Salt Bridge Switching from Arg290/Glu167 to Arg290/ATP Promotes the Closed-to-Open Transition of the P2X2 Receptor

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Received July 23, 2012; accepted October 5, 2012

ABSTRACT

P2X receptors are trimeric adenosine-5’-triphosphate (ATP)-gated cation channels involved in fast signal transduction in many cell types. In this study, we used homology modeling of the rat P2X2 receptor with the zebrafish P2X4 X-ray template to determine that the side chains of the Glu167 and Arg290 residues are in close spatial vicinity within the ATP-binding pocket when the rat P2X2 channel is closed. Through charge reversal mutation analysis and mutant cycle analysis, we obtained evidence that Glu167 and Arg290 form an electrostatic interaction. In addition, disulfide trapping indicated the close proximity of Glu167 and Arg290 when the channel is in the closed state, but not in the ATP-bound open state. Consistent with a gating-induced movement that disrupts the Glu167/Arg290 salt bridge, a comparison of the closed and open rat P2X2 receptor models revealed a significant rearrangement of the protein backbone and the side chains of the Glu167 and Arg290 residues during the closed-to-open transition. The associated release of the Glu167/Arg290 salt bridge during channel opening allows a strong ionic interaction between Arg290 and a γ-phosphate oxygen of ATP. We conclude from these results that the state-dependent salt bridge switching from Arg290/Glu167 to Arg290/ATP fulfills a dual role: to destabilize the closed state of the receptor and to promote the ionic coordination of ATP in the ATP-binding pocket.

Introduction

P2X receptors are adenosine-5’-triphosphate (ATP)-gated cation channels assembled from a repertoire of seven homologous subunits (P2X1 though P2X7) into homotrimers and heterotrimers (Nicke et al., 1999; North, 2002; Kaczmarek-Hajek et al., 2012). A crucial advance in the understanding of the P2X2 receptor at the molecular level is the resolution of the X-ray crystal structure of the zebrafish P2X4.1 (zfP2X4) receptor in the apo-closed and the ATP-bound open state (Hattori and Gouaux, 2012). The zfP2X4 protomer structure has a dolphin shape (Kawate et al., 2009; Hattori and Gouaux, 2012). The zfP2X4 protomer structure has a “dolphin” shape (Kawate et al., 2009) (see Fig. 1B) in which the intersubunit ATP-binding pocket is formed by the head domain, the upper body, and the left flipper of one subunit and the lower body and dorsal fin of an adjacent subunit. In the X-ray structure of the ATP-bound zfP2X4 receptor, the ATP molecule is accommodated into the binding pocket in a U-shaped configuration in which the β- and γ-phosphates are positioned toward the adenine ring (Hattori and Gouaux, 2012). This conformation allows the formation of a series of salt bridges and hydrogen bonds between the acidic phosphate oxygen atoms of ATP and a cluster of highly conserved basic and polar residues, including Lys70 and Lys72 of one subunit and Asn296, Arg298, and Lys316 of the adjacent subunit (Hattori and Gouaux, 2012). The corresponding residues have also been suggested to be involved in ATP binding in other P2X receptor subtypes based on the results of mutagenesis studies and computational homology modeling (Guérlet et al., 2008; Roberts et al., 2008, 2012; Ennion et al., 2000; Roberts and Evans, 2004; Fischer et al., 2007; Allsopp et al., 2011; Du et al., 2012).

Normal mode analysis and data from P2X2 mutants with introduced histidines, which allow Zn2+-mediated domain bridging, have showed that ATP binding induces the head domain and dorsal fin to move closer to each other, which leads to a tightening of the ATP-binding pocket (Jiang et al., 2012). This conformational closure of the intersubunit cleft between the head and dorsal fin domains was also observed in a comparison of the X-ray structures of the apo- and ATP-bound states of the zfP2X4 receptor (Hattori and Gouaux, 2012). The extracellular vestibule of the ATP-binding pocket is simultaneously enlarged by the outward flexion and rotation

ABBREVIATIONS: ATP, adenosine-5’-triphosphate; Cy5 NHS ester, Cy5 N-hydroxysuccinimide ester; DTT, dithiothreitol; IC50, 50% inhibitory concentration; αβ-meATP, αβ-methylene-ATP; MTS, methanethiosulfonate; MTSEA, 2-aminoethyl methanethiosulfonate; MTSES, 2-sulfonatoethyl methanethiosulfonate; NF770, 7,7’-(carbonylbis(mimino)-3,1-phenylene)(carbonylmimo)-3,1-(4-methyl-phenylene)(carbonylmimo)bis(1-methoxy-naphthalene-3,6-disulfonic acid); PDB, Protein Data Bank; TEVC, two-electrode voltage-clamp; zf, zebrafish.
of the lower body domains. This movement is transmitted to the directly linked transmembrane domains TM1 and TM2 to induce an iris-like movement of the TM helices, which ultimately opens the ion channel pore (Hattori and Gouaux, 2012). Additional evidence on the rotational conformational changes of TM2, which underlie the pore opening of the P2X receptors and allow ion access to the pore through lateral portals adjacent to the membrane, has also been obtained using mutagenesis data (Li et al., 2010; Cao et al., 2009; Kracun et al., 2010; Samways et al., 2011; Kawate et al., 2011). An extensive rearrangement within the extracellular domain, including the pulling apart of the $\beta$-strands of adjacent subunits, was further suggested by high-resolution electron microscopy and computational studies on the hP2X1 and zfP2X4 receptor, respectively (Du et al., 2012; Roberts et al., 2012).

We previously used the X-ray structure of the closed zfP2X4 receptor (Kawate et al., 2009) (PDB entry 3H9V) as a template to generate a homology model of the rP2X2 receptor. This model allowed us to identify the molecular determinants of the nanomolar potency interaction of the suramin-derivative NF770 with the P2X2 receptor (Wolf et al., 2011). The model predicted critical roles for Glu167 and Arg290 in ATP and NF770 binding, and these predictions were experimentally verified. In the present study, we investigated whether the close proximity of Glu167 and Arg290, which is predicted by our closed-state P2X2 model (Fig. 1A), allows an electrostatic interaction that may be important for receptor function. The residues Glu167 and Arg290 are conserved in all P2X receptor subtypes across several species, which indicates their potential importance in P2X receptor function. We provide evidence that a Glu167/Arg290 salt bridge is formed when the P2X2 receptor closes. This salt bridge is released when Glu167 and Arg290 move away from each other upon ATP binding and channel opening, which permits a strong ionic interaction between Arg290 and a $\gamma$-phosphate oxygen of ATP. By restricting the free movement of Glu167 and Arg290, this salt bridge switching may help guide the conformational transitions that define the closed and ATP-bound open states of the channel.

