Contributions of the C-terminal Domain to the Control of P2X Receptor Desensitization*

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The P2X purinergic receptor channels (P2XRs) differ among themselves with respect to the rates of desensitization during prolonged agonist stimulation. Here we studied the desensitization of recombinant channels by monitoring the changes in intracellular free Ca\(^{2+}\) concentration in cells stimulated with ATP, the native and common agonist for all P2XRs. The focus in our investigations was on the relevance of the P2XR C terminus in controlling receptor desensitization. When expressed in GT1 cells, the P2XRs desensitized with rates characteristic to each receptor subtype: P2X1R = P2X4R > P2X3R > P2X2aR > P2X2bR. A slow desensitization pattern of P2X2aR was mimicked partially by P2X3Ra and fully by P2X3R when the six-amino acid sequences of these channels located in the cytoplasmic C terminus were substituted with the corresponding arginine 371-proline 376 sequence of P2X2aR. Changing the total net charge in the six amino acids of P2X2Ra to a more positive pattern of P2X2bR was mimicked partially by P2X4R and fully by P2X4R when the six-amino acid sequences of P2X4R were substituted with the corresponding arginine 371 to proline 376 sequence of P2X2aR. Changing the total net charge in the six amino acids of P2X2Ra to a more positive direction also slowed the receptor desensitization. On the other hand, substitution of arginine 371-proline 376 sequence of P2X4Ra was mimicked partially by P2X3Ra and fully by P2X3R when the six-amino acid sequences of these channels located in the cytoplasmic C terminus were substituted with the corresponding arginine 371 to proline 376 sequence of P2X2aR with the corresponding sequences of P2X2Ra, P2X2Ra, and P2X2R increased the rate of receptor desensitization. Furthermore, heterologous polymerization of wild-type P2X2aRa and mutant P2X3Ra having the C-terminal six amino acids of P2X2aR at its analogous position resulted in a functional channel whose desensitization was significantly delayed. These results suggest that composition of the C-terminal six-amino acid sequence and its electrostatic force influence the rate of receptor desensitization.

ATP-gated receptor channels (P2XRs) are expressed in a number of tissues where they are involved in regulation of a wide variety of cellular functions, including central and peripheral neurotransmission, smooth muscle contraction, platelet activation, and hormone secretion (1–3). The regulation of these functions by the activated P2XRs requires or is susceptible to local and/or global changes in intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\textsubscript{i}) (3). Binding of extracellular ATP to P2XRs is associated with a rise in [Ca\(^{2+}\)]\textsubscript{i}, which is mediated by Ca\(^{2+}\) influx through these channels as well as by depolarization of cells and activation of voltage-gated Ca\(^{2+}\) channels. The pattern of [Ca\(^{2+}\)]\textsubscript{i}, signaling by native P2XRs is highly variable, depending on the cell type examined (3). In contrast to other Ca\(^{2+}\)-conducting channels, however, the relevant structural base of the P2XRs contributing to the control of [Ca\(^{2+}\)]\textsubscript{i}, signaling function has not been completely elucidated.

Using molecular cloning techniques, seven subunits of P2XRs have been obtained so far and are named P2X1R to P2X7R (4–10). They can form Ca\(^{2+}\)-permeable pores through homo- and heteropolymORIZATION (11, 12). Each subunit is proposed to have two putative transmembrane helices connected with a large extracellular loop, and both the N and C termini are located in the cytoplasm. From their N termini to the second transmembrane domain, the cloned subunits exhibit a relatively high level of amino acid (aa) sequence homology compared with their C termini, which are variable in lengths and show no apparent sequence homology except for the proximal region near the second transmembrane domain (1). During continuous exposure to agonist, current signals generated by recombinant P2XRs are desensitized gradually, an action that should effectively attenuate or terminate direct and indirect Ca\(^{2+}\) influx.

