Purinergic P2X<sub>2</sub> Receptor Desensitization Depends on Coupling between Ectodomain and C-Terminal Domain

MU-LAN HE, TAKA-AKI KOSHIMIZU, MELANIJA TOMIĆ, and STANKO S. STOJILKOVIC

Endocrinology and Reproduction Research Branch, National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, Maryland

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ABSTRACT

The wild-type P2X<sub>2</sub> purinergic receptor (P2X<sub>2</sub>aR) and its splice form lacking the intracellular Val<sup>177</sup>-Gln<sup>203</sup> C-terminal sequence (P2X<sub>2</sub>bR) respond to ATP stimulation with comparable EC<sub>50</sub> values and peak current/calcium responses but desensitize in a receptor-specific manner. P2X<sub>2</sub>aR desensitizes slowly and P2X<sub>2</sub>bR desensitizes rapidly. We studied the effects of different agonists, and of substituting the ectodomain, on the pattern of calcium signaling by P2X<sub>2</sub>aR and P2X<sub>2</sub>bR. Both receptors showed similar EC<sub>50</sub> values (estimated from the peak calcium response) and IC<sub>50</sub> values (estimated from the rate of calcium signal desensitization) for agonists, in the order 2-MeS-ATP ≪ ATP ≪ BzATP ≪ αβ-meATP, and the IC<sub>50</sub> values for agonists were shifted to the right compared with their EC<sub>50</sub> values. Furthermore, the ATP-induced receptor-subtype specific pattern of desensitization was mimicked by high- but not by low-efficacy agonists, suggesting a ligand-specific desensitization pattern. To test this hypothesis, we generated chimeric P2X<sub>2</sub>aR and P2X<sub>2</sub>bR containing the Val<sup>60</sup>-Phe<sup>301</sup> ectodomain sequence of P2X<sub>2</sub>bR and Val<sup>61</sup>-Phe<sup>313</sup> ectodomain sequence of P2X<sub>2</sub>bR instead the native Ile<sup>66</sup>-Tyr<sup>310</sup> sequence. The mutated P2X<sub>2</sub>aRΔX and P2X<sub>2</sub>bRΔX exhibited comparable EC<sub>50</sub> values for ATP, BzATP, and αβ-meATP in the submicromolar concentration range and desensitized in a receptor-specific and ligand-nonspecific manner. On the other hand, the chimeric P2X<sub>2</sub>aRΔX+X<sub>R</sub> exhibited decreased sensitivity for ATP and desensitized in a receptor-nonspecific manner. These results suggest that efficacy of agonists for the ligand-binding domain of P2X<sub>R</sub>Rs reflects the strength of desensitization controlled by their C-terminal structures.

During the prolonged agonist occupancy, ATP-gated purinergic receptor-channels (P2XRs) become refractory to the stimulus and cellular responses decline. This process, called desensitization, is common for ligand-gated channels and occurs because liganded receptors enter stable conformations through which ion permeation is blocked or attenuated. Based on the observed differences in their desensitization kinetics, homomeric P2XRs are generally divided into three groups: P2X<sub>1</sub>R and P2X<sub>3</sub>R desensitize very rapidly and P2X<sub>2</sub>R and P2X<sub>7</sub>R desensitize with a moderate rate, whereas P2X<sub>2a</sub>R, P2X<sub>2b</sub>R, and P2X<sub>3</sub>R show little or no desensitization (North and Barnard, 1997; Ralevic and Burnstock, 1998). Heteromultimerization results in channels that desensitize with different kinetics from those seen in cells expressing homomeric channels; the influence of participating subunits on channel desensitization pattern is well documented for P2X<sub>2</sub>R+P2X<sub>3</sub>R (Lewis et al., 1995; Radford et al., 1997). The differences in desensitization rates of P2XRs are reminiscent of those seen among subtypes of other ligand-gated receptor-channels (McBain and Mayer, 1994; Lerma et al., 2001).

The underlying molecular mechanisms of P2XR desensitization have been incompletely characterized. Calcium and other divalent cations influence the rate of desensitization and the rate of recovery from desensitization in native and cloned channels (Cook et al., 1998; Ding and Sachs, 2000). A highly conserved protein kinase C site located in the N terminus of P2XRs may control the rate of desensitization of P2X<sub>2</sub>R, P2X<sub>3</sub>R, and P2X<sub>7</sub>R (Boue-Grabot et al., 2001; Paukert et al., 2001; Ennion and Evans, 2002). Phosphorylation of a protein kinase A site in the C terminus of P2X<sub>2</sub>R may also participate in receptor desensitization (Chow and Wang, 1998). Experiments with chimeras composed of P2X<sub>2</sub>R and P2X<sub>2b</sub>R or P2X<sub>2a</sub>R subunits suggested that the rapid desensitization requires interactions between two transmembrane domains of receptor subunits (Werner et al., 1996). Several groups have also reported that the C-terminal splice variant of P2X<sub>2</sub>R, called P2X<sub>2</sub>ΔX, lacks a stretch of 69 residues and desensitizes faster than the full-length channel, called P2X<sub>2a</sub>R (Brandle et al., 1997; Simon et al., 1997; Ko-
shimizu et al., 1998b). The site-directed mutagenesis experiments suggested important roles of different residues in the C-terminal tail in P2X7R desensitization (Koshimizu et al., 1998a; Zhou et al., 1998; Smith et al., 1999). The variable C-terminal structures may also influence the desensitization rates of other members of P2XRs, including P2X3R and C-terminal structures may also influence the desensitization process during repetitive stimulation (Surprenant et al., 1996).

