Identification of Amino Acid Residues Contributing to Desensitization of the P2X₂ Receptor Channel*

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The P2X₂ receptor (P2X₂R) is a member of the ATP-gated ion channels that mediate Ca²⁺ entry in several tissues, including the brain, adrenal medulla, and pituitary. Alternative usage of cryptic splice sites in the primary P2X₂R transcript accounts for the existence of several transcript types, one of which (P2X₂–2R) encodes a functional channel. P2X₂–2R lacks a stretch of cytoplasmic C-terminal amino acids (Val³⁷⁰-Gln⁴³⁸) and exhibits rapid and complete desensitization, whereas P2X₂R desensitizes slowly and incompletely. The role of the C terminus in P2X₂R desensitization was studied by generating several channel mutants and monitoring intracellular free Ca²⁺ changes in transfected single GTI–7 neurons. Deletion studies indicated that the Arg³⁷¹-Thr³⁷²-Pro³⁷³ segment of the P2X₂R is required for sustained Ca²⁺ influx. To identify the important residues within this segment, three contiguous amino acids were sequentially changed to alanine. Only two of these replacement mutants, at Arg³⁷¹-Thr³⁷²-Pro³⁷³ and Lys³⁷⁴-His³⁷⁵-Pro³⁷⁶, had an enhanced rate of desensitization. Single amino acid deletions in the P2X₂R C terminus and a series of insertions of wild-type sequences into the corresponding spliced site identified four residues, Pro³⁷³-Lys³⁷⁴-His³⁷⁵-Pro³⁷⁶, required for sustained Ca²⁺ influx through agonist-occupied wild-type channels. Thus, it is likely that the Pro³⁷³-Pro³⁷⁶ sequence of P2X₂R represents a functional motif that is critical for the development of the slow desensitization profile observed in these channels. Consequently, deletion of this motif by alternative splicing provides an effective mechanism for generating a channel with controlled Ca²⁺ influx.

P2X receptors (P2XR) are ATP-gated cationic channels expressed in a variety of excitable cell types (1). Ligand binding to these channels activates an inward current, associated with an increase in the intracellular free Ca²⁺ concentrations ([Ca²⁺]ᵢ) (2). The cDNA cloning of ionotropic ATP receptors identified seven subunits (P₂X₁R to P₂X₇R), each of which individually can form a cation-permeable pore in heterologous expression systems (3–9). These channels share a common hydrophobicity profile of two putative transmembrane domains (M1 and M2) connected with a large hydrophilic loop and intracellular N and C termini (10). There is 50–65% amino acid sequence similarity overall between pairs of P2XR. In particular, the sequences from the N termini to the second transmembrane domains are relatively conserved, whereas the C-terminal tails display the least sequence similarity to each other (11). The physiological significance of the variations in the C-terminal structure has not been elucidated.

P2XR subtypes differ with respect to their ligand selectivity profiles, antagonist sensitivity, and desensitization rates (10, 12). Based on differences in the desensitization kinetics, these channels can be divided into two groups: rapidly desensitizing channels (P₂X₁R and P₂X₂R) and slow desensitizing channels (P₂X₃R, P₂X₅R, and P₂X₇R) (11). P₂X₄R expressed in oocytes exhibits strong desensitization, but when expressed in HEK293 cells this channel desensitizes slowly (7, 13, 14). Two experimental approaches have been used to study the molecular mechanism underlying P₂X₂R desensitization, the construction of chimeric channels between slowly and rapidly desensitizing subtypes and the co-expression of both types (5, 15). Chimeric studies suggest that the responsible domains for desensitization are localized within the two transmembrane regions of P₂X₁R and P₂X₇R (15). Co-transfection of the expression plasmids for these two channel types was also found to yield a P₂X₂R with altered desensitization and agonist selectivity properties, indicating that such channels are presumably heteropolymers (5). Recently, a new view about P₂X₂R desensitization has emerged. The P₂X₂R splice variant, termed P₂X₂–2R or P₂X₂bR (16–18), lacks a stretch of the C-terminal amino acids of the P₂X₂R molecule (Val³⁷⁰-Gln⁴³⁸) and encodes a functional channel that desensitizes faster than the wild-type. This observation suggests the importance of the spliced segment for prolonged Ca²⁺ influx through wild-type channels. The P₂X₂–2R and several other splice variants were observed in neuronal and pituitary tissues (17, 18). In pituitary somatotrophs, co-expression of spliced and wild-type P₂X₂R was shown to provide an effective mechanism for controlled Ca²⁺ influx (18).