**Materials and Methods**

**Chemicals.** The methanethiosulfonate (MTS) compounds 2-aminoethyl methanethiosulfonate (MTSEA) and 2-sulfonatoethyl methanethiosulfonate (MTSES) were purchased from Biotium (Hayward, CA). ATP (sodium salt) was purchased from Roche (Roche Diagnostics; Mannheim, Germany). The Cy5 NHS ester was purchased from GE.
The reversibility of the effects of H$_2$O$_2$ were tested by recording concentration cysteine reactivity experiments with oxidizing or reducing agents (Rettinger and Schmalzing, 2003; Hausmann et al., 2006). The constructs were verified using restriction analysis and nucleotide sequencing. The capped cRNAs were synthesized as previously described elsewhere (Schmalzing et al., 1991; Nicke et al., 1998) and injected into collagenase-defolliculated X. laevis oocytes in 50-nl aliquots (0.1 $\mu$g/µl) using a Nanoject 2000 injector (WPI, Sarasota, FL). The oocytes were cultured at 19°C in sterile oocyte Ringer’s solution (ORi: 90 mM NaCl, 1 mM KCl, 1 mM CaCl$_2$, 1 mM MgCl$_2$, and 10 mM HEPES, pH 7.4) supplemented with 50 $\mu$g/ml genanticin.

**Functional Analysis and Modification of Introduced Cysteines.** One to three days after the cRNA injection, the current responses were evoked through ATP at ambient temperature (21–24°C) in a nominally calcium-free ORi solution (designated Mg-ORi: 90 mM NaCl, 1 mM KCl, 2 mM MgCl$_2$, and 10 mM HEPES, pH 7.4) and recorded using a conventional two-electrode voltage-clamp (TEVC) with a Turbo TEC-05 amplifier (NPI Electronics, Tamm, Germany) at a holding potential of ~60 mV, as previously described elsewhere (Rettiger and Schmalzing, 2003; Hausmann et al., 2006). The cysteine reactivity experiments with oxidizing or reducing agents were performed using KCl agar bridge bath electrodes. In the ATP concentration–current response analysis, each P2X2 receptor-expressing oocyte was challenged in 30-second intervals with 15-second pulses of increasing concentrations of ATP.

To determine the effect of the MTS reagents, the oocytes expressing the desired rP2X2 construct were repeatedly stimulated with ATP until a stable current response was obtained. The ATP-evoked current amplitudes were recorded before and after a 120-second wash with Mg-ORi. To determine the effect of MTS on the ATP-bound P2X2 receptor, 3 mM ATP was applied for 3 seconds to open the P2X2 receptor; this step was followed by the coapplication of 3 mM ATP with either 1 mM MTSEA or 1 mM MTSES and a 120-second wash with Mg-ORi.

To indirectly monitor the disulfide bond formation, a minimum of two ATP-evoked current amplitudes were recorded before and after the application of a 120-second wash with 0.3% H$_2$O$_2$, which was followed by a 150-second wash with Mg-ORi. To examine the effect of H$_2$O$_2$ on the open P2X2 receptor, 3 mM ATP was applied for 3 seconds to open the receptor, followed by the coapplication of 3 mM ATP and 0.3% H$_2$O$_2$. The reversibility of the effects of H$_2$O$_2$ were tested by recording a minimum of two ATP-evoked current amplitudes from H$_2$O$_2$-treated oocytes before and after a 180-second application of 10 mM dithiothreitol (DTT), which was followed by a 150-second wash with Mg-ORi.

**Assembly and Plasma Membrane Expression of the P2X2 Receptor.** The assembly and plasma membrane expression of the various P2X2 receptor constructs were analyzed using blue native-PAGE (BN-PAGE) and SDS-PAGE, as previously described elsewhere (Hausmann et al., 2012; Fallah et al., 2011).

**Data Analysis.** The data were plotted and fitted using Prism5 (GraphPad Software Inc., San Diego, CA). The agonist concentration–response curves and EC$_{50}$ values were obtained by iteratively fitting the four-parameter Hill equation to the pooled data points from n oocytes:

$$I / I_{\text{max}} = \frac{top - bottom}{1 + \left( \frac{EC_{50}^{\text{mut}}}{ATP} \right)^n_{\text{Hill}}} + bottom,$$

where $I$ is the response evoked by the ATP concentration ([ATP]), $I_{\text{max}}$ is the maximal response, and $n_{\text{Hill}}$ is the Hill coefficient. The bottom level was constrained to zero. Each oocyte was challenged with seven increasing concentrations of ATP ranging from 1 µM to 1 mM or from 3 µM to 3 mM in logarithmically equal steps. The EC$_{50}$ values are presented as the geometric means and the corresponding 95% confidence intervals (95% CI). The mean current amplitudes and Hill slopes are presented as the arithmetic mean ± S.E.M. Error bars were omitted in the figures when these were smaller than the symbols used.

The effect of MTS or H$_2$O$_2$ treatment on the mean current amplitudes mediated by various P2X2 constructs was calculated as follows: ($I_{\text{mut}} / I_{\text{initial}}$) $\times$ 100, where $I_{\text{initial}}$ and $I_{\text{mut}}$ are the averaged ATP current amplitudes measured before and after the indicated treatment, respectively. The effect of the chemical reduction by DTT after H$_2$O$_2$ treatment was calculated as ($I_{\text{after H2O2 + DTT}} / I_{\text{mut}}$) $\times$ 100, where $I_{\text{after H2O2 + DTT}}$ represent the current amplitudes recorded before and after the application of DTT, respectively. Provided that a normal distribution could be verified by the D’Agostino-Pearson omnibus K2 normality test, one-way analysis of variance (ANOVA) followed by a post hoc Bonferroni’s multiple comparison test was used to determine the level of statistical significance between the means, as indicated in the figure legends.

The change in the Gibbs free energy ($\Delta G$) for each mutant (mut) was calculated as

$$\Delta G = -RT \ln \left( \frac{EC_{50}^{\text{mut}}}{EC_{50}^{\text{wt}}} \right),$$

where $EC_{50}^{\text{wt}}$ and $EC_{50}^{\text{mut}}$ represent the EC$_{50}$ values of the wt and mutant rP2X2 receptors, respectively. The constants $R$ and $T$ have the values of 1.987 cal/mol/K and 293 K, respectively. The interaction free energy, which is also designated as the coupling energy of the interaction of two residues ($\Delta G_{\text{INT}}$), was calculated as

$$\Delta G_{\text{INT}} = \Delta G_{\text{mut1+2}} - (\Delta G_{\text{mut1}} + \Delta G_{\text{mut2}}),$$

where $EC_{50}^{\text{mut1}}$ and $EC_{50}^{\text{mut12}}$ represent the EC$_{50}$ values of the single mutants and $EC_{50}^{\text{mut12}}$ represents the EC$_{50}$ value of the corresponding double mutant. The experimental error in the calculated $\Delta G$ and $\Delta G_{\text{INT}}$ values was determined using the lower and upper limits of the 95% confidence interval of the EC$_{50}$ values of the wt rP2X2 receptor and the respective mutants (Schreiber and Fersht, 1995).