Based on the observed differences in their current desensitization kinetics, recombinant P2XRs are generally divided into two groups: the rapidly desensitizing (P2X1R and P2X3R) and the slowly desensitizing (P2X2aR, P2X2bR, P2X3R, P2X4R, P2X5R, and P2X7R) (3). Experiments with chimera subunits composed of P2X2R and P2X3R or P2X4R or P2X5R subunits suggested that the rapid desensitization requires interactions between two transmembrane domains of receptor subunits (13). A large C terminus of P2X2aR has also been suggested to account for the non-desensitizing pattern of these channels during repetitive stimulation (10). Recently, a new view of P2XR desensitization has emerged; the C-terminal splice variant of P2X3R, termed P2X2aRa, was found to lack a stretch of 69 aa and to desensitize faster than the full-length P2X2aR, termed P2X2aR (14–16). Amino acids responsible for such a functional difference between the spliced and full-length channels are localized to the initial six residues (Arg371-Pro376) within the spliced segment (17).

Here, we examined the importance of the C-terminal 6-aa sequences of P2X2R, which correspond to Arg371-Pro376 of P2X2aR (Fig. 1), to the desensitization pattern of these channels. For this purpose, P2X2Rs and their mutants were expressed in GT1 immortalized neurons, and the impact of receptor desensitization on the pattern of [Ca\(^{2+}\)]\textsubscript{i}–mobilizing P2Y receptors is native for these cells (16). [Ca\(^{2+}\)]\textsubscript{i}–mobilizing P2Y receptors are native for these cells (16). Our results indicate that the pattern of receptor desensitization is unique for each homo- and heteropolymeric channel and that the structural differences in the C-terminal small region in part account for such variety of responses.
**EXPERIMENTAL PROCEDURES**

**Expression Constructs and Site-directed Mutagenesis**—Protein coding sequences for rat P2X1R (4), P2X2R (6), P2X3R (9), and P2X7R (16) were transferred to pcDNA3.1 (Invitrogen, Carlsbad, CA) at the P2X2aR or P2X3R sites, respectively. All restriction enzymes were obtained from Life Technologies (Gaithersburg, MD) and were treated with DNase I at 37 °C for 30 min. After heat inactivation of DNase I by incubating it at 70 °C for 15 min, first-strand cDNA was synthesized from 5 μg of total RNA by SuperScript II reverse transcriptase and oligo(dT)12–18 primers in a 20-μl reaction containing 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 2 mM MgCl2, and 0.5 units of Ex Taq polymerase (PanVera Corp., Madison WI). Primer annealing sites were selected from 3'- and 5'-untranslated regions and had the following sequences: X1 sense, 5'-GGCCTAACATGGGCTGCTCTGGTACGCTGCTCTCTCC-3'; X1 antisense, 5'-TCCATAAAGTCATCCTTCCATCTTAC-3'; X3 sense, 5'-TAAGTGGTGCTGAGCACTTCTTACTCC-3'; X3 antisense, 5'-GCAAGCGGATCCTGACATTAAG-3'; X6 sense, 5'-ATGTTGTGCGGCTGATCTGCTG-3'; X6 antisense, 5'-GCACTCTGTGCTGCTGCTGCTGCTG-3'; X7 sense, 5'-CCAAGCAGCAAGACGATG-3'; and X7 antisense, 5'-GGCTCGAATTATCCCCATAC-3'. In the X6 sense primer, a point mutation (underlined) was introduced to obtain optimum translation efficiency (20). PCR products were separated on a 1% agarose gel, recovered, and subcloned into pBluescript II vector (Stratagene, La Jolla, CA) at its SmaI site pretreated with 2 units of DpnI, generating pIRES/ATCC/pcDNA3.1. These plasmids were further used for evaluating the desensitization rates. The time course of [Ca2+]i response to ATP was fitted to one or two exponential functions using SigmaPlot 5.0 (Jandel Scientific software, San Rafael, CA) with the tolerance value set at 10−5. Significant differences were determined by either Student's t-test or one-way analysis of variance (ANOVA) followed by Scheffe's test, if applicable; p < 0.05 was considered as significantly different.

**RESULTS**

Desensitization Pattern of Homoplastic P2XRs—Our previous results have shown that GT1 cells are a suitable cell model for analyzing the P2X2, P2X3, and P2X4 desensitization by single cell [Ca2+]i measurements (16, 17). Here we show that all members of P2XRs, when expressed individually, responded to 100 μM ATP with a significant rise in [Ca2+]i. In cells transfected with P2X2 or P2X3 cDNA, however, preincubation of cultures with apyrase, an ectonucleotidase that degrades ATP, was necessary to detect any appreciable [Ca2+]i change. Preincubation of P2X2-expressing cells with this enzyme did not alter the pattern of [Ca2+]i response compared with that observed in controls (not shown).

