Identification of Negative Residues in the P2X₃ ATP Receptor Ectodomain as Structural Determinants for Desensitization and the Ca²⁺-sensing Modulatory Sites*

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Elsa Fabbretti‡, Elena Sokolova‡, Lara Masten, Marianna D’Arco, Alessandra Fabbro, Andrea Nistri§, and Rashid Giniatullin

From the Neurobiology Sector, International School for Advanced Studies (SISSA), Via Beirut 4, 34014 Trieste, Italy

On nociceptive neurons, one important mechanism to generate pain signals is the activation of P2X₃ receptors, which are membrane proteins gated by extracellular ATP. In the presence of the agonist, P2X₃ receptors rapidly desensitize and then recover slowly. One unique property of P2X₃ receptors is the recovery acceleration by extracellular Ca²⁺ that can play the role of the gain-setter of receptor function only when P2X₃ receptors are desensitized. To study negatively charged sites potentially responsible for this action of Ca²⁺, we mutated 15 non-conserved aspartate or glutamate residues in the P2X₃ receptor ectodomain with alanine and expressed such mutated receptors in human embryonic kidney cells studied with patch clamping. Unlike most mutants, D266A (P2X₃ receptor numbering) desensitized very slowly, indicating that this residue is important for generating desensitization. Recovery appeared structurally distinct from desensitization because E111A and D266A had a much faster recovery and D220A and D289A had a much slower one despite their standard desensitization. Furthermore, E161A, E187A, or E270A mutants showed lessened sensitivity to the action of extracellular Ca²⁺, suggesting that these determinants were important for the effect of this cation on desensitization recovery. This study is the first report identifying several negative residues in the P2X₃ receptor ectodomain differentially contributing to the general process of receptor desensitization. At least one residue was important to enable the development of rapid desensitization, whereas others controlled recovery from it or the facilitating action of Ca²⁺. Thus, these findings outline diverse potential molecular targets to modulate P2X₃ receptor function in relation to its functional state.

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Thus, Ca²⁺ can exert a profound, rapid action on the ability of P2X₃ receptors to transmit sensory inputs to the central nervous system. However, the precise sites mediating the effect of Ca²⁺ remain unknown and carry considerable interest for any attempts to manipulate transduction of pain signals.

Because on P2X₃ receptors the effects of Ca²⁺ are closely related to desensitization, our approach also provided an opportunity to explore the role of extracellular negative residues in controlling desensitization development and recovery from it as these processes are not completely understood as far as P2X₃ receptors are concerned.

For other P2X receptors, the development of desensitization is believed to be determined by receptor transmembrane and intracellular segments (10–15). However, recent experiments have indicated that the ectodomain not only controls agonist binding (16) but also desensitization via its coupling to the C-terminal domain (17). Furthermore, the chimeras of P2X₂ receptors containing the N-half of the P2X₃ receptor ectodomain develop desensitization, indicating that this region influences the desensitized conformation state and the process of recovery of receptor function (18).

The large family of ionotropic ATP receptors (P2X₁–7) shares a similar topology that comprises two transmembrane domains joined by one large extracellular loop with 10 disulfide bonds and intracellular N- and C-terminal regions (2, 5, 6). The facilitating action of extracellular Ca²⁺ is exclusively produced on desensitized P2X₃ receptors, perhaps via extracellular sites (3, 4). To identify the receptor region involved in this action and the amino acid sites important for it, we focused on negatively charged residues of the ectodomain of P2X₃ receptor that are not conserved in other P2X receptors. In fact, other subtypes of the P2X receptor family have either very slow desensitization (typical of the P2X₂ receptor class) (7, 8) or fast desensitization (e.g. the P2X₁ receptor class) not modulated by high extracellular Ca²⁺ (9).

Because on P2X₃ receptors the effects of Ca²⁺ are closely related to desensitization, our approach also provided an opportunity to explore the role of extracellular negative residues in controlling desensitization development and recovery from it as these processes are not completely understood as far as P2X₃ receptors are concerned.

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Because the latter study has narrowed the number of potential ectodomain residues important for desensitization (18), the present investigation based on single mutations of certain negatively charged amino acids allowed us to examine whether sites mediating recovery from desensitization may be distinct from those involved in the onset of desensitization.