**zfP2X4 Structure-Based Homology Models of the rP2X2 Receptor.** The detailed procedure for generating the homology models using the molecular modeling program MOE2008.10 (Molecular Operating Environment 2008; CCG, Montreal, Canada) has been previously described elsewhere (Wolff et al., 2011). To generate the closed- and open-state models of the rP2X2 receptor, we used as templates the X-ray structures of the zfP2X4 receptor in the apo-closed state (PDB entry 3H9V) (Kawate et al., 2009) and the ATP-bound open state (PDB entry 4DW1) (Hattori and Gouaux, 2012), respectively. The final sequence alignment used for the homology modeling is shown in Supplemental Fig. 1. A comparison of the 3H9V-based closed-state rP2X2 model with an identically generated closed-state rP2X2 model based on the recently refined PDB 4DWO structure (Hattori and Gouaux, 2012) revealed that the orientations and side chain directions of Glu167 and Arg290 were the same in both templates. For the sake of consistency, we used the 3H9V-based rP2X2 homology model (Wolff et al., 2011) in the present study. The PDB files...
of the rP2X2 homology modeling data shown in Figs. 1 and 8 are available in the supplemental data.

Results

The Charge-Reversal and Charge-Swap Mutants Suggest That Glu167 and Arg290 Form an Electrostatic Interaction. Our rP2X2 homology model (Wolf et al., 2011) predicts that the carboxylate carbon of Glu167 and the guanidinium carbon of Arg290 are separated by only ∼3.9 Å (Fig. 1A). Glu167 is located at the base of the cysteine-rich head domain directly C-terminal to the β9-strand, which connects the body domain and the cysteine-rich head domain in the upper region of the ATP-binding pocket (Fig. 1B). Arg290 is located in the β16-strand of the body domain at the base of the left flipper (Fig. 1B) and is part of the conserved “NFR” motif, which is known to be crucial for the activation of P2X receptors by ATP. To test for a possible electrostatic interaction between Glu167 and Arg290, we individually reversed the charges and swapped the charges to break and restore the ionic interactions, respectively. The charge reversal of Glu167 with Arg (E167R) resulted in a slight increase in the ATP potency and a 1.5-fold decrease in the mean current amplitude elicited by 1 mM ATP compared with the wt rP2X2 receptor (Fig. 2, A and B; Table 1). The charge reversal of Arg290 by Glu (R290E) decreased the ATP potency by a factor of 624 and decreased the mean current amplitude elicited by 1 mM ATP by 500-fold compared with the wt rP2X2 receptor (Fig. 2, A and B; Table 1). The pronounced effect of the charge reversal at Arg290 might be due in part to the crucial role of Arg290 in ATP binding (Roberts et al., 2008; Hattori and Gouaux, 2012). Biochemical analyses showed that the homotrimeric subunit assembly and the plasma membrane expression of the receptor were not affected by any of the charge-reversal mutations (Fig. 2C).

If the effects of the charge reversals at Glu167 and Arg290 are independent, the effect of the double reversal should be additive, that is, a total 374-fold decrease in the ATP potency and a total 714-fold decrease in the mean current amplitude. However, the charge-swapped mutant E167R,R290E–rP2X2 exhibited a 29- and 2.5-fold decrease in the ATP potency and the mean current amplitude elicited by 1 mM ATP, respectively, compared with the wt rP2X2 receptor (Fig. 2, A and B; Table 1). Thus, the charge swapping rescued the ATP potency by 12.9-fold compared with the effects of the single mutants. A similar 3.9- to 12.2-fold rescue of the ATP potency was observed in all possible charge-swap mutants that can be generated by exchanging Glu167 with Lys or Arg and Arg290 with Glu or Asp. These data are summarized in Table 1.

The Double-Mutant Cycle Analysis Supports an Electrostatic Interaction between Glu167 and Arg290. The functional rescue of the charge-swapped mutants compared with the single-charge reversal suggests that Glu167 and Arg290 form an electrostatic interaction with each other. To further investigate this interaction, we performed double-mutant cycle analysis, which consists of the calculation of the interaction energy between a pair of residues based on the Gibbs free energy change associated with the modification or mutation of these residues (Horovitz, 1996; Hidalgo and Mackinnon, 1995; Serrano et al., 1990; Carter et al., 1984; Schreiber and Fersht, 1995). If the residues do not interact, the change in the free energy of the double mutant should equal the sum of the free energy changes of the corresponding single mutations. In contrast, if the two residues interact, the change in the free energy of the double mutant will differ from the sum of the changes of the corresponding single mutations. Using the changes in the EC50 value, we determined that the additive sum of the changes in the free energy of the single mutants was different from the change in the free energy of the E167R,R290E–rP2X2 double mutant (Table 1). The calculated interaction energy of ∆ΔGINT of −1.45 ± 0.03 kcal/mol (Fig. 3) was significantly larger than the threshold value of ±0.35 kcal/mol that has been reported for noninteracting residues (Schreiber and Fersht, 1995). In general, a negative Gibbs free energy change due to an amino acid mutation indicates that the interacting residues have a stabilizing effect on the structure, whereas a positive free energy change indicates a destabilizing effect (Kumar and Nussinov, 1999). Thus, the interaction free energy ∆ΔGINT of −1.45 that was calculated from the experimentally determined EC50 values suggests that the interaction of Glu167 and Arg290 has a stabilizing effect.

In addition, we used the E167A and/or R290A mutants for double-mutant cycle analysis. The rationale for this experiment was that alanine replacement mutations are less likely to result in new interactions and are thus considered to be superior to other mutations in mutant cycle analysis (Faiman and Horovitz, 1996). The alanine substitution of Glu167 did not affect the ATP potency, whereas the alanine substitution of Arg290 decreased the ATP potency by more than 230-fold (Table 1). An interaction free energy (∆ΔGINT) of −1.11 ± 0.04 kcal/mol, which was also above the threshold of ±0.35 kcal/mol for noninteracting residues (Schreiber and Fersht, 1995), was calculated for the alanine double mutant E167A,R290A–rP2X2.