In this study, the structural elements in the P₂X₂R C terminus that are responsible for prolonged activation of wild-type channels were examined by generating diverse receptor mutants and monitoring [Ca²⁺]ᵢ in transfected single cells. For this purpose, several experimental approaches were employed. Initially, a series of C-terminal truncated mutants were produced to narrow the region(s) needed for the slow desensitization pattern of P₂X₂R. Subsequently, triple alanine replacement and single amino acid deletion mutants were constructed to precisely identify the amino acid sequence that is critical for long lasting Ca²⁺ signaling by wild-type channels. Finally, spliced amino acids from the C terminus of the wild-type channel were gradually added to the splice channel to regain the
slow desensitizing pattern of Ca\(^{2+}\) signaling in response to prolonged agonist stimulation. The results indicate that a polypeptide region containing Pro\(^{373}\), Leu\(^{374}\), His\(^{375}\), and Pro\(^{376}\) residues is important for prolonged Ca\(^{2+}\) influx in agonist-occupied wild-type channels.

**MATERIALS AND METHODS**

**Construction of Mutant P2X\(_R\)R—** A 1.5-kilobase pair cDNA fragment of the P2X\(_R\)R, obtained from rat pituitary by reverse transcription-PCR (18), was subcloned into pBluescript II vector (Stratagene, La Jolla, CA) in its XhoI/EcoRI site and used for PCR as a template to generate receptor mutants. For construction of C-terminal truncated receptors, in-frame premature stop codons were introduced in PCR primers at corresponding amino acid positions Ser\(^{431}\), Leu\(^{414}\), Pro\(^{392}\), and Arg\(^{371}\). For alanine-scanning mutagenesis, three contiguous amino acids were changed to alanine in the C-terminal portion starting from Arg\(^{371}\) to Ile\(^{374}\). New restriction sites for PstI were engineered to identify alanine mutant clones. A single amino acid deletion from the wild-type sequence and gradual additions of amino acids, which were spliced out from the wild-type, to the variant C terminus, were also generated by PCR. All PCR fragments for construction of mutant receptors were subcloned into pBluescript II and sequenced by the dideoxy chain termination method using Version 2.0 (Amersham Pharmacia Biotech). After confirming whole sequences of the PCR products, these mutations were cut with XbaI and XhoI and transferred to the C terminus of the P2X\(_R\)R expression vector, pME/P2X\(_R\)R (18), to substitute corresponding sequences.

**Cell Cultures and Expression Studies—** Cell culture and transfection of the GT1–7 neurons were performed as described previously (18). Briefly, GT1 neurons were cultured in Dulbecco's modified Eagle's Medium and Ham's F12 medium (1:1) supplemented with 10% fetal calf serum and 100 mg/ml ampicillin. On the day of transfection, 3 \(\mu\)g of the plasmid DNA was mixed with 7 \(\mu\)l of LipofectAMINE\(^{TM}\) in 3 ml of serum-free OPTI-MEM medium (Life Technologies, Inc.), incubated for 20 min at room temperature, and then applied to cells (10\(^{5}\) cells/60-mm dish). After 6 h of incubation, the medium was replaced with fresh culture medium and cells were allowed to grow for 24 h. For single cell calcium recordings, transfected cells were plated on poly-L-lysine-treated coverslips. Assays were performed 48 h after transfection.