MATERIALS AND METHODS

Mutagenesis of the P2X₃ Receptor—The pcDNA3-rP2X₃ plasmid was kindly provided by Prof. R. A. North (Sheffield University). The alignment of the amino acid sequences of P2X₁, P2X₃ and P2X₇, was deduced from NCBI accession numbers P47824 (rP2X₁), 2020424A (rP2X₇), and CAAR62894 (rP2X₃) (2).

The non-conserved, negatively charged residues of the extracellular domain of the P2X₃ receptor were mutated to the neutral amino acid alanine (Fig. 1). Single point mutations were introduced using the QuikChange mutagenesis kit (Stratagene, La Jolla, CA). Each mutated
plasmid P2X3 DNA was obtained with a single PCR reaction using specifically designed mirror-image oligonucleotides containing the mutation of interest. “Sense” and “antisense” oligonucleotides (Roche Applied Science) used for the mutagenesis are listed in Table I. For all of the mutants, the introduction of the correct mutation and the absence of spontaneous mutations were confirmed by automated DNA sequencing.

Cell Culture and Transfection—HEK 293T cells, supplied by the in-house SSISA cell bank, were maintained in culture in Dulbecco’s modified Eagle’s medium-Glutamax medium supplemented with 10% fetal calf serum and penicillin/streptomycin. For each transient transfection experiment, 5 × 10⁶ cells were plated and transfected 24 h later with the calcium/phosphate method using 1 μg of high quality purified P2X3 plasmid DNA (Sigma), either WT- or point-mutated. Transfected cells were used for further experiments 48 or 72 h later. Correct cell expression was confirmed with immunofluorescence and Western immunoblot assays as described previously (19).

Western Immunoblotting—Western immunoblots of transfected or untransfected HEK 293T cells were performed as recently reported (19). These cells were lysed using a buffer containing 100 μM Tris-HCl (pH 6.8), 200 μM dithiothreitol, 20% glycerol, and a mixture of protease inhibitors (Sigma).

Electrophysiological Recording—Details of the recording protocols can be found in Sokolova et al. (19). HEK 293T cells were continuously superfused with control solution containing (in mM) the following: 130 CsCl; 20 HEPES; 5 KCl; 1 MgCl₂; 2 CaCl₂; 20 glucose; and 10 HEPES with pH 6.8, 300 mOsm dithiothreitol, 4% SDS, 20% glycerol, and a mixture of protease inhibitors (Sigma) and separated on 10% polyacrylamide gel.

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After blocking with Tris-buffered saline containing milk, Tween 20, and protease inhibitors (Sigma). Western Immunoblotting—Western immunoblots of transfected or untransfected HEK 293T cells were performed as recently reported (19). These cells were lysed using a buffer containing 100 μM Tris-HCl (pH 6.8), 200 μM dithiothreitol, 20% glycerol, and a mixture of protease inhibitors (Sigma) and separated on 10% polyacrylamide gel.

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The basic properties of ac-S.E. (n) was accepted as indicative of statistically significant difference. The fitting function for recovery from desensitization as a function of time was as reported earlier (19).

RESULTS

Desensitization of Wild Type P2X₃ Receptors and Its Sensitivity to High Extracellular Ca²⁺—The basic properties of activation and desensitization of native P2X₃ receptors expressed by HEK 293T cells are shown in Fig. 2A. The inward current induced by 100 μM α,β-meATP peaked and then fully decayed.
Fig. 2. Extracellular Ca²⁺ transiently up-regulates WT P2X₃ receptors. A, inward currents induced by 100 μM α,β-meATP decayed fully to base line during a 2-s application of this agonist and possessed smaller amplitude when the agonist application was spaced at 30-s intervals. High extracellular Ca²⁺ (10 mM) strongly potentiated current peaks almost to control level. B, to measure recovery from desensitization, the α,β-meATP current amplitude (as percentage of non-desensitized current peak) was plotted versus time of testing subsequent agonist application. Full recovery was attained after ~100 s (time for 50% recovery was 24 s). Data are from 6–11 cells. C, time course of the enhancing action of high extracellular Ca²⁺ on α,β-meATP currents tested at 30-s intervals. The action of Ca²⁺ was rapidly lost on a washout with control solution (n = 11).