Here, we examined the interactions between the ectodomain and C-terminal domain in controlling the pattern of P2XR desensitization. Specifically, we studied the effects of altering the agonist and substituting the ectodomain on the pattern of calcium signaling by P2XRs. For this purpose, we used P2X2aR and P2X2bR because of their identical ectodomains and distinct desensitization patterns in response to ATP. The P2X2 receptor-subtype specific desensitization pattern was observed not only in current measurements, but also in single-cell calcium measurements (Koshimizu et al., 1998, 2000), indicating that such recordings are sufficient for studies with this particular receptor. These experiments revealed that the structure-dependent desensitization pattern of P2X2aR and P2X2bR reflects the efficacy of agonists for these receptors.

Materials and Methods

DNA Constructs. The coding sequences of the rat P2X2a, P2X3, P2X4, and P2X7 subunits were isolated by reverse transcription-PCR (Koshimizu et al., 1999), and subcloned into the bicistronic enhanced fluorescent protein expression vector pRES2-EGFP (BD Clontech, Palo Alto, CA) at the restriction enzyme sites of XhoI/EcoRI for P2X2a and P2X2b, and XhoI/EcoRI for P2X3 and P2X7. Chimeric subunits, termed P2X2a+X3EC and P2X2b+X3EC, contain extracellular domain from Val160 to Phe301 of P2X2 instead of the native Ile69-Tyr310 sequence of P2X2aR and P2X2bR (Fig. 1). To exchange the corresponding extracellular regions, two restriction endonuclease sites were introduced into both P2X2 and P2X3. The site-directed mutagenesis experiments with this particular receptor. These experiments revealed that the structure-dependent desensitization pattern of P2X2aR and P2X2bR instead the native sequence Ile69-Tyr310.

![Fig. 1. Schematic representation of the wild-type and chimeric constructs used in this study. White horizontal rectangles indicate P2X2aR and its splice variant lacking the Val177-Gln238 C-terminal sequence (shown in gray), called P2X2aR, black rectangles indicate P2X2R, and dotted rectangles indicate P2X4R. Vertical dashed rectangles indicate the positions of putative transmembrane domains TM1 and TM2. The constructed P2X2a+X3EC and P2X2b+X3EC chimeric receptors contain Val160-Phe301 extracellular domain of P2X2 instead the native sequence Ile69-Tyr310 sequence of P2X2aR and P2X2bR. The constructed P2X2a+X3EC and P2X2b+X3EC chimeric subunits contain Val160-Phe301 extracellular domain of P2X2 instead the native sequence Ile69-Tyr310.](https://molpharm.aspetjournals.org/doi/10.1183/09545704.00977710)
cells. Apyrase (Grade I; Sigma, St. Louis, MO) was used at 0.2 U/ml throughout the incubation process, loading with Fura-2 acetoxymethyl ester, and \([\text{Ca}^{2+}]_i\) recording in cells expressing P2X \(_2\)R, P2X \(_{2a}\)R, P2X \(_{2b}\)R, P2X \(_{2a}\)R, and their chimeras were done without apyrase. GFP was used as a marker for cells with P2XR expression as described previously (Koshimizu et al., 1999, 2000). Cells expressing GFP were optically detected by an emission signal at 520 nm when excited by 488-nm ultraviolet light and were not detectable by 340- and 380-nm excitations in the absence of Fura-2.

**Calculations.** To minimize the impact of receptor saturation kinetics on the \([\text{Ca}^{2+}]_i\) profiles, agonists were added rapidly and were continuously present during the recording. Thus, the rise in \([\text{Ca}^{2+}]_i\) predominantly reflects the bound-open equilibrium, whereas the decay represents the equilibration into desensitization state (Auerbach and Akk, 1998). The time course of the \([\text{Ca}^{2+}]_i\) was fitted to a single exponential function using Prism software (GraphPad Software, San Diego, CA). All values in the text are reported as mean ± S.E.M. Significant differences, with \(P < 0.05\), were determined by one-way analysis of variance with Newman-Keuls multiple comparison test. Concentration-response relationships were fitted to a four-parameter logistic equation using a nonlinear curve-fitting program (Kaleidagraph; Synergy Software, Reading, PA) that derives the EC\(_{50}\) and Hill values. Calcium recordings were done in 15 to 50 cells simultaneously, and each experiment was repeated three or more times to ensure the reproducibility of the findings.