**Measurements of Calcium Ion Concentration—** For single cell [Ca\(^{2+}\)], measurements, cells were incubated at 37 °C for 60 min with 2 \(\mu\)M fura-2/AM in phenol red- and ATP-free Dulbecco's modified Eagle's medium. The cells were subsequently washed with Krebs-Ringer solution containing 1.2 mM Ca\(^{2+}\) and kept for at least 1/4 h in this medium prior to measurements. All experiments were performed in cells bathed in Krebs-Ringer solution containing 1.2 mM Ca\(^{2+}\) and 100 nM nifedipine at room temperature. Nifedipine was added to exclude the incorporation of non-inactivating t-type Ca\(^{2+}\) channels, which are expressed in GT1–7 cells (19). Coverslips with cells were mounted on the stage of an Axioskop 135 microscope (Carl Zeiss, Oberkochen, Germany) attached to the Attocor Digital Fluorescence Microscopy System (Atto Instruments, Rockville, MD). Cells were examined under a 40× oil immersion objective during exposure to alternating 340 and 380 nm light beams, and the intensity of light emission at 520 nm was measured. The ratio of light intensities, \(F_{340}/F_{380}\), which reflects changes in Ca\(^{2+}\) concentration, was simultaneously followed in several single cells.

**Calculations—** The statistical significance of mono- and multiexponential fits were assessed according to the "extra sum of squares" principle; \(p < 0.01\) was considered significant (GraphPad Prism, GraphPad Software, San Diego, CA). The results for desensitization rate were expressed as means ± S.E. Student's t-test was used for statistical comparison among means where applicable. Differences in a \(p\) value less than 0.01 were considered significant. In all figures showing [Ca\(^{2+}\)], changes, the tracings are normalized with respect to the maximum in the amplitude (100%).

**RESULTS AND DISCUSSION**

The presence of multiple splice variants of P2X\(_R\)R in various cell types has been reported by several groups (16–18). One of these transcripts, P2X\(_R\) \(_{-}\), forms a functional channel when expressed in Xenopus oocytes, GT1–7, and HEK293 cells. In our study, the immortalized GT1–7 neurons were used as an expression system because of the following three reasons. First, these cells express neither P2Y calcium-mobilizing receptors nor P2X receptor channels, as documented by the inability of 100 \(\mu\)M ATP to rise [Ca\(^{2+}\)], in cells bathed in Ca\(^{2+}\)-deficient as well as Ca\(^{2+}\)-containing medium. Second, the pattern of single cell [Ca\(^{2+}\)] responses and the profiles of isolated Ca\(^{2+}\) currents sufficiently demonstrated a marked difference in the desensitization kinetics between wild-type and splice variant subunits (18). Since the rates of desensitization for these channels were comparable in both Ca\(^{2+}\)-current and [Ca\(^{2+}\)] measurements, here we employed only [Ca\(^{2+}\)] measurements. Finally, our preliminary experiments for RNA and protein levels confirmed that further splicing of RNA transcripts for the wild-type channel molecule does not occur in these neuronal cells, allowing channel subunit-specific characterization.

The P2X\(_R\) \(_{-}\) lacks a stretch of C-terminal amino acids (Val\(^{370}\)-Glu\(^{373}\)), indicated by dashed lines in Fig. 1A. Comparison between the wild-type and spliced channels suggested that they do not differ in terms of their activation properties, EC\(_{50}\) values to ATP, maximum current/Ca\(^{2+}\) responses to ATP, or
the recovery times from desensitization (18). They did, however, exhibit different rates of desensitization (Fig. 1B). The spliced channels P2X2R desensitized relatively rapidly and completely (within 2–3 min), whereas the wild-type channels desensitized slowly and incompletely (16–18). Thus, the presence of the Val370-Gln438 sequence in wild-type channels is critical for their slow desensitization.