As negative controls, we mutated the oppositely charged residues Asp57 and Arg275 of the rP2X2 receptor, which are separated by more than 30 Å according to our homology model. Consistent with their noninteraction, the ATP potency of the charge-swapped D57R,R275D–rP2X2 double mutant differed by only ∼1.6-fold from the additive sum of the ATP potencies of the charge-reversal mutants D57R–rP2X2 and R275D–rP2X2. The double-mutant cycle analysis yielded a low interaction energy (∆ΔGINT) of 0.32 ± 0.07 kcal/mol (Fig. 3; Table 1). In addition, the alanine mutants of Asp57 and Arg275 yielded a nonsignificant interaction energy (∆ΔGINT) of −0.31 ± 0.04 kcal/mol, which is below the ±0.35 kcal/mol calculated threshold energy (Schreiber and Fersht, 1995) (Table 1).

The Modification of the Introduced Cysteines with Charged MTS Reagents Supports a Functionally Important Electrostatic Interaction between Residues 167 and 290. To provide further evidence on the electrostatic nature of the interaction between Glu167 and Arg290, we substituted Glu167 and Arg290 individually with cysteine residues. As a background for these mutations, we used an rP2X2 mutant (designated rP2X2C9,348,430S) in which the three cysteine residues, which are located in the cytoplasmic N-terminal domain (Cys9), the C-terminal end of TM2 (Cys348), and the C-terminal domain (Cys430), were replaced by serines. The three Cys-to-Ser mutations were functionally silent in terms of ATP potency and mean current amplitudes (Fig. 2A; Table 1), but prevented the spontaneous intersubunit cross-linking that is observed in wt rP2X2 (Jiang et al., 2010;
Li et al., 2008). Nonreducing SDS-PAGE analysis verified that the rP2X2C9,348,430S receptor did not form any intersubunit cross-links and showed efficient plasma membrane expression of the rP2X2C9,348,430S receptor and the single and double E167C and R290C mutants (Fig. 2D). The E167C mutation had no effect on the ATP potency and decreased the mean current amplitude by a factor of 2. The R290C substitution resulted in a 71-fold decrease in the ATP potency and a 10-fold decrease in the mean current amplitude compared with the parental rP2X2C9,348,430S receptor (Fig. 2A; Table 1). The strong effect of the R290C mutation might be due to the crucial role of Arg290 in ATP binding (Hattori and Gouaux, 2012).

To reintroduce a charge at positions 167 and 290 post-translationally, we used the positively and negatively charged MTS reagents MTSEA and MTSES, respectively; these reagents share a similar molecular mass and thiol reactivity. Accordingly, the differences in the current responses to ATP after treatment with MTSEA or MTSES can be attributed to the addition of a positive or negative charge at the introduced cysteine, respectively. The treatment of the parental rP2X2C9,348,430S receptor with 1 mM MTSEA or MTSES for 120 seconds had almost no effect on the current amplitudes (Fig. 4, left panel; Table 2) or the ATP potency (Table 2). The restoration of the original charges at E167C or R290C by MTSES or MTSEA, respectively, resulted in a partial rescue of the receptor function, which was observed as a 2-fold increase in the EC50 (both mutants, Table 2) and a 2.8-fold increase in the current amplitude (R290C mutant; Fig. 4, right panel; Table 2). In contrast, the introduction of an opposite charge at each position further decreased the function of the receptor, which was observed as a 3-fold and a 1.4-fold increase in the EC50 value of the E167C and R290C mutants (Table 2), respectively, and a 7.5-fold decrease in the mean current amplitude obtained with the R290C mutant (Fig. 4, right panel; Table 2).

These data support the functional importance of the electrostatic interaction of Glu167 and Arg290. The partial restoration of the function of the R290C mutant obtained

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**Fig. 2.** Functional and biochemical characterization of the Glu167 and Arg290 rP2X2 mutants. (A) Bar graph showing the mean current amplitudes (± S.E.M.) elicited by 1 mM ATP in oocytes expressing the indicated wt or mutant rP2X2 receptor. The results for the cysteine mutants, in which cysteines were introduced into the rPX2C9,348,430S receptor background (lacks nonextracellular cysteine residues), are shown as gray bars. (B) ATP concentration–response curves obtained with the indicated wt or mutant rP2X2 receptors. The absolute current amplitudes are indicated. ■, wt-rP2X2, EC50 = 11.4 (95% CI 9.3–14.9) μM, nH = 1.5 ± 0.4, n = 9; ▲, E167R-rP2X2, EC50 = 6.3 (95% CI 5.4–7.6) μM, nH = 1.8 ± 0.2, n = 6; ■, R290E-rP2X2, EC50 = 7192 (95% CI 6393–8607) μM, nH = 2.6 ± 0.3, n = 6; ▲, E167R,R290E-rP2X2, EC50 = 328 (95% CI 288–371) μM, nH = 2.6 ± 0.2, n = 6. (C) The indicated charge-reversal mutants show a wt-like homotrimeric assembly and cell surface abundance. The proteins were purified under non-denaturing conditions from *X. laevis* oocytes using Ni-NTA chromatography, resolved by BN-PAGE (upper panel) or reducing SDS-PAGE (lower panel), and visualized using Typhoon fluorescence scanning. “SDS” in lane 1 indicates the migration of the rP2X2 protein after partial denaturation after a 1-hour incubation with 0.1% SDS before BN-PAGE. The open circles in the left margin indicate the numbers of rP2X2 subunits that correspond to each protein band. (D) The indicated proteins were purified as in C, resolved using nonreducing SDS-PAGE and visualized using Typhoon fluorescence scanning. The rP2X2C9,348,430S receptor and the indicated cysteine mutants are efficiently expressed at the cell surface and do not exhibit intersubunit cross-linking. The asterisk indicates a nonspecific background band that was present in some samples. The numbers in the left margins in C and D refer to the molecular masses of the marker proteins (in kiloDaltons).
through the restoration of the positive charge through the MTSEA reagent indicates that the specific side chain of arginine itself might also be functionally important. The marked changes in the ATP-induced current amplitudes of the R290C mutant treated with MTSEA or MTSES suggest that the charge in position 290 plays a critical role not only in ATP binding (Hattori and Gouaux, 2012) but also in channel gating.

**Disulfide Trapping Confirms the Proximity of Residues 167 and 290.** To confirm the ∼3 Å spatial proximity between Glu167 and Arg290 that was predicted by our homology model (Fig. 1), we substituted Glu167 and Arg290 with cysteines and tested whether these cysteines could be oxidatively cross-linked. The ATP-induced currents recorded from the oocytes that expressed the cysteine double mutant E167C,R290C-rP2X2 were statistically significantly (P < 0.001) lower after a 120-second exposure to 0.3% H2O2 (Fig. 5). The H2O2-induced inhibition of the ATP-evoked current could be partially reversed by the subsequent incubation of the R290C mutant treated with MTSEA or MTSES (see Fig. 2B and Table 1).