The averaged peak [Ca2+]i amplitudes induced by supramaximal concentrations of ATP apparently differed among P2XRs. When stimulated with 100 μM ATP, the amplitudes of [Ca2+]i, were comparable among cells expressing P2X2, P2X3, and P2X4, whereas 500 μM ATP was required to reach the comparable [Ca2+]i response in P2X2-transfected cells. The peak [Ca2+]i responses caused by activated P2X2 and P2X3R with 100 μM ATP were about one-third those observed in cultures expressing P2X2. A small fraction (less than 10%) of cells transfected with P2X2 or P2X3 cDNA, however, preincubated with apyrase, an ectonucleotidase that degrades ATP, was necessary to detect any appreciable [Ca2+]i change. Preincubation of P2X2-expressing cells with this enzyme did not alter the pattern of [Ca2+]i response compared with that observed in controls (not shown).

As illustrated in Fig. 2A, the receptors also differed in their capacities to sustain [Ca2+]i signaling during continuous stimulation with ATP. The P2X2R- and P2X3R-expressing cells completely terminated [Ca2+]i signaling within 1–2 min of stimulation with 100 μM ATP. The [Ca2+]i responses caused by...
activation of P2X<sub>2b</sub>R and P2X<sub>4</sub>R showed a relatively slow decrease compared with those generated by the P2X<sub>1</sub>R and P2X<sub>3</sub>R. In contrast, P2X<sub>2a</sub>R- and P2X<sub>7</sub>R-expressing cells induced long-lasting [Ca<sup>2+</sup>]<sub>i</sub> signals. The P2X<sub>2a</sub>R-induced [Ca<sup>2+</sup>]<sub>i</sub> signals usually decreased to one-third of maximum response, and this steady-state plateau level was reached within 10 min of stimulation. The P2X<sub>7</sub>R-expressing cells showed no obvious decline in [Ca<sup>2+</sup>]<sub>i</sub> response for more than 10 min. In all cases, except for P2X<sub>7</sub>R, single exponential functions were sufficient to describe the decline rates of [Ca<sup>2+</sup>]<sub>i</sub> responses. The mean values of calculated rate constants are shown in Fig. 2B. The rank order of desensitization rates derived from these data (P2X<sub>1</sub>R > P2X<sub>3</sub>R > P2X<sub>2b</sub>R > P2X<sub>4</sub>R > P2X<sub>2a</sub>R > P2X<sub>7</sub>R) was highly comparable with that observed in current measurements (22), confirming the validity of single cell [Ca<sup>2+</sup>]<sub>i</sub> measurements as a method for comparative evaluation of receptor desensitization.

**Role of a C-terminal 6-aa Sequence in Receptor Desensitization**—Recently, we found that the Arg<sub>371</sub>-Pro<sub>376</sub> sequence, located in the cytoplasmic region of P2X<sub>2a</sub>R, is necessary for the slow desensitizing pattern of these receptors (17). As shown in Fig. 1, this 6-aa region is located near the second putative transmembrane domain, and the C-terminal difference in amino acid sequences among the members of P2XRs starts from this region. To study the possible role of structural diversity of this region in the control of receptor desensitization, the Arg<sub>371</sub>-Pro<sub>376</sub> sequence of P2X<sub>2a</sub>R was introduced to P2X<sub>3</sub>R and P2X<sub>4</sub>R instead of the native Thr<sub>362</sub>-Lys<sub>367</sub> and Glu<sub>376</sub>-Gly<sub>381</sub> sequences, respectively. Such C-terminal chimeric subunits were termed P2X<sub>3</sub>/X<sub>2a</sub>R and P2X<sub>4</sub>/X<sub>2a</sub>R (Figs. 1 and 3). Cells expressing P2X<sub>3</sub>/X<sub>2a</sub>R mutant responded to 100 µM ATP with an apparent delay in desensitization of [Ca<sup>2+</sup>]<sub>i</sub> signals compared with the wild-type P2X<sub>3</sub>R (Fig. 3A). Furthermore, the averaged spike [Ca<sup>2+</sup>]<sub>i</sub> amplitude was significantly higher in cells expressing P2X<sub>3</sub>/X<sub>2a</sub>R than in cells expressing P2X<sub>3</sub>R (F<sub>B</sub>/F<sub>350</sub> = 1.6 ± 0.1 (n = 31) versus 2 ± 0.05 (n = 34), respectively). As seen in wild-type P2X<sub>2a</sub>R, the addition of apyrase to the bath solution was necessary to detect the [Ca<sup>2+</sup>]<sub>i</sub> change in