Table II
Current amplitude and desensitization onset of single point mutants of P2X₃ receptors

| Mutant     | μA            | τ₁    | τ₂    |
|------------|---------------|-------|-------|
| Wild type P2X | 1222 ± 178 (n = 43) | 123 ± 9 (n = 21) | 1346 ± 190 (n = 21) |
| D53A       | 660 ± 117 (n = 11)  | 133 ± 18 (n = 11) | 1395 ± 300 (n = 11) |
| E57A       | 681 ± 138 (n = 22)  | 146 ± 10 (n = 15) | 1016 ± 125 (n = 15) |
| E100A      | 935 ± 212 (n = 11)  | 139 ± 13 (n = 9)  | 1343 ± 231 (n = 10) |
| E111A      | 1014 ± 294 (n = 13) | 133 ± 9 (n = 11)  | 1425 ± 417 (n = 13) |
| E124A      | 1092 ± 377 (n = 10) | 119 ± 12 (n = 9)  | 1395 ± 350 (n = 10) |
| E161A      | 1106 ± 257 (n = 13) | 126 ± 17 (n = 11) | 1197 ± 245 (n = 13) |
| E187A      | 1139 ± 150 (n = 16) | 113 ± 11 (n = 14) | 1416 ± 209 (n = 15) |
| E197A      | 1351 ± 373 (n = 9)  | 111 ± 16 (n = 10) | 739 ± 170 (n = 10)  |
| D199A      | 1680 ± 318 (n = 9)  | 135 ± 10 (n = 11) | 1480 ± 148 (n = 11) |
| E208A      | 1048 ± 245 (n = 10) | 145 ± 21 (n = 9)  | 950 ± 84 (n = 10)   |
| D220A      | 110 ± 15 (n = 34)   | 100 ± 9 (n = 12)  | 828 ± 164 (n = 14)  |
| D266A      | 636 ± 122 (n = 19)  | 127 ± 15 (n = 14) | 1416 ± 225 (n = 18) |
| E270A      | 1233 ± 211 (n = 20) | 115 ± 16 (n = 15) | 1039 ± 180 (n = 15) |
| E293A      | 1478 ± 87 (n = 16)  | 117 ± 13 (n = 12) | 1031 ± 217 (n = 12) |

* p < 0.05.

with biexponential time course to base line (average data of current amplitude and decay are in Table II), indicating full desensitization. Response recovery was complete after a 6-min washout and partial (60 ± 4%; n = 21) when agonist applications were spaced at 30-s intervals. In this case, the protocol of applying agonist pulses at varying intervals after the desensitizing response enabled quantification of recovery from desensitization, which showed the typical sigmoidal time course (Fig. 2B) previously observed with native P2X₃ receptors (19). Membrane currents evoked when P2X₃ receptors were desensitized could be readily enhanced by a high extracellular Ca²⁺ solution (50 ± 6%; n = 11, Fig. 2A) in a prompt and persistent fashion (Fig. 2C). Hence, the P2X₃ receptors expressed by HEK 293T cells appeared to display all of the main properties (including sensitivity to high extracellular Ca²⁺) of native receptors of dorsal root ganglion nociceptors (3, 4).

Effect of Single Amino Acid Mutations on the Amplitude of P2X₃ Receptor Currents and Their Desensitization—We examined the extracellular loop for negatively charged sites potentially involved in mediating the action of Ca²⁺ and Mg²⁺ (3, 4) and identified 15 non-conserved amino acids (Fig. 1), which were subjected to mutation. Table II compares the functional properties of the WT and mutated P2X₃ receptors following the application of 100 μM α,β-meATP. Although the majority of
mutants generated current amplitudes very similar to the WT (Table II), the responses recorded from mutants D53A, E57A, D220A, and D266A had a significantly smaller amplitude. Examples of control currents (pulse₁) recorded from mutants E111A, D220A, and D266A are given in Fig. 3A, b–d. Although the E111A response was closely similar to that of WT, D220A generated responses of very limited size (note larger current calibration) but with a shape analogous to WT. Conversely, the D266A response was considerably slower because it produced currents with monoexponential decay, suggesting that, in this case, the onset of receptor desensitization was largely impaired. All of the other mutants had \( r_{1,2} \) values close to those of WT (see Table II), indicating that, in those cases, receptor desensitization developed unabatedly.