To elucidate the functional role of the C-terminal tail in control of the rate of desensitization, four truncated P2XR were constructed and expressed in GT1–7 neurons (Fig. 1A). Removal of the putative cytoplasmic tail up to Pro392 resulted in mutant receptors that lost the last 81 amino acids of P2XR and did not alter the pattern of [Ca\(^{2+}\)] responses to 100 \(\mu\)M ATP (Fig. 1B, three central tracings). This argues against the potential importance of the proline-rich segment (20), observed only within the P2XR subunits, in activation and desensitization of these channels. In contrast, removal of the polypeptide sequence to Arg371 resulted in desensitization rates that were indistinguishable from those observed in P2X2R (Fig. 1, two bottom tracings). The truncated channel also showed a significant reduction in the amplitude of Ca\(^{2+}\) response to 100 \(\mu\)M ATP ([Ca\(^{2+}\)], ratio: P2XR = 3.12 ± 0.24 (n = 47) versus P2X2R-Arg371 = 1.42 ± 0.18 (n = 18)), indicating that the activation properties of this truncated channel were altered. Since the first 69 amino acids in this segment are missing in the spliced channels, these results also indicate that the C-terminal tail of the P2X2R can substitute for the Arg371-Ile391 segment but only in a transient activation of these channels.

To localize the C-terminal region responsible for the desensitization properties of the wild-type channel, specific amino acid(s) in the Arg371-Ile391 segment were mutated, whereas the residual part of the C-terminal was retained. For this purpose, we used the alanine-scanning mutagenesis method and constructed seven mutant channels, in which three contiguous amino acids were substituted for triple alanine residues: RTP373/AAA, KHP376/AAA, SSR379/AAA, WPV1852/AAA, TLA385/AAA, LVL388/AAA, and GQI391/AAA (Fig. 2A). All constructs were functional in terms of the portion of cells expressing channels and the amplitudes of [Ca\(^{2+}\)], in response to 100 \(\mu\)M ATP (not shown). The first five mutant channels (SSR379/AAA, WPV1852/AAA, TLA385/AAA, LVL388/AAA, and GQI391/AAA) also showed no change in the rate of desensitization when compared with wild-type channels. In contrast, RTP373/AAA and KHP376/AAA mutants had a significant (p < 0.01) increase in the rate of desensitization compared with wild-type channels (Fig. 2, B and C). Although the RTP373/AAA channel resulted in a faster decline in [Ca\(^{2+}\)], than the KHP376/AAA channel, neither rate of desensitization for these mutant channels fully reached that of the spliced channels (Fig. 2, B and C). This result indicates that the critical amino acids for development of a slow desensitizing pattern of Ca\(^{2+}\) signaling by P2XR are located within the C-terminal segment Arg371-Pro376 (Fig. 2A, gray area).

When the amino acid sequences for the seven subunits of the P2XR family were aligned according to their hydrophobicities, the putative cytoplasmic C termini show very little similarity in terms of amino acid moiety and length (11). However, the polypeptide sequence Arg371-Pro376 of P2XR contains three conserved residues, Arg371, Pro373, and Lys374, among four members of slow desensitizing receptors, P2X2R, P2X3R, P2X4R, and P2X7R (Fig. 3A). These residues are not present in P2X2R, which desensitizes slowly when expressed in HEK293 cells (21), but rapidly when expressed in Xenopus oocytes (13, 14). Furthermore, threonine at position 372 in the P2X2R tail is a potential phosphorylation site that can be modified by protein kinase C, (R/K)XX(T/S)X(R/K), or type II calmodulin-dependent kinase, (R/K)XX(T/S)X (X represents any amino acid). To address the role of the conserved residues, as well as the Thr372 residue, in the desensitization pattern of the wild-type channel, mutant receptors with single amino acid deletions from the wild-type C-terminal were compared with the splicing variant P2X2–2R or wild-type receptor. Elimination of four individual residues in this segment indicated the importance of two amino acids, Pro373 and Lys374, in the desensitization pattern of wild-type channels. As shown in Fig. 3, B and C, both mutants exhibited an enhanced rate of desensitization when compared with wild-type channels, but neither channel alone was able to mimic the pattern of desensitization of the spliced channel. On the other hand, deletions of Arg371 or Thr372 resulted in no detectable change in the desensitization rate.

