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Size of side-chain at channel pore mouth affects Ca^{2+} block of $P2X_2$ receptor

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Abstract

Effects of amino acid replacement at the channel pore mouth of $P2X_2$ receptor/channel on multivalent cation channel block were investigated. When Asn^{333} was replaced with various amino acid residues with neutral side chains (Gly, Ala, Val, Leu and Ile), the block by Ca^{2+} was attenuated according to the sizes of the side chains. The block by La^{3+} was also greatest with the Gly-substituted mutant, but this preference was not found for the block by other multivalent cations tested. The side chain at the channel pore mouth may interfere with the access of Ca^{2+} block by steric hindrance.

Keywords: $P2X_2$ receptor; Ion channel; Ca^{2+}; Multivalent cation block; Site-directed mutagenesis; *Xenopus* oocyte

1. Introduction

P2X receptors are ion channel-forming proteins which are activated by extracellular ATP, and its roles in excitatory neurotransmission have been demonstrated in various tissues (Burnstock, 1997; Khah, 2001). To date, at least seven subclasses ($P2X_{1-7}$) have been cloned, and they have been shown to form homo- or heteromeric receptors which act as functional ion channels (North and Surprenant, 2000). The analysis of the hydropathy profiles of amino acid sequences of P2X receptors has shown that each subclass consists of two transmembrane domains (TM1 and TM2) and one long extracellular domain between them (E1). A line of experimental evidence supports the contribution of TM2 to the formation of the channel pore (Rassendren et al., 1997; Egan et al., 1998; Migita et al., 2001), and recent findings have also suggested the contribution of TM1 to the pore formation (Jiang et al., 2001; Haines et al., 2001). An asparagine residue at the position 333 in TM2 of $P2X_2$ receptor (Asn^{333}) is believed to exist near the outer mouth of the channel pore, and serve as a key residue which determines single-channel conductance (Nakazawa et al., 1998a). Asn^{333} appears to contribute to formation of the channel pore because the dilation of the channel pore upon long-lasting receptor activation was accelerated when this residue was replaced with alanine (Virginio et al., 1999). Ca^{2+} and other divalent cations (Nakazawa and Hess, 1993; Ding and Sachs, 1999, 2000; Negulyaev and Markwardt, 2000) and trivalent cations (Nakazawa et al., 1997) are known to inhibit ionic current permeating through P2X receptor/channels. In the present study, we replaced Asn^{333} of $P2X_2$ receptor/channel with various amino acids, and investigated the block by Ca^{2+} and other multivalent cations of these mutant channels to elucidate the interaction between these ions and the channel pore mouth.

2. Materials and methods

Mutants of $P2X_2$ receptor constructed from the cloned rat $P2X_2$ receptor (Brake et al., 1994) were kindly supplied by Prof. R.A. North, except for N333I, N333V, N333L and

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N333I, which were constructed by site-directed mutagenesis in our laboratory as described (Nakazawa et al., 1998b). Channels were expressed in *Xenopus* oocytes and ionic currents permeating through them were measured as previously described (Nakazawa and Ohno, 1996; Nakazawa et al., 1998b). Oocytes were bathed in ND96 solution containing (in mM) NaCl 96, KCl 2, $CaCl_2$ 1.8, $MgCl_2$ 1, HEPES 5 (pH 7.5 with NaOH). ATP (adenosine 5′ -triphosphate disodium salt; Sigma, St. Louis, MO, USA) was applied by superfusion for about 6 s with a regular interval of 1 min. All the divalent and trivalent cations used were chloride salts of reagent grade. The trivalents cations and Mn^{2+} were dissolved in standard ND96 solution. When block by Ca^{2+} or Mg^{2+} was assessed, these cations were dissolved in Ca^{2+}-free, Mg^{2+}-free ND96 solution. The current amplitude in the presence of trivalent cations and Mn^{2+} was normalized to that in the absence of these cations. Under divalent cation-free condition, *Xenopus* oocytes become electrically too leaky to record current responses to ATP because of the opening of divalent cation-sensitive nonselective cation channels (Arellano et al., 1995; Zhang et al., 1998). Thus, for the current block by Ca^{2+} or Mg^{2+}, the current amplitude was normalized to that in the presence of 0.18 mM Ca^{2+} or Mg^{2+}, respectively. Statistical analysis was first made by the analysis of variance (ANOVA) and, when *F*-values were statistically significant, the comparison of groups was made by Tukey's test. When statistical significance was stated, *F*- and *P*-values were listed in corresponding figure legends.

