} \) and \( I_{\text{after}} \) represent responses before and after \( \text{H}_2\text{O}_2 \) application, respectively, and \( I_{\text{after H}_2\text{O}_2/\text{DTT}} \) represents those after \( \text{H}_2\text{O}_2 \) and DTT applications. Note that \( I_{\text{after H}_2\text{O}_2/\text{DTT}} \) is larger than \( I_{\text{before}} \). Bottom, the same protocol applied on the P2X2-2T receptor has no effect on responses evoked by 10 \( \mu \text{M} \) ATP. **B**, average of the data using the protocol shown in **A** for E63C/R274C (\( n = 11 \)), E63C (3 \( \mu \text{M} \) ATP, \( n = 4 \)), R274C (3 \( \mu \text{M} \), \( n = 6 \)), and P2X2-2T (\( n = 5 \)). The dashed lines indicate the 2\% levels for inhibition and potentiation, which correspond to \( \pm 12 \) and \( \pm 26\% \), respectively. *, values are significantly different from E63C, R274C, and P2X2-2T.
Critical Electrostatic Interaction for ATP Function in P2X2R

As discussed earlier (31), increasing \( P_o \) should decrease the value of EC_{50} for agonists, by increasing the gating equilibrium constant (defined as opening rate constant/shutting rate constant). We found indeed that the ATP EC_{50} values for R274C and D57K were significantly lower than that of their respective controls (Tables 1 and 3). Although further experiments, such as single channel analysis, may shed light on this mechanism, these data suggest, but do not prove, that the mutation increases the open probability of the ion channel \( P_o \). This in turn might increase current densities, and the resulting compensating effect would be that although surface expression is low, \( I_{max} \) would remain unchanged.

In the crystal structure of zfP2X4R, which probably represents the closed state of the ion channel (12), Arg^{280} (homologous to Arg^{274} in rP2X2) interacts with the adjacent subunit yet through another residue located on strand \( \beta9 \), Asn^{204} (supplemental Fig. S3). Interestingly, Arg^{274} is conserved only in subtypes that display low ATP sensitivity (P2X2, P2X4, and P2X7), whereas in subtypes showing higher ATP sensitivity (P2X1 and P2X3), smaller residues, not prone to contact the adjacent subunit, are found (Fig. 1B). Regardless of the mechanism by which the difference in ATP sensitivity occurs (an effect on ATP binding affinity or gating), an attractive hypothesis would be that the absence of the arginine in P2X1R and P2X3R influence at least partially ATP sensitivities. Although this point deserves to be addressed for P2X1 and P2X3, a recent work has shown that mutating Arg^{276} in murine P2X7 (corresponding to Arg^{274} in rP2X2) into alanine also resulted in a gain-of-function phenotype (32). Finally, a natural polymorphism exists for this residue in human P2X7, although its phenotype is still undefined (33), emphasizing this region as a potential target for allosteric regulation of human P2XR.

We found that the presence of ATP reduces the rate of disulfide bond formation. Two mechanisms are possible. First, ATP binding directly prevents disulfide bond formation through steric hindrance; second, the ATP-binding site and the action site of \( \text{H}_2\text{O}_2 \) are topologically distinct, but interact with each other through an allosteric cross-talk. Although we cannot firmly rule out the first possibility because the structure of zfP2X4 was solved in the absence of ATP, we favor the second possibility for the reason discussed above. We propose that disulfide bond formation is unfavorable during activation contributions to the gating equilibrium between the closed and open states. Although the two mechanisms remain possible, we favor the second one for the following reason. In light of the recent crystal structure of zfP2X4 (12) and of our homology model, compelling evidence has shown that residues important for ATP binding (26–30) are positioned close to each other, in a deep intersubunit pocket, located more than 20 Å from the investigated area (Fig. 7A). New crystal structures in the presence of ATP are now needed to definitively localize the ATP-binding pocket.