The functional properties of the P2X2R C-terminal were further analyzed by mutagenesis studies, in which amino acid residues spliced out from the wild-type tail were gradually added back to the splice site at the P2X2–2R C terminus (Fig. 4A). The common 34 amino acid end for P2X2R and P2X2–2R was also kept in these mutant receptors. In all mutants, the amplitudes of [Ca\(^{2+}\)], in response to 100 \(\mu\)M ATP were comparable with that of the wild-type channel. Additions of three amino acids in P2X2–2R + V-T372 to the splice site did not result in an apparent change in the desensitization rate. Additional
insertions of four amino acids from the wild-type sequence resulted in a gradual decrease in the rate of desensitization when compared with that of the spliced channels (P2X2-V-P373, P2X2-V-K374, P2X2-V-H375, and P2X2-V-P376 in Fig. 4, B and C). These data indicate that insertion of the Pro373-Lys374-His375-Pro376 sequence is required for sustained Ca\textsuperscript{2+} influx. Thus, it is likely that the conserved Pro 373 and Lys374 residues comprise a part of the functional region that is critical for development of the slow desensitization pattern of P2X2R.

These results indicate that desensitization rates in [Ca\textsuperscript{2+}]\textsubscript{i} responses following activation of wild-type, spliced, and mutant P2X2R channels correlate with the C-terminal structures of expressed channels. Earlier studies have shown comparable rates of desensitization for wild-type and spliced channels in isolated Ca\textsuperscript{2+} current and [Ca\textsuperscript{2+}]\textsubscript{i} measurements (18), as well as in total current measurements (16). This does not exclude the need for current measurements through these non-selective cation channels and their mutants in future experiments. However, control of Ca\textsuperscript{2+} influx through P2X2R channels is physiologically important because this ion represents an intracellular messenger that regulates a number of cellular functions, including plasma membrane excitability, hormone secretion, de novo protein synthesis, and apoptosis (22).

In this respect, the wild-type channel induces a prolonged, high amplitude Ca\textsuperscript{2+} signal, similar to that observed in apoptotic cells (23). Conversely, deletion of the Pro373-Lys374-His375-Pro376 sequence as a consequence of alternative splicing generates a channel capable of limiting Ca\textsuperscript{2+} influx into the cells during prolonged agonist activation. Furthermore, the co-expression of wild-type and spliced channels provides an effective mechanism to sustain Ca\textsuperscript{2+} signaling but protects the cells from overloading with Ca\textsuperscript{2+} (18). For example, pituitary somatotrophs express both spliced and wild-type channel subunits, resulting in rapid but incomplete desensitization. This leads to the biphasic pattern of Ca\textsuperscript{2+} signaling, which is com-
posed of an early spike phase and sustained plateau phase (18). The probable heteromeric assembly of wild-type P2X2R with rapidly desensitizing P2X3R may also compose a channel with controlled cationic influx (24), but this mechanism is potentially available only for cells that express both subunits.

In conclusion, the presence of specific residues in the C terminus of P2X2R, with two conserved amino acids contributing significantly to the development of a sustained Ca\(^{2+}\) influx through these channels, may indicate a common mechanism of desensitization for the P2XR family. The Lys\(^{375}\) residue is common to four slow desensitizing channels, P2X2R, P2X5R, P2X6R, and P2X7R, whereas Pro\(^{374}\) is only present in three of them, P2X2R, P2X6R, and P2X7R. On the other hand, the rapidly desensitizing P2X1R and P2X3R do not contain these two amino acids (11). These residues are also not present in P2X4R, a channel that desensitizes rapidly when expressed in oocytes (13, 14). The removal of the Pro\(^{373}\)-Pro\(^{376}\) segment by splicing resulted in the rapidly desensitizing P2X2–2R (16–18). Certainly, future experiments with insertion of this segment into rapidly desensitizing channels and its deletion from slow desensitizing P2XR will provide important insight into the functional regulation of P2XR by their C termini.

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