3. Results

By increasing extracellular Ca^{2+}, ionic current activated by 30 μM ATP was decreased (Fig. 1A). Fig. 1B compares

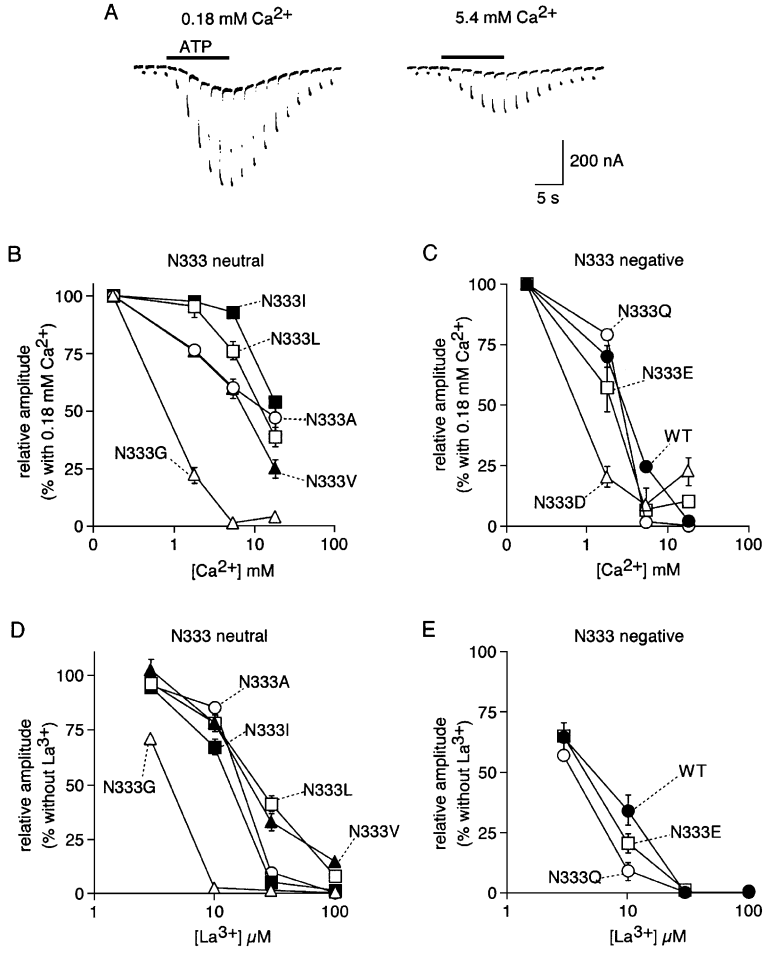

Fig. 1. (A) Ionic currrent activated by 30 μM ATP in a *Xenopus* oocyte expressing the wild type $P2X_2$ receptor/channel in the presence of 0.18 (left) or 5.4 mM Ca^{2+} (right). The oocyte was held at −50 mV and stepped for 400 ms to −80 mV every 2 s. (B–E) Concentration–response curve for Ca^{2+} (B, C) and La^{3+} (D, E) block on channels with neutral (B, D) or negatively polarized and charged (C, E) amino acid residues at the position 333. Current was measured as in (A), and responses at −80 mV were normalized to those in the presence of 0.18 mM Ca^{2+} or in the absence of the La^{3+} (see Section 2). The normalized responses are plotted against the logarithm of Ca^{2+} or La^{3+} concentrations. Each symbol and bar represent the mean and S.E. obtained from four to six oocytes tested. The values from the statistical analysis of the block by 5.4 mM Ca^{2+} are $F=135.6$ ($P<0.001$ by ANOVA) and $P<0.001$ (N333G vs. N333A), $P<0.01$ (N333A vs. N333L, N333V vs. N333I, N333L vs. N333I) or $P>0.05$ (N333A vs. N333V), and those of the block by 10 μM La^{3+} are $F=183.7$ ($P<0.001$ by ANOVA) and $P<0.001$ (N333G vs. N333A, N333G vs. N333V, N333G vs. N333G vs. N333L, N333G vs. N333I).

the block by Ca^{2+} of ionic current through $P2X_2$ receptor/channel mutants that possess amino acid residues with neutral side chains at position 333 (N333G, N333A, N333V, N333L and N333I). Among these neutral mutants, N333G was the most sensitive to Ca^{2+}, and the sensitivity was lowered almost completely according to the size of the side chains (Gly>Ala≅Val>Leu>Ile). This order was also statistically assured when the block by 5.4 mM Ca^{2+} was compared among these mutants. The block by La^{3+} was also greatest with N333G; the remaining neutral mutants uniformly exhibited lower sensitivities (Fig. 1D). The block by 10 μM La^{3+} was significantly greater with N333G than with the remaining four mutants.

Fig. 1C compares the block by Ca^{2+} of the channels that possess amino acid residues with negatively polarized (WT and N333Q) or charged (N333D and N333E) residues at the position 333. With introducing aspartic acid at the position 333, the block by Ca^{2+} was enhanced, suggesting that a negative charge at this position increases Ca^{2+} sensitivity. However, such enhancement was not observed with the introduction of glutamic acid. As for the block by La^{3+}, the block was not augmented by the introduction of glutamic acid (Fig. 1E). The effect of La^{3+} on N333D channel was not examined because the ATP-evoked current permeating through this channel became too small to analyze the blocking effect quantitatively in the presence of 1.8 mM Ca^{2+}, as seen in Fig. 1C.