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**Fig. 2. Pattern of calcium signaling by homopolymeric P2XRs transiently expressed in GT1 cells.** A, representative tracings of ATP-induced [Ca<sup>2+</sup>]<sub>i</sub> signals, with normalized amplitudes of [Ca<sup>2+</sup>]<sub>i</sub> responses (y axes). The P2X<sub>2</sub>R-expressing cells were stimulated with 500 µM ATP, whereas the other receptor-expressing cells were stimulated with 100 µM ATP. B, the calculated desensitization rates were derived from single exponential fittings. The bars shown are the means ± S.E. derived from the number of trials indicated above the bars. *, p < 0.05 between P2X<sub>2b</sub>R and P2X<sub>3</sub>R; ** p < 0.05 between P2X<sub>2a</sub>R and P2X<sub>3</sub>R; and *** p < 0.05 between P2X<sub>4</sub>R and P2X<sub>2a</sub>R.

**Fig. 3. Calcium signaling in GT1 cells expressing chimeric P2XRs bearing the Arg<sup>371</sup>-Pro<sup>376</sup> sequence of P2X<sub>2a</sub>R.** A, pattern of ATP-induced calcium signals in wild-type P2X<sub>3</sub>R and mutant P2X<sub>3</sub>/X<sub>2a</sub>R. Cells were stimulated with 100 µM ATP in the presence of 20 µg/ml apyrase. B, pattern of Ca<sup>2+</sup> signaling in cells expressing wild-type P2X<sub>4</sub>R and mutant P2X<sub>4</sub>/X<sub>2a</sub>R. The tracings shown in A and B are computer-derived means from 8–10 single cell recordings. C, comparison of the desensitization rates for native and mutant receptors with or without Arg<sup>371</sup>-Pro<sup>376</sup> sequence. The bars shown are mean ± S.E. derived from the number of trials indicated above the bars. *p < 0.05 or higher among pairs.
P2X Receptor Desensitization

P2X3/R-expressing cells, indicating that sensitivity of P2X3R to ATP was not largely affected by introducing Arg371-Pro376 sequence to its C terminus.

Cells expressing P2X3/X2aR also showed a slower desensitization rate compared with the cells transfected with the wild-type P2X3R (Fig. 3B). The calculated time constants for the desensitization rates were 12 ± 2 ms⁻¹ (n = 12) for P2X3R versus 4 ± 0.7 ms⁻¹ (n = 12) for P2X3/X2aR. The averaged peak [Ca²⁺], amplitudes induced by 100 μM ATP were elevated in the P2X3/X2aR-expressing cells, indicating that sensitivity of P2X3R desensitization. This agrees with literature data (13) suggesting that the transmembrane domains are critical for the fast desensitization process of P2X3R and P2X3R signaling.

We have also analyzed the relevance of P2X3R C-terminal 6-aa sequence on receptor desensitization. The optimized results of the amino acid sequence alignments for P2XRs revealed that ValProGluValAsp and Arg-Glu-Pro-Glu-Ala are critical for P2X3R desensitization. This suggests that the transmembrane domains are critical for the fast desensitization process of P2X3R and P2X3R signaling.