### Table 1

<table>
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<tr>
<th>rP2X2</th>
<th>EC50 (95% CI)</th>
<th>EC50^mut/EC50^wt</th>
<th>n_{Hill}</th>
<th>1 mM ATP</th>
<th>I_{max}^mut/I_{max}^wt</th>
<th>n</th>
<th>ΔΔG</th>
<th>ΔΔG_{INT}</th>
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<td>wt</td>
<td>11.4 (9.2–14.9)</td>
<td>15 ± 0.4</td>
<td>11.4 ± 0.7</td>
<td>1.0 ± 0.0</td>
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<td>0.6</td>
<td>2.6 ± 0.2</td>
<td>23.4 ± 1.7</td>
<td>0.4</td>
<td>6</td>
<td>6</td>
<td>1.95 ± 0.07</td>
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<tr>
<td>R290E</td>
<td>7192 (6393–8607)</td>
<td>29</td>
<td>26.2 ± 0.3</td>
<td>4.6 ± 5.1</td>
<td>0.5</td>
<td>5</td>
<td>0.25 ± 0.02</td>
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<td>E167R, R290E</td>
<td>328 (288–371)</td>
<td>29</td>
<td>2.6 ± 0.2</td>
<td>3.4 ± 0.1</td>
<td>4</td>
<td>6</td>
<td>1.23 ± 0.08</td>
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<td>17.5 (14.6–25.6)</td>
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<td>19.0 ± 0.9</td>
<td>4.1 ± 2.3</td>
<td>0.1</td>
<td>5</td>
<td>2.53 ± 0.09</td>
<td>1.46 ± 0.05</td>
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<td>R290D</td>
<td>3538 (2815–4689)</td>
<td>308</td>
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<td>0.08 ± 0.01</td>
<td>0.001</td>
<td>4</td>
<td>3.34 ± 0.01</td>
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<td>E167K, R290D</td>
<td>533 (491–638)</td>
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<td>2.5 ± 0.3</td>
<td>2.6 ± 0.4</td>
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<td>1.9 ± 0.3</td>
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<td>547 (509–652)</td>
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<td>2.4 ± 0.3</td>
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<td>wt</td>
<td>11.4 (8.3–15.5)</td>
<td>1</td>
<td>1.5 ± 0.3</td>
<td>51.0 ± 3.4</td>
<td>1.0</td>
<td>8</td>
<td>3.17 ± 0.01</td>
<td></td>
</tr>
<tr>
<td>E167A, R290A</td>
<td>2663 (2054–3528)</td>
<td>234</td>
<td>2.6 ± 0.3</td>
<td>2.4 ± 1.9</td>
<td>0.04</td>
<td>8</td>
<td>2.06 ± 0.02</td>
<td>-1.11 ± 0.03</td>
</tr>
<tr>
<td>E167D</td>
<td>24.7 (17.9–34.1)</td>
<td>2</td>
<td>1.0 ± 0.2</td>
<td>17.3 ± 2.2</td>
<td>0.3</td>
<td>5</td>
<td>0.44 ± 0.04</td>
<td></td>
</tr>
<tr>
<td>R290K</td>
<td>172 (137–209)</td>
<td>15</td>
<td>1.8 ± 0.3</td>
<td>6.2 ± 1.9</td>
<td>0.1</td>
<td>5</td>
<td>1.58 ± 0.02</td>
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<tr>
<td>E167D, R290K</td>
<td>182 (126–253)</td>
<td>16</td>
<td>1.4 ± 0.5</td>
<td>14.1 ± 7.1</td>
<td>0.3</td>
<td>5</td>
<td>1.61 ± 0.06</td>
<td>-0.41 ± 0.04</td>
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<tr>
<td>D57R</td>
<td>5.1 (3.7–7.1)</td>
<td>0.5</td>
<td>1.3 ± 0.3</td>
<td>61.1 ± 5.5</td>
<td>1.1</td>
<td>10</td>
<td>-0.47 ± 0.05</td>
<td></td>
</tr>
<tr>
<td>D57A</td>
<td>12.8 (9.8–16.6)</td>
<td>1.1</td>
<td>1.3 ± 0.2</td>
<td>54.9 ± 4.2</td>
<td>1.0</td>
<td>9</td>
<td>0.07 ± 0.02</td>
<td></td>
</tr>
<tr>
<td>R275A</td>
<td>139 (117–164)</td>
<td>12</td>
<td>2.0 ± 0.3</td>
<td>39.5 ± 3.6</td>
<td>0.7</td>
<td>10</td>
<td>1.45 ± 0.04</td>
<td></td>
</tr>
<tr>
<td>D57A, R275A</td>
<td>91 (80–104)</td>
<td>8</td>
<td>1.6 ± 0.1</td>
<td>84.6 ± 3.9</td>
<td>1.6</td>
<td>10</td>
<td>1.21 ± 0.06</td>
<td>-0.31 ± 0.04</td>
</tr>
<tr>
<td>rP2X2^mut^1/2</td>
<td>387 (348–4308)</td>
<td>33</td>
<td>2.5 ± 0.3</td>
<td>13.3 ± 0.7</td>
<td>0.2</td>
<td>10</td>
<td>2.94 ± 0.09</td>
<td></td>
</tr>
<tr>
<td>E167C^mut^1/2</td>
<td>10.5 (7.5–14.1)</td>
<td>1^b</td>
<td>1.5 ± 0.5</td>
<td>28.6 ± 4.8</td>
<td>0.5^b</td>
<td>7</td>
<td>0.03 ± 0.03</td>
<td></td>
</tr>
<tr>
<td>R290C^mut^1/2</td>
<td>729 (637–839)</td>
<td>71^b</td>
<td>2.6 ± 0.4</td>
<td>3.0 ± 0.9</td>
<td>0.05^b</td>
<td>7</td>
<td>0.03 ± 0.03</td>
<td></td>
</tr>
<tr>
<td>E167C, R290C^mut^1/2</td>
<td>837 (784–910)</td>
<td>82^b</td>
<td>2.6 ± 0.4</td>
<td>8.4 ± 2.4</td>
<td>0.2^b</td>
<td>8</td>
<td>0.03 ± 0.03</td>
<td></td>
</tr>
</tbody>
</table>

ATP, adenosine-5'-triphosphate; ΔΔG, calculated Gibbs free energy changes of the mutant; ΔΔG_{INT}, calculated interaction free energy (see Materials and Methods); I_{max}, mean current amplitude elicited by 1 mM ATP; n, number of independent measurements; n_{Hill}, Hill coefficient.