For some mutants (namely D57K and R274C) we found that current densities were not significantly different from those of their respective controls, but cell surface expression of these mutants was very low. The reason for this unusual observation is not clear, but one possibility might be related to a compensatory mechanism that involves the open probability (\( P_o \)) of the WT P2X2 ion channel. At a saturating concentration of ATP, this value was found to be lower than unity (0.6) as determined by single channel analysis in HEK-293 cells (23). This means that ATP does not gate all the time the ion channel and consequently behaves as a low efficacy agonist on the WT P2X2 receptor. This also means that \( P_o \) can theoretically be increased.
Critical Electrostatic Interaction for ATP Function in P2X2R

FIGURE 7. Glu63 and Arg274 form a salt bridge in a rat P2X2R molecular model. A, location of the identified residues viewed laterally. The upper part (top) and bottom (base) of the β-sandwich, along with the TM domain, are indicated. The star marks one of the three fenestrations between subunits. Also indicated are the residues previously shown to be important for ATP action (Lys192, Lys71, and Lys589) and ion permeation (Thr336, Thr339, and Ser340), the β-strand numbers, and one of the three TM2s. Also shown is the distance between the investigated region and the putative ATP-binding site. B, slab view of the bottom part of the ectodomain from the extracellular side (indicated by a dashed bar in A), along the ion pore axis, showing the pair of interacting residues at the subunit interface. Also shown is the distance between the α-carbons. Each subunit is depicted in a different color. C, close-up view of the salt bridge formed between the negatively charged Glu63 residue from one subunit (shown in yellow) and the positively charged Arg274 residue from another (blue).

because the separation and/or orientation between the engineered cysteines are different from those in the absence of ATP (i.e., closed state). This suggests that in the WT receptor Glu63 and Arg274 move away from each other during gating. At present, we cannot determine which state is stabilized or destabilized by the salt bridge, but the fact that the rate of disulfide bond formation is reduced in the presence of ATP along with the fact that charge reversals produce gain-of-function phenotypes indicate that the putative salt bridge stabilizes the closed rather than the open state of the receptor. Additional experiments, such as a single channel analysis, will be needed to clarify this hypothesis.

Our results show that allosteric motions of adjacent subunits at the level of Glu63 and Arg274 occur as a key part of the gating process of P2X2R. Other groups have previously highlighted other critical interfacial regions of the receptor: the upper part of the TM domain (34), the putative ATP-binding site (35, 36), and the allosteric zinc-binding site (37). In addition, a very recent report has shown that the opening of the ion channel involves anticlockwise rotation and separation of TM2 (38), providing evidence that α-helices framing the ion channel pore are submitted to structural reorganization during gating. It thus appears that the subunit–subunit interface is critical for the gating process in P2XR. In light of the zfP2X4 crystal structure, a molecular mechanism, previously anticipated (12), can now be extended: ATP binding at the subunit interface, close to residues shown beforehand to be important for ATP binding (e.g., Lys69, Lys71, and Lys808) causes rearrangements of the extensive subunit-subunit contacts, in particular those located at the upper region of each β-sandwich body (top domain in Fig. 7A). This in turn triggers movement of one rigid β-sandwich relative to another, allowing the base of the extracellular body domain, directly connected to the upper region of the TM domain through post-M1 and pre-M2 loops, to change the conformation of the transmembrane helices (Fig. 7A). This conformational change ultimately causes the helices to move to an open conformation, enabling residues identified as part of the ion selective pore (39, 40) (Thr336, Thr339, and Ser340) to face the ion-conducting pathway. The putative salt bridge identified here is located at an ideal interfacial position. Coming from the TM domain, it is the first extracellular interaction of the base domain that contacts two adjacent subunits through strand β1 of one β-sandwich and strand β12 of another. Moreover, the salt bridge is located just above fenestrations, large openings between subunits thought to represent one of the access routes for ions (12) (Fig. 7A). Thus, the presence of this putative electrostatic interaction at this special location might represent a powerful means for influencing conformational changes between subunits involved in gating motions of P2X2R.

Acknowledgments—We thank Prof. M. Goeldner and Drs. A. Specht and S. O’Regan for critical reading of the manuscript. We are grateful to Drs. F. Rassendren and P. Séguela for generously providing the P2X2 plasmids.

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