Identification of Residues Contributing to the Receptor Desensitization—The in-frame splicing of the P2X3αC terminus starts at Val370 (indicated by an arrow in Fig. 1) and effectively removes three positively charged residues from the Arg371-Pro376 sequence of P2X3αR (Fig. 1B). Also, a 18-aa stretch from Cys362 to Val379 of P2X3αR is not related to any other subunit C termini. Thus, an insertion was needed in the alignment (1). Substitution of Val392-Pro397 sequence with the corresponding Glu376-Gly381 sequence of P2X4R generated a functional P2X4R-Arg371-Pro376 sequence of P2X3αR. However, a 18-aa stretch from Cys362 to Val379 of P2X3αR is not related to any other subunit C termini. Thus, an insertion was needed in the alignment (1). Substitution of Val392-Pro397 sequence with the corresponding Glu376-Gly381 sequence of P2X4R generated a functional P2X4R-Arg371-Pro376 sequence of P2X3αR. This suggests that the C-terminal 6-aa sequence of P2X3R subunit is not functionally equivalent to those found in other subunits in terms of controlling receptor desensitization.

| Receptor | Time constant (ms⁻¹) |
|----------|----------------------|
| P2X3R    | 47 ± 6 (n = 15)      |
| P2X3R/X4R| 7 ± 0.5 (n = 35)    |
| P2X3R    | 24 ± 4 (n = 35)     |
| P2X3R/X2aR| 9 ± 0.4 (n = 25) |
| P2X3R/X1R| 12 ± 2 (n = 12)    |
| P2X3R/X4| 10 ± 0.8 (n = 20) |

*p < 0.05 vs. wild-type channels.

To test this, we next evaluated the effects of single amino acid substitution in the P2X3αC terminus on the pattern of ATP-induced [Ca²⁺] response. The negatively charged residues in the 6-aa sequence of P2X3αR were replaced with neutral ones, or the basic residues of the Arg371-Pro376 sequence were introduced. As shown on Fig. 5, both elimination of negatively
charged residues from and introduction of positively charged residue to the position of Glu^376 in P2X₄R decreased the rate of desensitization. Mutation of Glu^376 to a basic lysine residue also resulted in a significant decrease in the rate of desensitization. However, the point mutations at Glu^376 or Glu^379 could not produce the same extent of desensitization as seen in cells expressing wild-type P2X₂aR and mutant P2X₂aR/F₂aR. In addition, we constructed two mutant P2X₄R subunits having 3-aa substitutions, with native amino moieties of P2X₄R changed to neutral (P2X₄E376Q/D377Q/E379Q) or three basic amino acids of P2X₄R introduced at the corresponding sites of P2X₄R (P2X₄RE376R/E379K/Q380H). Both triple mutants desensitized slower than the wild-type P2X₄R (Fig. 5). Thus, changing the amino acid composition and the net charge of this particular C-terminal segment altered the duration of Ca^{2+} influx induced by P2X₄R.

Within the Arg^371-Pro^376 of P2X₂aR, Thr^372 represents an optimum phosphorylation site, (K/R)XX(T/S), for the type II calcium/calcmodulin-dependent protein kinase (23). At the analogous position of Thr^372 in P2X₄R, either glutamate or aspartate was found in P2X₂R, P2X₂bR, P2X₃R, and P2X₄R (Fig. 1). We speculated that phosphorylation of the single Thr^372 residue or introduction of a negatively charged moiety at this position may change the desensitization rate of P2X₃R. However, substitution of this residue with glutamate or with glutamine did not affect the desensitization rate of mutant channels compared with that of the wild type. The desensitization constants for glutamate- and glutamine-containing mutants were 4 ± 0.3 ms⁻¹ (n = 35) and 5 ± 0.3 ms⁻¹ (n = 21), respectively, and 4 ± 0.2 ms⁻¹ (n = 26) for wild-type channels. Also, no obvious difference was observed in the maximum amplitude of [Ca^{2+}]ᵢ response and the EC₅₀ values for ATP between P2X₃R and each C-terminal mutant receptors examined (not shown). Desensitization Pattern of Heteroplymeric P2XRs—The co-expression of P2X₃R and P2X₄R has been shown to form a heteroplymeric P2XR channel, which exhibits a distinct pharmacological profile and desensitization pattern from those seen in homoplymeric channels (11, 12). We used this particular subunit combination to analyze the impact of the C-terminal structure on the [Ca^{2+}]ᵢ-signaling pattern of heteroplymeric channels. GT1 neurons expressing only P2X₃R responded to 20 µM AMP-CPP with a rise in [Ca^{2+}]ᵢ, the amplitude and desensitization rate of which were highly comparable with that observed in 100 µM ATP-stimulated cells (Fig. 2). When co-expressed, these two subunits generated a channel that also responded to 20 µM AMP-CPP but with the amplitude of response comparable with that observed in homoplymeric P2X₃R stimulated with 100 µM ATP (Fig. 6A). In contrast, cells co-transfected with an empty vector and cDNA for P2X₂aR did not respond to this agonist (Fig. 6A).