**Results**

**P2X\(_{2a}\)R and P2X\(_{2b}\)R Exhibit Similar EC\(_{50}\) Values for Agonists.** The native and chimeric P2XRs were subcloned into GFP-expression vector pIRES2-EGFP, and the relative transfection efficiency of P2XRs constructs was estimated in single cells by analyzing the intensity of fluorescence signals, as described previously (Koshimizu et al., 2000). In the presence of fixed amount of expression constructs and comparable post-transfection times, the percentage of GFP+ ATP-positive cells varied between 45 and 60% and was independent on the channel type expressed. When the average GFP fluorescence was similar for each set of cells (about 60 arbitrary units), the mean amplitude of peak \([\text{Ca}^{2+}]_i\) to 100 μM ATP were highly reproducible for the same channel types. No repetitive stimulation was done to avoid the possible impact of desensitization on the amplitude and pattern of \([\text{Ca}^{2+}]_i\) signals. Also, in all experiments agonists were added rapidly to the coverslip dish to minimize the impact of agonist diffusion on the profile of \([\text{Ca}^{2+}]_i\) signals.

Under these experimental conditions, P2X\(_{2a}\)R and P2X\(_{2b}\)R responded to ATP stimulation, as well as to 2-MeS-ATP, ATP\(_S\), BzATP, and αβ-meATP stimulation, with a rapid rise in \([\text{Ca}^{2+}]_i\), followed by a gradual decline to the steady plateau levels. Figure 2 shows typical patterns of \([\text{Ca}^{2+}]_i\),

![Fig. 2](https://www.aspetjournals.org/content/p2x-receptors-downloads-downloads/1189)

*Fig. 2.* Comparison of the effects of ATP and two analogs, BzATP and αβ-meATP, on the peak calcium response and rates of signal desensitization in cells expressing P2X\(_{2a}\)R and P2X\(_{2b}\)R. In this and following figures, experimental records are shown by open circles (mean values from at least 15 traces in representative experiments) and fitted curves by full lines. A single exponential function was sufficient to describe the desensitization rates. The fitted function is extrapolated for clarity. Agonists were added in concentrations indicated below traces and were continuously present during the recording.
signals in response to stimulation with increasing ATP (left), BzATP (middle), and αβ-meATP (right) concentrations. At high agonist concentrations, the peak $[Ca^{2+}]_i$ responses induced by ATP, BzATP, and αβ-meATP (Fig. 2), as well as by 2-MeS-ATP and ATPγS (data not shown), were comparable. Figure 3 illustrates the sigmoidal concentration-dependence of ATP, BzATP, and αβ-meATP on the amplitude of $[Ca^{2+}]_i$ responses, shown as the mean values of peak response minus baseline. The dotted lines and numbers above the lines illustrate the EC50 values for these agonists. The calculated EC50 values for 2-MeS-ATP were slightly lower and for ATPγS slightly higher compared with ATP. Thus, both receptors show similar EC50 values for agonists, in the order: 2-MeS-ATP > ATP > αβ-meATP.

The C-Terminal-Dependent Desensitization Pattern of P2X2R Is Ligand-Specific. The desensitization rates of $[Ca^{2+}]_i$ signals generated by two receptors were also dependent on agonist concentrations. Figure 2, left, shows typical desensitization profiles in P2X2aR- and P2X2bR-expressing cells stimulated with increasing ATP concentrations. Consistent with the relevance of C-terminal domain structure of P2X2R in control of receptor desensitization (Brandle et al., 1997; Simon et al., 1997; Koshimizu et al., 1998b), P2X2bR desensitized more rapidly than P2X2aR (Fig. 2, left). Stimulation with increasing BzATP and αβ-meATP concentrations also produced a progressive increase in the rates of signal desensitization (Fig. 2, middle and right). Furthermore, P2X2bR-expressing cells desensitized more rapidly than P2X2aR-expressing cells during the prolonged stimulation with high concentrations of BzATP, whereas signals generated by two receptors desensitized with comparable rates in response to αβ-meATP.

The ligand- and receptor-specificity of signal desensitization is summarized in Fig. 4. The calculated order of agonist concentrations that induce half-maximum rate of signal desensitization (IC50 values) was 2-MeS-ATP ≈ ATP ≈ ATPγS ≈ BzATP ≈ αβ-meATP, and was identical to the EC50 values order. However, the IC50 values for agonists were shifted to the right compared with EC50 values. For example, the EC50 values for ATP were 2 and 3 µM for P2X2aR and P2X2bR (Fig. 3), respectively, whereas the IC50 values for the same agonist were 26 and 29 µM, respectively (Fig. 4, A–C). In parallel to the concentration-dependence of peak $[Ca^{2+}]_i$ responses, the rates of P2X2bR desensitization reached comparable levels at saturating ATP, BzATP, and αβ-meATP concentrations (Fig. 4, A–C, dotted line). In contrast to the activation of channels, BzATP was unable to mimic the action of ATP on the rates of P2X2aR desensitization when added in the 1 to 1000 µM concentration range. Furthermore, the receptor subtype-specific pattern of signal desensitization was completely lost in cells stimulated with αβ-meATP, a low potency agonist.