**Mutated Receptors Display Different Recovery from Desensitization**—Mutants could be classified into three groups as far as recovery from desensitization was concerned. Fig. 3A shows examples of current recovery (compare pulse₂ records taken 30 s after the corresponding pulse₁ traces) after \( \alpha,\beta\)-meATP-induced desensitization. The first group (Fig. 3B) comprised 10 mutants (of 15) with recovery time not significantly different from the WT recovery. The second group (Fig. 3C) showed significantly faster recovery and included E111A (with onset of desensitization similar to the WT P2X₃; see also Fig. 3Ab) and D266A (with very slow onset of desensitization; Fig. 3Ad). The third group comprised mutants with much slower recovery (Fig. 3C), namely D220A (Fig. 3Ac) and E289A, both of them with onset characteristics close to WT.

In summary, by single point mutations of certain non-conserved, negatively charged amino acids in the extracellular loop of P2X₃ receptor, we observed that Glu-111, Asp-220, Asp-266, and Glu-289 differentially contributed to the process of recovery from desensitization.

**Effect of Single Point Mutations on the Ability by Extracellular Ca\(^{2+}\) to Facilitate Recovery from Desensitization**—Using the same protocol shown in Fig. 2A, we investigated how single point mutations of the extracellular loop of P2X₃ receptors might affect the effectiveness of high extracellular Ca\(^{2+}\) to facilitate responses to \( \alpha,\beta\)-meATP. On WT receptors activated by repeated applications of \( \alpha,\beta\)-meATP to produce stable, low amplitude currents, the application of 10 mM Ca\(^{2+}\) potentiated (~150%) the agonist-induced current with respect to the one in
responses were evoked by applying 100 μM α,β-meATP applied every 30 s, A, examples of WT P2X receptors, which provided EC50 (1.4 ± 0.4 μM; n = 6) and nH (1.2) values very similar to those of native receptors of rat dorsal root ganglion neurons (19). Fig. 6A shows that E111A, E161A, E187A, and E270A produced maximal current amplitudes not significantly different from WT. Although the EC50 values of these four mutants were relatively close to those of WT, they all remained significantly larger but with similar nH values (Table III). On the other hand, Fig. 6B shows that D266A and D220A had lower potency and efficacy than WT (see Tables II and III). The reduced ability of D266A and D220A to respond to α,β-meATP could not be overcome even by applying a 10-fold larger agonist concentration (1 mM), suggesting that such mutations probably impaired channel gating rather than

standard saline solution as exemplified in Fig. 4A.

Most mutants displayed the same degree of Ca2+ modulation as the WT P2X3, (see open bars in Fig. 4B). However, six mutants did not (Fig. 4B, filled bars). In particular, E161A, E187A, and E270A generated responses significantly less sensitive to high Ca2+ (despite the fact that their recovery from desensitization was similar to that of WT). Even more strikingly, D266A, D220A, and E111A completely lost sensitivity to high Ca2+. In the latter case, we wondered whether the lack of sensitivity to high Ca2+ was related to differential recovery from desensitization (see Fig. 3C). To this end, we applied α,β-meATP at different intervals because, for each mutant, the response amplitude became ~50% of the control (obtained at a 6-min interval) as in the case of WT receptors. On E111A, 10 mM Ca2+ produced clear potentiation when responses were evoked by applying α,β-meATP every 10 s instead of every 30 s (Fig. 5A). The same approach was used for D220A receptors tested at 120-s intervals and D266A receptors tested at 5-s intervals. These results summarized in the histograms of Fig. 5B indicate that currents generated by E111A and D220A could then display strong Ca2+-dependent potentiation, whereas those produced by D266A remained Ca2+-insensitive.