Under the continuous presence of AMP-CPP, the subsequent application of 100 µM ATP further increased [Ca^{2+}]ᵢ, suggesting the existence of two channels with distinct pharmacological features in a co-transfected cell: AMP-CPP-sensitive and -insensitive (Fig. 6A). When the co-transfected cells were initially stimulated with 100 µM ATP, the subsequent addition of 100 µM AMP-CPP failed to induce any change in [Ca^{2+}]ᵢ (not shown). Therefore, all P2XR-expressed cells were sensitive to ATP and desensitized completely during the initial stimulation. This suggests that the AMP-CPP-mediated rise in [Ca^{2+}]ᵢ was initiated by the activation of heteroplymeric P2X₃R + P2X₄R, which desensitized more rapidly than the homoplymeric P2X₃R but slowly when compared with P2X₄R (Figs. 2 and 6).

The co-expression of the P2X₃R and C-terminal mutant P2X₃R/F₂aR also resulted in the formation of AMP-CPP-sensitive heteroplymeric channels (Fig. 6). However, the desensiti-
zation of $[\text{Ca}^{2+}]_i$, signals generated by these channels was significantly slower than in $\text{P2X}_3\text{R} + \text{P2X}_4\text{R}$-expressing cells ($9.5 \pm 0.3$ ($n = 63$) versus $13.5 \pm 0.9$ ($n = 34$), respectively). These results indicate that the C-terminal structure of the participating subunits influences the desensitization of heteropolymeric $\text{P2X}_3\text{R} + \text{P2X}_4\text{R}$ channels in a manner comparable with that observed in homopolymeric channels. It is also important to stress that the exact subunit stoichiometry of the heteropolymeric $\text{P2X}_{3\alpha\gamma} + \text{P2X}_4\text{R}$ and $\text{P2X}_{3\gamma\gamma} + \text{P2X}_4\text{R}$ in our experiments is not known and might vary within the cells. To overcome the possible impact of such heterogeneity on conclusions drawn from these experiments, the number of recordings from which means were derived was elevated compared with other experiments (see above).

**Discussion**

In this study, we employed GT1 neurons as an expression system to analyze the impact of receptor desensitization on the Ca$^{2+}$-signaling function of recombinant P2XRs and to identify the role of C terminus in receptor desensitization. The time scale for $[\text{Ca}^{2+}]_i$ measurements in our experiments was set to be sufficiently long (5–10 min) to incorporate the consequence of a pore dilation effect during ATP stimulation reported by others (24, 25) on Ca$^{2+}$ signaling. Under such recording conditions, activation of all homopolymeric P2XR by ATP caused a rise in $[\text{Ca}^{2+}]_i$, the pattern of which was highly specific for each particular channel. Receptors differed among themselves with respect to the amplitude of Ca$^{2+}$ response. $[\text{Ca}^{2+}]_i$ signals initiated by the homopolymeric P2XRs also desensitized with rates characteristic to each receptor subtype: $\text{P2X}_1\text{R} = \text{P2X}_2\text{R} + \text{P2X}_3\text{R} > \text{P2X}_4\text{R} > \text{P2X}_{3\gamma\gamma} > \text{P2X}_{2\alpha\gamma} > \text{P2X}_7\text{R} > \text{P2X}_2\text{R} > \text{P2X}_2\text{R}$. The kinetics of receptor desensitization estimated in single cell $[\text{Ca}^{2+}]_i$, measurements should be interpreted with two reservations. First, voltage-gated calcium channels, which are expressed by GT1 cells (18), were not silenced during $[\text{Ca}^{2+}]_i$ recording. Second, although the rank orders for receptor desensitization estimated in current and $[\text{Ca}^{2+}]_i$, measurements were highly comparable, the time needed to reach the steady desensitized states for P2XRs was significantly longer when estimated in $[\text{Ca}^{2+}]_i$, measurements (22). This probably reflects the slow kinetics of Ca$^{2+}$ elimination from the cytoplasm, which was additionally enhanced by the integration of voltage-gated Ca$^{2+}$ influx during receptor stimulation. Thus, $[\text{Ca}^{2+}]_i$, recordings are of limited use for studies on the dynamics of channel behavior, and the calculated rates should be interpreted only as a relative indicator of the status of receptor desensitization, especially when compared with results obtained in electrophysiological measurements.