The ligand-specific P2X2bR desensitization pattern was further illustrated in Fig. 4, D–F. When P2X2bR-expressing cells were stimulated with high (500 µM) agonist concentrations for a prolonged time, they responded with comparable amplitudes of $[Ca^{2+}]_i$ spikes but with variable rates of signal desensitization (Fig. 4, D and E). There was an inverse relationship between the EC50 values for agonists and the rates of receptor desensitization estimated at 500 µM concentrations (Fig. 4F). These data indicate that the C terminus-specific desensitization pattern was affected when low potency agonists were used.

Increase in P2X2R Sensitivity for Agonists Facilitates Desensitization. We further examined the agonist-specific desensitization pattern of P2X2R by producing the chimeric receptors with increased and decreased sensitivity for agonists. To make P2X2R with high sensitivity for agonists, we constructed chimeric receptors containing the Val60-Phe301 extracellular domain sequence of P2X3R in control of receptor desensitization (Brandle et al., 1997; Simon et al., 1997; Koshimizu et al., 1998b), P2X2bR desensitized more rapidly than P2X2aR (Fig. 2, left). Stimulation with increasing BzATP and αβ-meATP concentrations also produced a progressive increase in the rates of signal desensitization (Fig. 2, middle and right). Furthermore, P2X2bR-expressing cells desensitized more rapidly than P2X2aR-expressing cells during the prolonged stimulation with high concentrations of BzATP, whereas signals generated by two receptors desensitized with comparable rates in response to αβ-meATP.

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same shift in EC$_{50}$ values for two agonists (not shown). On the other hand, the peak amplitude of [Ca$^{2+}$]i responses in cells expressing chimeric receptors was 2- to 3-fold higher than that observed in P2X$_2$R-expressing cells (Fig. 5B).

Molecular changes in the ectodomain of P2X$_{2a}$R and P2X$_{2b}$R also affected the rates of receptor desensitization. Figure 6, A and B, illustrate typical calcium signal profiles in cells expressing native P2X$_{2a}$R and P2X$_{2b}$R and chimeric P2X$_{2a}$+$X_3$EC receptors during prolonged stimulation with 100 µM αβ-meATP and 100 µM ATP. Numbers above traces show the mean values for rates of signal desensitization, which are significantly different compared with native P2X$_{2a}$R and P2X$_{2b}$R. A significant increase in the rates of P2X$_{2a}$R+$X_3$EC and P2X$_{2b}$R+$X_3$EC desensitization was also observed during stimulation with BzATP. Figure 7 shows typical calcium profiles in P2X$_{2a}$R-, P2X$_{2a}$+$X_3$EC-, and P2X$_{3}$R-expressing cells stimulated with increasing concentrations of BzATP.

In contrast with native P2X$_2$Rs, P2X$_{2a}$+$X_3$EC chimera desensitized with comparable rates when stimulated with equimolar ATP, αβ-meATP, and BzATP concentrations (Figs. 6 and 7). P2X$_{2a}$+$X_3$EC chimera also desensitized with comparable rates when stimulated with 1 µM ATP, BzATP, and αβ-meATP (Fig. 8). Furthermore, the C terminus-dependent pattern of accelerated desensitization was preserved for ATP stimulation and was developed for αβ-meATP and BzATP stimulation. As shown in Table 1, in all doses studied, there was a significant difference in the rates of P2X$_{2a}$+$X_3$EC and P2X$_{2b}$+$X_3$EC receptor desensitization. These results support the hypothesis that an increase in the EC$_{50}$ values for agonists introduced by substitution of the extracellular domain results in a loss of ligand-specificity of receptor desensitization.

**Decrease in P2X$_2$R Sensitivity for ATP Blocks C Terminal-Dependent Desensitization.** To further test the hypothesis about the relevance of ectodomain for C-terminal structure-dependent desensitization, we made chimeric P2X$_{2a}$R and P2X$_{2b}$R with lower sensitivity to ATP and higher sensitivity to BzATP, compared with the wild-type channels. This was achieved by constructing P2X$_{2a}$+$X_7$EC and

![Fig. 4](image-url)
P2X2b\textsubscript{a}+X\textsubscript{3}EC chimeric receptors containing the Val\textsuperscript{61}-Phe\textsuperscript{313} extracellular domain sequence of P2X7R instead of the native Ile\textsuperscript{66}-Tyr\textsuperscript{310} sequence. In accordance with the literature (Surprenant et al., 1996), native P2X\textsubscript{7}R expressed in GT1 neurons responded to BzATP stimulation with a rapid and non-desensitizing rise in [Ca\textsuperscript{2+}]\textsubscript{i} (Fig. 9A), with a calculated EC\textsubscript{50} of 8 \mu M (Fig. 9D). In a majority of cells, ATP also induced similar patterns of [Ca\textsuperscript{2+}]\textsubscript{i} signaling, albeit of smaller amplitude, whereas a fraction of cells (about 30\% in response to 500 and 1000 \mu M ATP) responded with atypical [Ca\textsuperscript{2+}]\textsubscript{i} pro-