These data show that Glu-161, Glu-187, and Glu-270 contributed to the facilitating action by Ca2+. For E111A, D220A, and D266A, their low sensitivity to Ca2+ seemed to be secondary to primary changes in receptor desensitization properties.

**Fig. 4.** Differential effectiveness of high extracellular Ca2+ on currents from mutated P2X3 receptors. All of the responses were evoked by 100 μM α,β-meATP applied every 30 s. A, examples of WT P2X receptors (see open bars in Fig. 4B). However, six mutants did not (Fig. 4B, filled bars). In particular, E161A, E187A, and E270A generated responses significantly less sensitive to high Ca2+ (despite the fact that their recovery from desensitization was similar to that of WT). Even more strikingly, D266A, D220A, and E111A completely lost sensitivity to high Ca2+. In the latter case, we wondered whether the lack of sensitivity to high Ca2+ was related to differential recovery from desensitization (see Fig. 3C). To this end, we applied α,β-meATP at different intervals because, for each mutant, the response amplitude became ~50% of the control (obtained at a 6-min interval) as in the case of WT receptors. On E111A, 10 mM Ca2+ produced clear potentiation when responses were evoked by applying α,β-meATP every 10 s instead of every 30 s (Fig. 5A). The same approach was used for D220A receptors tested at 120-s intervals and D266A receptors tested at 5-s intervals. These results summarized in the histograms of Fig. 5B indicate that currents generated by E111A and D220A could then display strong Ca2+-dependent potentiation, whereas those produced by D266A remained Ca2+-insensitive.

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**Potency and Efficacy of the Agonists on Mutants with Changed Ca2+ Sensitivity**—To explore this issue, we first constructed an α,β-meATP dose-response relation for WT P2X3 receptors, which provided EC50 (1.4 ± 0.4 μM; n = 6) and nH (1.2) values very similar to those of native receptors of rat dorsal root ganglion neurons (19). Fig. 6A shows that E111A, E161A, E187A, and E270A produced maximal current amplitudes not significantly different from WT. Although the EC50 values of these four mutants were relatively close to those of WT, they all remained significantly larger but with similar nH values (Table III). On the other hand, Fig. 6B shows that D266A and D220A had lower potency and efficacy than WT (see Tables II and III). The reduced ability of D266A and D220A to respond to α,β-meATP could not be overcome even by applying a 10-fold larger agonist concentration (1 mM), suggesting that such mutations probably impaired channel gating rather than
Finally, we tested E293A because this mutant had standard desensitization properties and sensitivity to Ca$^{2+}$ (Table II and Fig. 4B). Also in this case, the EC$_{50}$ value was slightly but significantly larger than that for the WT (Table III).

Fig. 6C shows an example of Western blot analysis of WT, D220A, and D266A P2X$_3$ receptor expression. It is apparent that, in each case, there was the comparable pattern of P2X$_3$ agonist binding (see comparable data with P2X$_2$ receptors) (21). Finally, we tested E293A because this mutant had standard desensitization properties and sensitivity to Ca$^{2+}$ (Table II and Fig. 4B). Also in this case, the EC$_{50}$ value was slightly but significantly larger than that for the WT (Table III).

**TABLE III**

| Mutant | EC$_{50}$ | $n_H$ |
|--------|----------|-------|
| WT     | 1.4 ± 0.5 | 1.1 ± 0.2 |
| E111A  | 5.9 ± 1.2$^a$ | 1.7 ± 0.5 |
| E161A  | 5.4 ± 1.3$^a$ | 1.7 ± 0.4 |
| E187A  | 5.3 ± 1.2$^a$ | 1.0 ± 0.3 |
| D220A  | 7.2 ± 1.2$^a$ | 1.2 ± 0.3 |
| D266A  | 28 ± 2.9$^a$ | 1.5 ± 0.4 |
| E270A  | 5.3 ± 1.5$^a$ | 1.3 ± 0.1 |
| E293A  | 4.5 ± 1.7$^a$ | 1.1 ± 0.1 |

$^a p < 0.05$. receptor protein, indicating that the large disparity in agonist sensitivity could not be simply due to insufficient receptor expression. Similar levels of P2X$_3$ receptor expression were also detected for all of the other mutants (data not shown).