**a** Generated using the rP2X2^mut^1/2 receptor.

**b** Normalized to the rP2X2^mut^1/2 receptor.

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**Fig. 3.** Double-mutant cycle analysis of Glu167/Arg290 and Asp57/Arg275 rP2X2 mutants. The ATP EC50 values of the indicated rP2X2 receptor constructs were derived from the concentration-response curves (see Fig. 2B and Table 1). Significant interaction free energies greater than ±0.35 kcal/mol (Schreiber and Frsht, 1995) were obtained with the Glu167/Arg290 pair of residues but not the Asp57/Arg275 pair of residues, which are separated by more than 30 Å in our rP2X2 receptor homology model.
H₂O₂ treatment. This is consistent with experiments in which DTT application increased the ATP-induced current amplitude of the E167C,R290C-rP2X2C9,348,430S receptor without prior H₂O₂ addition (data not shown). These data suggest that the two cysteines can spontaneously form a disulfide bond. Similarly, variable levels of spontaneous disulfide bond formation have been previously observed in different batches of oocytes (Liu et al., 2006). Neither H₂O₂ nor DTT produced a statistically significant change in the current amplitudes (E167C or R290C). Overall, these data demonstrate that H₂O₂ treatment did not significantly affect the EC₅₀ value suggests, but does not prove, that the disulfide bond formation affected the channel gating rather than the ATP binding.

We further examined whether the disulfide bond formation between E167C and R290C occurs in the open state of the rP2X2C9,348,430S receptor. We therefore tested the ability of H₂O₂ to promote cysteine cross-linking in the presence of 3 mM ATP, which is a concentration that approximately corresponds to the EC₉₀ value of the E167C,R290C-rP2X2C9,348,430S receptor (Fig. 5C; Table 1). When H₂O₂ was applied to the open E167C, R290C-rP2X2C9,348,430S channel, only a marginal inhibition of the current amplitude was observed in the continuous presence of ATP (Fig. 6A). In addition, subsequent repetitive stimulations with ATP provided no evidence of the current reduction that is indicative of an inhibitory disulfide bond formation similar to that observed when H₂O₂ was applied in the absence of ATP (Fig. 6A). This finding suggests that the presence of ATP prevents the disulfide bond formation between E167C and R290C by hindering the contact between the two cysteine residues either by steric hindrance or by a conformational increase in the distance between 167C and 290C during ATP-induced channel gating.

We attempted to discriminate between these two possibilities by testing whether the competitive rP2X2 receptor antagonist NF770 blocks the ability of H₂O₂ to promote disulfide formation in the E167C,R290C-rP2X2C9,348,430S receptor. We therefore tested the ability of H₂O₂ to promote cysteine cross-linking in the absence of ATP before and after H₂O₂ treatment. The EC₅₀ value of the E167C,R290C-rP2X2C9,348,430S receptor without channel gating, we performed an ATP concentration-response analysis before and after H₂O₂ treatment. The ATP potencies and agonist-induced current responses of the indicated P2X2 receptor mutants before and after MTS treatment are shown in Table 2.

### Table 2

<table>
<thead>
<tr>
<th>rP2X2</th>
<th>MTS</th>
<th>EC₅₀ (95% CI)</th>
<th>nH</th>
<th>1 mM ATP</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>rP2X2C9,348,430S</td>
<td>—</td>
<td>8.5 (6.4–10.2)</td>
<td>1.6 ± 0.3</td>
<td>54.1 ± 5.3</td>
<td>6</td>
</tr>
<tr>
<td>E167C-rP2X2C9,348,430S</td>
<td>—</td>
<td>12.0 (9.1–15.3)</td>
<td>1.7 ± 0.2</td>
<td>24.9 ± 4.9</td>
<td>6</td>
</tr>
<tr>
<td>R290C-rP2X2C9,348,430S</td>
<td>—</td>
<td>39.6 (33.9–46.1)</td>
<td>1.2 ± 0.3</td>
<td>19.9 ± 1.6</td>
<td>5</td>
</tr>
<tr>
<td>MTS</td>
<td>8.6 (7.9–9.5)</td>
<td>1.4 ± 0.3</td>
<td>55.7 ± 4.7</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>MTS</td>
<td>7.9 (6.9–8.7)</td>
<td>1.6 ± 0.3</td>
<td>47.6 ± 5.8</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>MTS</td>
<td>11.6 (9.8–13.5)</td>
<td>1.8 ± 0.2</td>
<td>29.0 ± 4.9</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>MTS</td>
<td>39.6 (33.9–46.1)</td>
<td>1.2 ± 0.3</td>
<td>19.9 ± 1.6</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>MTS</td>
<td>6.6 (5.8–8.1)</td>
<td>2.3 ± 0.3</td>
<td>29.0 ± 4.9</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>MTS</td>
<td>437 (411–473)</td>
<td>1.8 ± 0.6</td>
<td>8.3 ± 1.0</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>MTS</td>
<td>1284 (1229–1343)</td>
<td>2.3 ± 0.2</td>
<td>0.4 ± 0.1</td>
<td>6</td>
<td></td>
</tr>
</tbody>
</table>

**ATP:** adenosine-5′-triphosphate; CI, confidence interval; MTS, methanethiosulfonate; n, number of independent measurements; nH, Hill coefficient.
receptor even if the channel is closed. We have shown that NF770 fits tightly into the ATP-binding pocket and interacts strongly with Arg290 through a methoxy group (Wolf et al., 2011). Thus, if steric hindrance is responsible for the noninhibitory effect of H2O2 in the presence of ATP, then NF770, similarly to ATP, should diminish the H2O2-induced disulfide cross-linking of E167C and R290C. Upon coapplication with 3 mM ATP, 10 μM NF770 inhibited the ATP-induced current amplitude of the E167C,R290C-rP2X2C9,348,430S mutant by ∼50%. After NF770 washout, the magnitude of the current response to ATP was the same as the current obtained before exposure to NF770 (Fig. 6, B and C). When 10 μM NF770 was coapplied with H2O2 to the closed channel, which was followed by the washout of both compounds, the current response to ATP was diminished to ∼41% (39–44%), which is similar to the extent that was observed when H2O2 was applied in the absence of NF770 (Fig. 6, B and D).

We also examined the extent of inhibitory disulfide bond formation when 3 mM ATP was coapplied with 10 μM NF770 and H2O2 (Fig. 6C). The current amplitude remained stable as long as ATP, NF770, and H2O2 were applied together. After the washout of the NF770 and H2O2 mix, the ATP-induced current amplitude was determined to be 20% smaller than that obtained with the previous H2O2 treatment (Fig. 6, C and D). The data indicate that the fraction of channels that were maintained in the closed state by the competitive inhibition of NF770 was sensitive to the inhibitory disulfide bond formed between E167C and R290C. The observed 20% reduction in the current amplitude corresponds to a ∼40% inhibition of 50% of the E167C,R290C-rP2X2C9,348,430S channels that were maintained in the closed, cross-linkable state by NF770. In other words, the same ∼40% proportion of closed channels became oxidatively blocked regardless of whether all or some of the channels were in the closed conformation. Overall, there seems to be a proportional relationship between the ratio of open to closed channels and the extent of channel inhibition by oxidation. Thus, the NF770 data suggest that the open channel state, as opposed to steric hindrance by a ligand, prevents the disulfide bond formation in the presence of ATP.