Fig. 5. Influence of the substitution of ectodomain at P2X\textsubscript{2}Rs on agonistic potency of ATP (left), BzATP (middle), and \alpha\beta-meATP (right). A, change in the EC\textsubscript{50} values for agonists in cells expressing P2X\textsubscript{2}a+X\textsubscript{3}EC receptors. The results shown are means ± S.E.M. B, comparison of the peak amplitude of [Ca\textsuperscript{2+}]\textsubscript{i} signals in P2X\textsubscript{2}a+X\textsubscript{3}EC-expressing cells. P2X\textsubscript{2}a+X\textsubscript{3}EC receptors showed comparable leftward shifts in EC\textsubscript{50} values for three agonists and a decrease in peak amplitude of [Ca\textsuperscript{2+}]\textsubscript{i} responses. P2X\textsubscript{2}R+X\textsubscript{3}EC chimeras were constructed as described under Materials and Methods.

P2X\textsubscript{2}a+X\textsubscript{3}EC chimeric receptors containing the Val\textsuperscript{61}-Phe\textsuperscript{313} extracellular domain sequence of P2X-R instead of the native Ile\textsuperscript{66}-Tyr\textsuperscript{310} sequence. In accordance with the literature (Surprenant et al., 1996), native P2X-R expressed in GT1 neurons responded to BzATP stimulation with a rapid and non-desensitizing rise in [Ca\textsuperscript{2+}]\textsubscript{i} (Fig. 9A), with a calculated EC\textsubscript{50} of 8 \mu M (Fig. 9D). In a majority of cells, ATP also induced similar patterns of [Ca\textsuperscript{2+}]\textsubscript{i} signaling, albeit of smaller amplitude, whereas a fraction of cells (about 30\% in response to 500 and 1000 \mu M ATP) responded with atypical [Ca\textsuperscript{2+}]\textsubscript{i} pro-

Fig. 6. The pattern of desensitization in cells expressing wild-type P2X\textsubscript{3}R and P2X\textsubscript{2}aR and P2X\textsubscript{2}a+X\textsubscript{3}EC chimera. A and B, comparison of the effects of 100 \mu M \alpha\beta-meATP (A) and 100 \mu M ATP (B) on the rates of calcium signal desensitization in GT1 neurons expressing P2X\textsubscript{3}R (left traces) P2X\textsubscript{2}aR+X\textsubscript{3}EC chimera (central traces), and P2X\textsubscript{2}aR (right traces). Traces shown are representative from three to eight independent experiments, each done in at least 15 cells. Numbers above traces represent mean ± S.E.M. values of desensitization rates. Asterisks indicate significant differences compared with desensitization rates for P2X\textsubscript{3}R-and P2X\textsubscript{2}aR-expressing cells.
files (Fig. 9B). In all concentrations studied, ATP was less effective compared with 65 \( \mu \)M BzATP (Fig. 9C), and the estimated EC\(_{50}\) was 485 \( \mu \)M (Fig. 9G).

Like P2X\(_7\)R, chimeric P2X\(_{2a}\)+X\(_3\)EC and P2X\(_{2b}\)+X\(_3\)EC receptors showed an inverse sensitivity for BzATP and ATP. The BzATP dose-response curve for chimeric channels was highly similar to that of P2X\(_7\)R and was leftward shifted for about half-log concentration compared with the wild-type channels (Fig. 9D). This was accompanied with dramatic increase in the rates of receptor desensitization (Fig. 9E, horizontal arrow). The influence of C-terminal structure on rates and level of receptor desensitization was preserved in chimeric receptors (Fig. 9F).

The chimeric channels showed a rightward shift in the EC\(_{50}\) values for ATP, but the agonistic potency of ATP at the chimeric receptor was closer to the ATP potency at wild-type P2X\(_2\)R than at wild-type P2X\(_7\)R (Fig. 9G). This indicates the relevance of P2X\(_2\)R transmembrane domains and/or their flanking Lys\(^{53}\)-Ser\(^{65}\) and Gly\(^{311}\)-Ser\(^{326}\) sequences for ATP potency. There was a difference in the rates of P2X\(_{2a}\)+X\(_3\)EC and P2X\(_{2b}\)+X\(_3\)EC desensitization (Fig. 9H, arrow). P2X\(_{2a}\)+X\(_3\)EC receptors desensitized faster than native P2X\(_{2a}\)R (data not shown) and both receptors responded to 500 \( \mu \)M ATP with similar rates of desensitization (Fig. 9I). Table 2 illustrates the lack of C-terminal-specific desensitization for P2X\(_{2a}\)+X\(_3\)EC receptors when stimulated with ATP. Thus, a small decrease in the agonist potency resulted in a loss of P2X\(_2\)R subtype-specific desensitization pattern.