**DISCUSSION**

The principal finding of this study is the identification of non-conserved, negatively charged residues in the extracellular loop of P2X$_3$ receptors involved in sensing Ca$^{2+}$. Furthermore, we observed distinct ectodomain residues responsible for desensitization development or for recovery from it. Thus, the present data might suggest new targets for novel analgesic strategies based on discrete regulation of P2X$_3$ receptor states.

Single Point Mutations of the P2X$_3$ Receptor Strongly Affect Its Ca$^{2+}$ Sensitivity—The strong and rapid desensitization of P2X$_3$ receptors followed by rather slow recovery (2, 3) is an important process to regulate the activity of such receptors. Because extracellular Ca$^{2+}$ can powerfully facilitate recovery of receptor function whenever the receptor is desensitized (3), understanding the mechanisms underlying this phenomenon is a major issue, especially because extracellular Ca$^{2+}$ concentrations can rapidly fluctuate during intense neuronal activity (22) and thus modulate P2X$_3$ receptor function. Although previous data suggested the action of Ca$^{2+}$ to be on the extracellular loop of P2X$_3$ receptors (3, 4), supportive evidence was only indirect. By focusing on non-conserved negatively charged residues that should be potential sites for Ca$^{2+}$ binding (6), the present data indicated Glu-161, Glu-187, and Glu-270 to be a major issue, especially because extracellular Ca$^{2+}$ were structurally and operationally distinct from those controlling desensitization.
A special case was observed with the D266A mutant (with a rather slow recovery process), because extracellular Ca\(^{2+}\) could not improve the recovery of D266A, even after changing the extent of the desensitization state.

In conclusion, the finding that slow onset or very fast recovery was accompanied by reduced sensitivity to Ca\(^{2+}\) is consistent with data showing that non-desensitized P2X\(_3\) receptors are almost insensitive to Ca\(^{2+}\) (3, 4).

Structural Determinants of Desensitization Onset—Studies of chimeras of P2X\(_2\)/P2X\(_3\) and P2X\(_1\)/P2X\(_2\) receptors have indicated that the onset of desensitization is controlled by a concerted interaction between transmembrane and intracellular domains (10, 15–17, 23). Whereas recent investigations using chimeras demonstrated the role of the ectodomain N-region to stabilize the desensitized receptor conformation (18), this study showed that a single negative residue (Asp-266) was important to control the desensitizing properties of the P2X\(_3\) receptor because its mutation conferred unusually slow onset and rapid recovery. Thus, it seems probable that the multiple regions of P2X\(_3\) receptors are involved in shaping development of desensitization.

In accordance with recent data on P2X\(_4\) receptors (24), this report indicated that a single mutation in the extracellular domain could control agonist potency, which was reduced in the tested mutants, a phenomenon not attributable to insufficient receptor protein expression. However, decreased receptor sensitivity was not necessarily intertwined with the faster development of receptor desensitization because it was possible to observe very slow desensitization together with reduced agonist potency (e.g., D266A), normal desensitization with reduced agonist potency (see Table III), and rather weak efficacy coupled with standard desensitization (D220A).

All together, these results suggested a complex interplay between the agonist binding sites (and activated channels) and the extracellular and intracellular domains controlling the onset of desensitization.

Structural Determinants of Recovery from Desensitization—Our previous work with distinct P2X\(_3\) agonists (19) suggested the onset of desensitization and recovery to be governed by independent mechanisms since agonists with identical receptor activation and onset of desensitization generated different rates of recovery. Desensitization recovery in chimeric P2X\(_1\)/P2X\(_3\) receptors is controlled by the extracellular domain (18, 23), indicating a special protein structure regulating this process. Consistent with this notion, we observed that mutations in the extracellular loop, which left the onset of desensitization unaltered with respect to WT, displayed either faster (E111A) or slower (E289A and D220A) recovery from desensitization.

Since our previous work has suggested that desensitization is a multi-step process involving several receptor conformational states (19), it seems plausible to hypothesize that the ability to generate (or exit from) discrete conformations is governed by distinct molecular determinants within the P2X\(_3\) receptor protein.