To exclude the possibility that ATP sterically blocks access to the cysteine side chains introduced by the E167C and R290C mutations, we examined whether the presence of ATP allows the modification of single cysteine mutants by the cationic or anionic MTS reagents. MTSES produced the same small but reproducible increase in the E167C-rP2X2C9,348,430S receptor-mediated current amplitude in the absence or presence of 3 mM ATP (Fig. 7, left panel). Also the MTSEA-induced increase in the R290C-rP2X2C9,348,430S receptor-mediated current amplitude was the same in the absence and presence of 3 mM ATP (Fig. 7, right panel). Overall, the data suggest that the reduced level of cross-linking in the ATP-bound open state results from a conformational increase in concentration–response analysis yielded EC50 values of 850 (95% CI 6983–1034) μM (nH = 2.7 ± 0.8, Imax = 8.4 ± 0.6 μA, n = 6) and 1002 (95% CI 772–1301) μM (nH = 2.8 ± 0.8, Imax = 5.8 ± 0.6 μA, n = 6) for the nonoxidized (■) and H2O2-oxidized (▲) E167C,R290C-rP2X2C9,348,430S mutants, respectively. The absolute current amplitudes are plotted.
in the distance between Glu167 and Arg290 upon P2X2 receptor activation.

**Discussion**

Ionic interactions, which are also designated as salt bridges, have been identified within and between subunits and have been shown to be critical for gating, pore opening, and stability in various ion channels. In the superfamily of Cys-loop receptors, intrasubunit salt bridges are responsible for coupling agonist binding to channel gating (Kash et al., 2003) and for stabilizing the ligand-bound state by restricting the loop C mobility of GABA<sub>A</sub> receptors (Venkatachalan and Czajkowski, 2008). An intersubunit salt bridge at the β/α interface of the GABA<sub>A</sub> receptor was determined to stabilize the ligand-bound state (Laha and Wagner, 2011). In the monomeric outer-membrane protein A (OmpA) channel in *Escherichia coli*, switching between two salt bridges within the pore defines the closed and open states of the channel (Moroni and Thiel, 2006; Hong et al., 2006).

In this study, we provide different evidence to support the existence of a functionally important electrostatic interaction between Glu167 and Arg290 in the ATP-binding pocket of the closed rP2X2 receptor that is broken upon channel opening: 1) the charge swapping of Glu167 and Arg290 partially rescued the decrease in the receptor function that was caused by the single charge-reversal mutations; 2) the recharging of E167C with the negatively charged MTSES and the recharging of R290C with the positively charged MTSEA partially rescued the rP2X2 receptor function; 3) the modification of E167C or R290C with the respective oppositely charged MTS reagent led to a further decline in the receptor function; 4) the charge-swap (E167R,R290E-rP2X2) and double-charge neutralization (E167A,R290A-rP2X2) mutants nonadditively altered the ATP potency, and the double-mutant cycle analysis revealed significant interaction free energies, which indicated that Glu167 and Arg290 are energetically coupled; and 5) the cysteine substitution of Glu167 and Arg290 resulted in oxidative intrasubunit disulfide cross-linking between these two residues in the closed but not the open state of the P2X2 receptor channel. Based on these data along with the partial blocking of the ATP-induced current responses after disulfide formation, we infer that the salt bridge between Glu167 and Arg290 serves to stabilize the closed state of the receptor.

We consider it unlikely that the Glu167/Arg290 salt bridge is buried and hence water inaccessible for several reasons: 1) according to the rP2X2 homology model, the Glu167 and Arg290 residues line the surface of the ATP-binding pocket, and 2) both the E167C and the R290C mutants react readily
with the charged MTS reagents, which strongly argues against buried positions of the engineered cysteine residues. The hydrophobic solubility of a cysteine residue is sufficiently high to not prevent the burial of the engineered cysteines in the protein core (Nagano et al., 1999; Hessa et al., 2005). In addition, both cysteine residues in the E167C,R290C-rP2X2\textsubscript{C9,348,430} double mutant were readily accessible for oxidative cross-linking, and the disulfide bond that was formed between them could be readily cleaved by chemical reduction. The relatively low $\Delta G_{\text{INT}}$ of less than $|2|$ kcal/mol is also consistent with a localization of the Glu167/Arg290 salt bridge in a hydrophilic environment. Salt bridges within water-accessible, hydrophilic environments are generally known to be of lower strength than ionic interactions within buried hydrophobic regions of the protein interior (Kumar and Nussinov, 1999). Altogether, the negative $\Delta G_{\text{INT}}$ deduced from the mutant cycle analysis can be most readily assigned to a surface-localized, water-exposed salt bridge that stabilizes the closed state of the rP2X2 receptor.

We note that the breaking of a closed-state, stabilizing salt bridge by charge neutralization may be expected to favor channel opening and thus increase the agonist potency. In the large data set analyzed in this study, the E167A mutation did not significantly change the ATP potency of the rP2X2 receptor in either direction. However, breaking a salt bridge by simply mutating one of the pair of charges most likely changes a number of interactions within the protein that contribute to its stability (Serrano et al., 1990). In the case of the rP2X2 receptor, the charge neutralization of Glu167 releases Arg290 from its coupling to the residue at position 167. The uncoupled Arg290 may undergo compensatory interactions with other acidic residues that may cancel out the destabilizing effect of the E167A mutation. Only the double-mutant cycle allows one to isolate the energy of the electrostatic interaction against a complicated background (Horovitz et al., 1990; Serrano et al., 1990). Therefore, the unchanged ATP potency of the E167A mutant does not argue against a stabilization of the closed state of the rP2X2 receptor by the Glu167/Arg290 salt bridge.

The observed disulfide bond formation between E167C and R290C strongly indicates that the distance between these two residues is less than 4.6 Å, which is the maximum distance between the C\textsubscript{\gamma}-carbons of cysteines that can be linked by a disulfide bond (Sowdhamini et al., 1989; Careaga and Falke, 1992a, 1992b). We propose that a gating movement of the E167C and R290C residues upon channel activation by ATP, and not steric hindrance by bound ATP, accounts for the impaired disulfide bond formation between these two residues in the presence of ATP for the following reasons: 1) the oxidative disulfide bridge formation did not affect the ATP potency but reduced the ATP efficacy, which suggests that the disulfide bond interfered with channel gating and not ATP binding; 2) the competitive antagonist NF770, which interacts with several basic residues in the ATP-binding pocket in a manner similar to the phosphate oxygen atoms of ATP (Wolf et al., 2011), did not affect the oxidative disulfide formation; and 3) ATP did not prevent the reaction of E167C and R290C with the MTS reagents, which suggests that the bound ATP does not block the access of the introduced cysteines to the thiol-reactive reagents. Overall, these data suggest that the reduced level of cross-linking in the ATP-bound open-channel state results from a spatial rearrangement of the Glu167 and Arg290 residues in the open-channel state, which is a result of the increased separation of these residues during channel gating.