**Discussion**

Two main hypotheses emerged from previous work on desensitization of P2XRs, one based on the structure of channels, and the other based on the actions of intracellular messengers. The dual control of P2XR may well be expected from studies on such allosteric proteins, and is reminiscent of those seen with other ligand-gated and voltage-gated channels. For example, desensitization of glutamate receptors depends on N-terminal domain (Krupp et al., 1998), the flip-flop cassette (Sommer et al., 1990), and M3–M4 domain (Partin et al., 1995), as well as on intracellular messengers in the postsynaptic cells, including Ca\(^{2+}\) (Krupp et al., 1996).

The desensitization properties of AMPA receptors can be modified by alternative splicing and mRNA editing, and by heteromeric assembly of channels (Sommer et al., 1990; Robert et al., 2001). In cyclic nucleotide-gated channels, the agonist-binding domain is in the C terminus, and the N-terminal domain alters the efficacy of agonists through in-
interactions with the ligand-binding site by a Ca\(^{2+}\)-calmodulin-sensitive mechanism (Tibbs et al., 1997; Varnum and Zagotta, 1997). The structure of intracellular domains of voltage-gated channels are also critical for their voltage-dependent inactivation, whereas the functional control of these channels is mediated by various intracellular messengers (Hille, 1991).

Here we focused on the mechanism of C-terminal structure-dependent P2X\(_{2a}\)R desensitization. Two sister receptors, P2X\(_{2a}\)R and P2X\(_{2b}\)R, exhibit comparable activation profiles for ATP and peak current/\([\text{Ca}^{2+}]_i\) responses but desensitize with different rates. P2X\(_{2a}\)Rs desensitize slowly and partially, whereas P2X\(_{2b}\)Rs desensitize rapidly and to the steady levels significantly lower than that of P2X\(_{2b}\)R (Brandt et al., 1997; Simon et al., 1997; Koshimizu et al., 1998b). Similar EC\(_{50}\) values for ATP are consistent with identical structure of extracellular domains for these receptors. Different rates of receptor desensitization, on the other hand, indicate the relevance of Val\(^{370}\)-Gln\(^{388}\) C-terminal sequence, deleted in P2X\(_{2b}\)R, for receptor-desensitization. In our experiments, potency of several agonists for P2X\(_{2a}\)R and P2X\(_{2b}\)R were in an order (2-MeS-ATP ñ ATP ñ ATPS < BzATP ñ αβ-meATP) comparable with results obtained by others (reviewed in Railevic and Burnstock, 1998).

We also show that these two receptors desensitized in a concentration-dependent manner and with the same order of agonists. However, the IC\(_{50}\) values for desensitization were right-shifted compared with the EC\(_{50}\) values for activation of channels. This is a novel finding for P2XRbs but has been shown for other ligand-gated channels. For example, the extent of AMPA receptor desensitization increases with agonist concentrations (Vyklicky et al., 1991). The half-maximal activation of kainate channels occurs at glutamate concentrations of 330 \(\mu\)M, whereas the half-maximal steady state desensitization occurs at ligand concentrations 20 times lower. A similar ratio was also observed for GluR6 homomers when kainate was used as an agonist (Lerma et al., 2001). Also, concentrations required to desensitize Torpedo californica receptors are nearly 1000-fold lower than those required for activation (Corringer et al., 1998). A dual aspect of agonist pharmacology may contribute to the shaping of synaptic currents and modulating the fraction of activatable channels (Jones and Westbrook, 1996). However, the slow and incomplete inactivation of P2X\(_{2a}\)R and the right-shifted IC\(_{50}\) for ATP argue against such a role of receptor desensitization in neurons expressing these channels.

The receptor subtype-specific desensitization pattern was observed in response to ATP, the native agonist for these channels but also in response to stimulation with two analog agonists, 2-MeS-ATP and ATPS. However, the receptorspecificity of desensitization was less obvious when stimulated with BzATP and was lost when receptors were stimulated with αβ-meATP. Furthermore, the ligand-specific desensitization patterns were recorded at maximal agonist concentrations, where peak amplitudes in \([\text{Ca}^{2+}]_i\) responses but desensitize were comparable in P2X\(_{2a}\)R and P2X\(_{2b}\)R. Consistent with a role of agonist-binding domains in desensitization of other ligand-gated channels, both AMPA and glutamate maximally activate AMPA receptors, whereas kainate and domoate act as partial agonists, and produce much less desensitization than glutamate (Patneau and Mayer, 1990; Patneau et al., 1993; Swanson et al., 1997; Armstrong and Gouaux, 2000). The novel aspect in ligand-specific receptor desensitization emerging from this study is in coupling between ectodomain and C-terminal domain. In general, there was a parallelism between the rates of P2X\(_{2a}\)R and P2X\(_{2b}\)R desensitization and the EC\(_{50}\) values for agonists. This suggests that C-terminal-dependent desensitization pattern is not an “all-or-none”

![Fig. 8. Agonist-induced calcium signaling in GT1 cells expressing P2X\(_{2a}\)R and P2X\(_{2b}\)+X\(_3\)EC receptors. A to C, typical patterns of P2X\(_{2a}\)R+X\(_3\)EC desensitization in response to 1 \(\mu\)M ATP(A), 1 \(\mu\)M BzATP (B), and 1 \(\mu\)M αβ-meATP (C). Traces shown are representative from five to six independent experiments. Mean ± S.E.M. values for receptor desensitization kinetics are shown in Table 1.