However, we previously found a marked and comparable difference in the desensitization rates of $\text{P2X}_{3\alpha\gamma}$ and $\text{P2X}_{3\gamma\gamma}$ in both current recordings and single cell $[\text{Ca}^{2+}]_i$, measurements, the latter done under conditions where voltage-gated calcium channels were silent (16). In addition, the amplitudes of $[\text{Ca}^{2+}]_i$, response induced by these channels were significantly reduced when Ca$^{2+}$ influx though L-type Ca$^{2+}$ channels was blocked by nifedipine, but the estimated rate of receptor desensitization was not affected (17). Finally, the rank order of receptor desensitization estimated in $[\text{Ca}^{2+}]_i$, measurements was highly comparable to those observed by others in current measurements for $\text{P2X}_1\text{R}, \text{P2X}_2\text{R, P2X}_{3\alpha\gamma}, \text{P2X}_{3\gamma\gamma}$, and $\text{P2X}_2\text{R}$ (22).

In our experimental conditions, the rapidly desensitizing $\text{P2X}_1\text{R}$ and $\text{P2X}_2\text{R}$ were unable to induce a measurable increase in $[\text{Ca}^{2+}]_i$, when GT1 cells were bathed in physiological solution, but the inclusion of apyrase in extracellular space recovered receptors from the desensitized state. This suggests that the spontaneous release or pathological leakage of cellular ATP may prevent the fast-desensitizing receptors from responding to ATP stimulation with an increase in $[\text{Ca}^{2+}]_i$. $\text{P2X}_3\text{R}$ exhibited fast desensitization when expressed in oocytes and slow desensitization when expressed in HEK293 cells (19, 26, 27), indicating that experimental conditions, including a difference in amphibian and mammalian expression systems, may change the recombinant channel behavior (24). In our expression system, $\text{P2X}_3\text{R}$ completely desensitized during continuous agonist stimulation. With respect to the rate of desensitization, this channel should be considered as a relatively slow desensitizing one.

These observations support the validity of $[\text{Ca}^{2+}]_i$, measurements as an indicator of P2X receptor desensitization. Since the pattern of $[\text{Ca}^{2+}]_i$, response initiated by P2XR represents signals that encode the activity of these receptors for controlling cellular functions, the time course for desensitization estimated by single cell $[\text{Ca}^{2+}]_i$, measurements is highly relevant. Also, activation of voltage-gated Ca$^{2+}$ influx is a physiological mechanism by which P2XRs amplify $[\text{Ca}^{2+}]_i$, signals in excitable cells in addition to conducting Ca$^{2+}$ through their pores. All together, these results indicates that selective expression of P2XR subunits in an excitable cell can serve as an effective mechanism for generating specific Ca$^{2+}$ signals.

Our results further indicate that the variable subunit C termini in part accounts for the observed differences in the desensitization of channels. The structural element controlling the desensitization pattern of $\text{P2X}_{3\alpha\gamma}$ and $\text{P2X}_4\text{R}$ appears to be exclusively localized to the 6-aa C-terminal sequence. Within the Arg$^{371}$-Pro$^{376}$ of $\text{P2X}_2\text{R}$, Thr$^{372}$ represents an optimum phosphorylation site, (R/K)XX(T/S), for type II calcium/calmodulin-dependent protein kinase and is not present in P2X4R. Nonetheless, this residue is not responsible for the difference in the desensitization of these two channels, as documented in experiments with single amino acid mutations.