A ~0.5-Å increase in the side chain distance between the carboxylate carbon of Glu163 and the guanidinium carbon of Arg298 upon the closed-to-open transition is also indicated by a comparison of the apo-closed-state (4DW0) and the ATP-bound structures. The head domain and the C-terminal base of the left "flipper" are located in the upper right and lower left corner, respectively. The polypeptide backbones of the two adjacent rP2X2 subunits that contribute to the ATP-binding site are colored in orange or pink, and selected residues are depicted as sticks. Specific atoms of the amino acid side chains and ATP are colored (carbons are light gray or yellow, oxygens are red, nitrogens are blue, hydrogens are dark gray, and phosphates are pink). A comparison of the closed- and open-state models indicates substantial movement of the protein backbone containing the Glu167 and Arg290 residues, which includes a marked rearrangement of their side chains. In the closed state, Arg290 is found to be mainly in an ionic interaction with Glu167 (dashed light blue lines marked by a gray arrowhead, left panel). In contrast, in the ATP-bound open state, Arg290 is observed mainly in a strong ionic interaction with a γ-phosphate oxygen of ATP (dashed light blue lines marked by a gray arrow, right panel). The thickness of the center of the dashed blue lines indicates the strength of the ionic interaction, which was assessed using the MOE2008.10 program.

![Apo closed state](image.png) ![ATP-bound open state](image.png)

**Fig. 8.** Comparison of the closed- and open-state rP2X2 receptor homology models. Selected details of the interior of the intrasubunit ATP-binding pocket of the apo-closed state (left panel) and the ATP-bound open state (right panel) of the homology-modeled rP2X2 receptor (viewed from the side, parallel to the membrane plane) are presented. The models are based on the closed (PDB entry 3H9V) and open (PDB entry 4DW1) zfP2X4 X-ray structures. The head domain and the C-terminal base of the left "flipper" are located in the upper right and lower left corner, respectively. The polypeptide backbones of the two adjacent rP2X2 subunits that contribute to the ATP-binding site are colored in orange or pink, and selected residues are depicted as sticks. Specific atoms of the amino acid side chains and ATP are colored (carbons are light gray or yellow, oxygens are red, nitrogens are blue, hydrogens are dark gray, and phosphates are pink). A comparison of the closed- and open-state models indicates substantial movement of the protein backbone containing the Glu167 and Arg290 residues, which includes a marked rearrangement of their side chains. In the closed state, Arg290 is found to be mainly in an ionic interaction with Glu167 (dashed light blue lines marked by a gray arrowhead, left panel). In contrast, in the ATP-bound open state, Arg290 is observed mainly in a strong ionic interaction with a γ-phosphate oxygen of ATP (dashed light blue lines marked by a gray arrow, right panel). The thickness of the center of the dashed blue lines indicates the strength of the ionic interaction, which was assessed using the MOE2008.10 program.
open-state (4DW1) X-ray structures of the zP2X4 receptor. The Glu163 and Arg299 residues of the zP2X4 receptor correspond to the Glu167 and Arg290 residues of the rP2X2 receptor, respectively. Moreover, molecular dynamics simulations suggest that the β7-strand and the β-sheet of the β13- to β14-strands, which contain Glu163 and Arg298, respectively, undergo significant movement during channel gating (Du et al., 2012).

The comparison of the rP2X2 receptor homology models, which were based on the closed- and open-state crystal structures of the zP2X4 receptor, reveal substantial movement of the protein backbone containing Glu167 and Arg290 as well as a marked rearrangement of their side chains in the closed-to-open state transition (Fig. 8). The closed-state rP2X2 model shows that Arg290 is mainly in ionic contact with Glu167 (dashed blue lines marked by a gray arrowhead; Fig. 8, left panel). In contrast, in the ATP-bound open-state model of the rP2X2 receptor, Arg290 is mainly detected to form a strong ionic interaction with a γ-phosphate oxygen of ATP (dashed blue lines marked by a gray arrow; Fig. 8, right panel). Thus, because of the spatial rearrangement, the electrostatic coupling between Glu167 and Arg290 is released, which results in a new ionic interaction between Arg290 and ATP. The importance of Arg290 in ATP binding through the coordination of a γ-phosphate oxygen of ATP is indicated in the P2X2 receptor model and supported by the site-directed mutagenesis of Arg290. The charge neutralization at position 290 resulted in a marked decrease in the ATP potency; this effect was further decreased by charge reversal mutants with glutamic acid or aspartic acid. Thus, our results imply that Arg290 has a dual state-dependent role in the P2X2 receptor function: the Arg290/Glu167 salt bridge stabilizes the closed state of the receptor, whereas the Arg290/ATP interaction is of crucial importance for the coordination of ATP in the open ATP-bound state.

Authorship Contributions

Participated in research design: Hausmann, Schmalzing

Conducted experiments: Hausmann, Günther, Kless, Kuhlmann, Markwardt.

Contributed new reagents or analytic tools: Kassack.

Performed data analysis: Hausmann, Günther, Kless, Kuhlmann, Bahrenberg, Markwardt.

Wrote or contributed to the writing of the manuscript: Hausmann, Schmalzing.

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Molecular Pharmacology

Salt bridge switching from Arg290/Glu167 to Arg290/ATP promotes the closed-to-open transition of the P2X2 receptor

Ralf Hausmann, Janka Günther, Achim Kless, Daniel Kuhlmann, Matthias U. Kassack, Gregor Bahrenberg, Fritz Markwardt, and Günther Schmalzing

Supplemental Figure 1. Primary sequence alignment between the template (zfP2X4) and the model (rP2X2). Shown is the sequence alignment of the zebrafish P2X4 subunit (zfP2X4, UniProt entry Q6NYR1) and the rat P2X2 subunit (rP2X2, UniProt entry P49653) used for the generation of the apo-closed state and ATP-bound open state rP2X2 receptor homology models. The sequence alignment was performed using a modified version of the Needleman-Wunsch algorithm (Needleman and Wunsch, 1970). In this approach, the alignments are computed by optimizing a function based on residue similarity scores. The function uses the BLOSUM62 amino acid substitution matrix (Henikoff and Henikoff, 1992) and gap penalties and was constrained, in this case, by the 10 conserved extracellular cysteine residues (indicated in yellow), which were adjusted and fixed manually.

References