**TABLE 1**

<table>
<thead>
<tr>
<th>Agonist</th>
<th>P2X(_{2a})+X(_3)EC</th>
<th>P2X(_{2b})+X(_3)EC</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATP, 1 (\mu)M</td>
<td>0.0188 ± 0.0012 (5)</td>
<td>0.0609 ± 0.00696 (8)*</td>
</tr>
<tr>
<td>ATP, 10 (\mu)M</td>
<td>0.0172 ± 0.0013 (5)</td>
<td>0.0598 ± 0.00375 (5)*</td>
</tr>
<tr>
<td>αβ-meATP, 1 (\mu)M</td>
<td>0.0193 ± 0.0014 (4)</td>
<td>0.0614 ± 0.0024 (4)*</td>
</tr>
<tr>
<td>BzATP, 1 (\mu)M</td>
<td>0.0143 ± 0.0011 (7)</td>
<td>0.0660 ± 0.00655 (10)*</td>
</tr>
<tr>
<td>BzATP, 10 (\mu)M</td>
<td>0.0187 ± 0.0015 (11)</td>
<td>0.0674 ± 0.0079 (7)*</td>
</tr>
<tr>
<td>BzATP, 100 (\mu)M</td>
<td>0.0183 ± 0.0011 (9)</td>
<td>0.0609 ± 0.0057 (5)*</td>
</tr>
</tbody>
</table>

*, \(P < 0.05\).
phenomenon but a graded process that probably depends on ligand binding affinity and/or activation efficacy.

The relevance of ectodomain structure on C-terminal-dependent desensitization pattern was further confirmed in experiments with chimeric channels. The agonist-specific desensitization pattern of P2X2Rs was lost by changing the native binding site of these channels with the P2X3R extracellular domain. Both chimeras, P2X2a\textsubscript{H11001}X3EC and P2X2b\textsubscript{H11001}X3EC, exhibited about 30-, 25-, and 150-fold increase in the EC\textsubscript{50} values for ATP, BzATP and α\textbeta-meATP, respectively. The rates of desensitization for both chimeric receptors also increased for 2–3-fold. However, the C-terminal structure-dependent desensitization pattern was preserved; like native receptors, P2X2b\textsubscript{H11001}X3EC receptor desensitized more rapidly.

**Fig. 9.** Characterization of calcium signaling pattern by chimeric P2X2Rs containing the P2X7R ectodomain. A to C, characterization of agonist-induced calcium response in P2X7R-expressing cells. Concentration-dependent effects of BzATP (A) and ATP (B) on [Ca\textsuperscript{2+}]\textsubscript{i} response. Numbers indicate concentrations of agonists that were continuously present during the recording. C, comparison of the agonistic potency of ATP and BzATP. D to F, comparison of the BzATP effects on peak [Ca\textsuperscript{2+}]\textsubscript{i} response (D and rates of receptor desensitization (E and F) in cells expressing P2X2R, P2X7R, and P2X2\textsubscript{bH11001}X7EC receptors. G to I, comparison of the ATP effects on peak [Ca\textsuperscript{2+}]\textsubscript{i} response (G) and rates of receptor desensitization (H and I) in cells expressing P2X2R, P2X7R, and P2X2\textsubscript{bH11001}X7EC receptors. No difference in peak [Ca\textsuperscript{2+}]\textsubscript{i} responses were observed between P2X2a\textsubscript{H11001}X7EC and P2X2b\textsubscript{H11001}X7EC and these results are shown combined. Arrows indicate differences in the EC\textsubscript{50} values (D and G) and half-times for receptor desensitization (E and H). The results shown are means ± S.E.M. with three to eleven experiments per dose. In all, horizontal bars above and below traces indicate the duration of agonist stimulation.
than P2X$_{2a}$+X$_3$EC receptor finally. The ligand-specific and receptor subtype-specific desensitization patterns reversed in cells expressing P2X$_{2a}$+X$_3$EC and P2X$_{2a}$+X$_7$EC receptors. Such chimeras showed lower sensitivity for ATP, compared with native P2X$_{2a}$Rs and desensitized with comparable rates and higher sensitivity to BzATP accompanied with the C terminus-specific desensitization pattern.