It is likely that total net charge in the Arg$^{371}$-Pro$^{376}$ sequence of $\text{P2X}_{3\gamma\gamma}$ and in the equivalent sequences of $\text{P2X}_2\text{R}$ and $\text{P2X}_3\text{R}$ is an important determinant of the extent of Ca$^{2+}$ influx during sustained ATP stimulation. In a case of mutant $\text{P2X}_{2\alpha\gamma}/X\text{R}$, the replacement of three positive net charges with three negative ones in the equivalent segment increased the rate of desensitization compared with $\text{P2X}_{2\alpha\gamma}/X\text{R}$ mutant receptors with two and one negative residue, respectively. The relevance of single mutations at the charged residues to the charged residues to the desensitization of these two channels, as documented in experiments with single amino acid mutations.

In parallel to electrophysiological measurements (11, 12), $[\text{Ca}^{2+}]_i$, measurements showed that heteropolymerization of $\text{P2X}_{3\alpha\gamma}$ and $\text{P2X}_{2\alpha\gamma}$ results in a channel sensitive to AMP-CPP, which desensitizes with a pattern different from those seen in cells expressing homopolymeric channels. The contribution of each subunit C terminus to $[\text{Ca}^{2+}]_i$-signaling profile was additive in this combination of subunits. Furthermore, desensitization of the heteropolymeric channels composed of $\text{P2X}_{3\gamma\gamma}$ and $\text{P2X}_4\text{R}$ subunit was delayed by the C-terminal mutation intro-
duced only in the P2X<sub>7</sub>R subunit. Based on the putative pseudosymmetrical orientation of the channel subunits, heteropolymeric P2X<sub>3</sub>R + P2X<sub>7</sub>R could have the 6-aa C-terminal region of each subunit at an equivalent position around the pore axis. Such a ring-like structure made by the C-terminal-charged residues could determine the pattern of P2XR signaling.

In contrast to the other receptor subunits, mutation at the C-terminal 6-aa sequence of P2X<sub>7</sub>R did not result in modulation of receptor desensitization. This is not a surprise, since the structure of P2X<sub>7</sub>R C terminus is distantly related to others. It is the longest C terminus among P2XRs and contains the structural basis underlying the unique feature of this channel, the cell-lytic pore formation. It also shows the least amino acid sequence similarity compared with the other P2XR C termini (10). The amino acid sequence alignment of P2XRs revealed that an 18-aa stretch of P2X<sub>7</sub>R, with no similarity to others, is positioned proximal to the Val<sup>392</sup>-Pro<sup>397</sup> sequence, which corresponds to the Arg<sup>371</sup>-Pro<sup>376</sup> sequence of P2X<sub>2a</sub>R (1). This insertion should effectively move the charged 6-aa residues away from the internal mouth of the pore.

A role of the C terminus in receptor signaling is not unique for P2XRs. The contribution of the C terminus to channel gating was reported for other channels, including acetylcholine-gated channels, inward rectifier potassium channels, and mechanosensitive channels (28–31). In the case of inwardly rectifying potassium channels, which have the same topological architecture as P2XRs, the important determinants of the pore block by Mg<sup>2+</sup> and polyamines are mediated by negatively charged residues located in the C terminus and the second transmembrane region (29). The mechanism for P2XR desensitization by the charged C-terminal small segment needs to be further examined by means of structural and biophysical studies.

In conclusion, we show here that the activation of P2XRs by ATP leads to an increase in [Ca<sup>2+</sup>]<sub>i</sub>, the pattern of which is highly specific for each channel expressed and determined by the rate of receptor desensitization. The variable C termini of receptor subunits in part account for the observed difference in desensitization rates of homo- and heteropolymeric receptors. The charged residues in the 6-aa C-terminal sequence appear to serve as a common factor influencing the desensitization rates of P2X<sub>3</sub>R, P2X<sub>2a</sub>R, P2X<sub>2b</sub>R, and P2X<sub>4</sub>R. The structural element responsible for the difference in desensitization rates among P2X<sub>3</sub>R, P2X<sub>2a</sub>R, and P2X<sub>4</sub>R is exclusively localized to the 6-aa C-terminal sequence. In the case of P2X<sub>7</sub>R and P2X<sub>3</sub>R subunits, the structure other than the C termini also participates in the control of desensitization. Finally, P2X<sub>7</sub>R has a distinct C terminus, as well as a distinct pattern of [Ca<sup>2+</sup>]<sub>i</sub> signaling, and further experiments are required to establish the possible relationship between the structure of this terminus and the sustained Ca<sup>2+</sup> influx.

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