At the present time, it is difficult to discuss the possible molecular mechanism of interactions between the ectodomain and C-terminal domain in development of desensitization. Calcium measurements used in our study provide several advantages. P2XR-generated calcium signals mediate the action of these receptors on cellular functions, including neurotransmission, hormone secretion, transcriptional regulation, and protein synthesis (Berridge, 1993). Thus, calcium rather than current profiles reflect the importance of a particular pattern of signaling on cellular functions. P2X$_{2a}$Rs conduct calcium and the addition of nifedipine blocks the indirect (through voltage-gated L-type calcium channels) action of activated receptors in our expression system (Koshimizu et al., 2000), reflecting Ca$^{2+}$ influx function of these channels. Single-cell calcium measurements can be done simultaneously in many cells, leading to better statistics, which are critical for interpretation of EC$_{50}$ and rates of desensitization. Measurements of GFP intensities also provide an effective mechanism for selection of cells with comparable expression of P2X$_{2a}$Rs and more reliable data on peak desensitization. Measurements of GFP intensities also provide the interpretation of activation and desensitization properties of channels, because of calcium handling mechanism of the cells used in experiments.

Other limitation comes from the fact that the ligand-binding domain structure and the crystal structure of P2X$_{2a}$Rs have not been identified, in contrast to glutamate channels (Sun et al., 2002). In our chimeric receptors, the extracellular loop is derived almost entirely from P2X$_{2a}$-R and P2X$_{2a}$-R. Consistent with this, the ATP potency of the chimeric P2X$_{2a}$+X$_3$EC receptors matches the ATP potency at native P2X$_{2a}$-R rather than native P2X$_{2a}$-R. However, the ATP potency at the P2X$_{2a}$+X$_3$EC receptors is closer to the ATP potency at the parental P2X$_{2a}$-R rather than P2X$_{2a}$-R. These contradictory results suggest the relevance of flanking P2X$_{2a}$ sequences on agonistic potency of ATP. We may speculate that these sequences act as “dominant-positive” domains to offset atypical low sensitivity of P2X$_{2a}$ for native agonist. In accordance with this view, it has been reported recently that point mutations in the first transmembrane domain, specifically Phe$^{4+}$, affect the ligand-selectivity of rat P2X$_{2a}$-R (Jiang et al., 2001).

In conclusion, our results show that homomeric P2X$_{2a}$-R and P2X$_{2a}$-R exhibit highly comparable EC$_{50}$ values for receptor activation by various agonists, but desensitize in a receptor- and agonist-specific manner. Pharmacological manipulations with activation of these receptors and molecular manipulations with their ectodomains indicate that the efficacy of agonists reflects the ligand-specificity of receptor desensitization; highly potent agonists trigger P2X$_{2a}$-R subtype specific C terminus-mediated desensitization, whereas agonists with lower potency are less effective or ineffective. Thus, it seems that conformational changes needed for activation of P2X$_{2a}$Rs are not always sufficient to trigger C terminus-controlled desensitization. These findings provide a solid base for further biophysical investigations on hypothesis that the affinity of agonists for receptors determines the strength of molecular conformational changes needed for development of C-terminal-controlled channel desensitization.

References


TABLE 2

The lack of C terminus-specific desensitization of chimeric P2X$_{2a}$-R and P2X$_{2a}$-R containing the extracellular domain of P2X$_{2a}$-R. Data shown are means ± S.E.M. for rates of inactivation of two chimeric receptors. Numbers in brackets indicate number of experiments, each performed in at least 15 cells.

<table>
<thead>
<tr>
<th>Agonist</th>
<th>P2X$_{2a}$+X$_3$EC</th>
<th>P2X$_{2a}$+X$_7$EC</th>
<th>s$^{-1}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATP, 100 μM</td>
<td>0.0125 ± 0.0040 (8)</td>
<td>0.0160 ± 0.0007 (6)</td>
<td></td>
</tr>
<tr>
<td>ATP, 500 μM</td>
<td>0.0221 ± 0.0016 (4)</td>
<td>0.0242 ± 0.0005 (5)</td>
<td></td>
</tr>
<tr>
<td>ATP, 1000 μM</td>
<td>0.0212 ± 0.0015 (4)</td>
<td>0.0259 ± 0.0016 (3)</td>
<td></td>
</tr>
</tbody>
</table>

In conclusion, our results show that homomeric P2X$_{2a}$-R and P2X$_{2a}$-R exhibit highly comparable EC$_{50}$ values for receptor activation by various agonists, but desensitize in a receptor- and agonist-specific manner. Pharmacological manipulations with activation of these receptors and molecular manipulations with their ectodomains indicate that the efficacy of agonists reflects the ligand-specificity of receptor desensitization; highly potent agonists trigger P2X$_{2a}$-R subtype specific C terminus-mediated desensitization, whereas agonists with lower potency are less effective or ineffective. Thus, it seems that conformational changes needed for activation of P2X$_{2a}$Rs are not always sufficient to trigger C terminus-controlled desensitization. These findings provide a solid base for further biophysical investigations on hypothesis that the affinity of agonists for receptors determines the strength of molecular conformational changes needed for development of C-terminal-controlled channel desensitization.


Address correspondence to: Dr. Stanko Stojilkovic, SCS/ERRB/NICHD, Bldg. 49, Room 6A-36, 49 Convent Drive, Bethesda, MD 20892-4510. E-mail: stankos@helix.nih.gov