Tests were made to determine the size-dependence found for the Ca^{2+} block was also found for the block by other divalent cations. Fig. 2A shows the block by Mg^{2+} of the

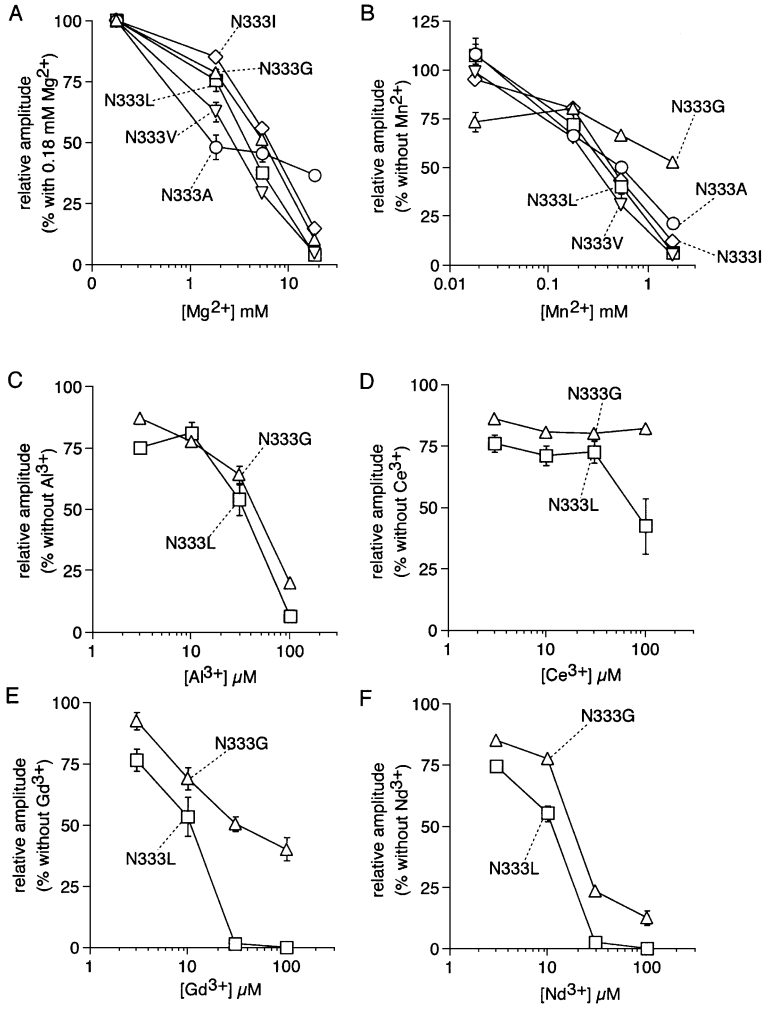

Fig. 2. Block by various multivalent cations of N333 neutral mutants. Current was measured as in Fig. 1A. Each symbol and bar represent the mean and S.E. obtained from four to five oocytes tested. (A, B) Concentration–response curve for Mg^{2+} (A) and Mn^{2+} block (B) on channels with neutral amino acid residues at the position 333. Current responses at −80 mV were normalized to those in the presence of 0.18 mM Mg^{2+} (A) or in the absence of Mn^{2+} (B). The normalized responses are plotted against the logarithm of Mg^{2+} or Mn^{2+} concentrations. The values from the statistical analysis of the block by 1.8 mM Mg^{2+} are $F=21.0$ ($P<0.001$ by ANOVA) and $P<0.001$ (N333A vs. N333L, N333A vs. N333G, N333A vs. N333I), $P<0.01$ (N333V vs. N333I), $P<0.05$ (N333A vs. N333V, N333V vs. N333I) or $P>0.05$ (N333V vs. N333L). (C–F) Effects of Al^{3+} (C), Ce^{3+} (D), Gd^{3+} (E) and Nd^{3+} (F) on N333G and N333L mutant channels. Current responses at −80 mV were normalized to those in the absence of trivalent cations. The normalized responses are plotted against the logarithm of trivalent cation concentrations.

neutral mutants of $P2X_2$ receptor/channel. When the magnitude of the block by 1.8 mM Mg^{2+} was compared, the block was apparently reduced in the order of Ala>Val>Leu>Gly>Ile. When statistically analyzed, the block by 1.8 mM Mg^{2+} with N333A was greater than with the remaining four mutants, and that with N333V was greater than with N333L, N333G or N333I. The results suggest that the size-dependence was found for the Mg^{2+} block except the Gly substitution. On the other hand, no size-dependence was found for the block by Mn^{2+} of these neutral mutants (Fig. 2B).

The effects of various trivalent cations were compared between N333G and N333L to determine whether or not selective block of N333G channel similar to that observed with La^{3+} was found. Among four trivalent cations tested (Al^{3+}, Ce^{3+}, Gd^{3+} and Nd^{3+}), none of them preferentially blocked N333G channel; the cations rather preferentially blocked N333L channel (Fig. 2C).

4. Discussion

By comparing the effects on the mutants possessing neutral amino acid residues at the position 333, we have demonstrated that the size of amino acid residues at this position affects the block by Ca^{2+} of $P2X_2$ receptor/channel. P2X receptor/channel in rat pheochromocytoma PC12 cells, the properties of which resemble those of the cloned $P2X_2$ receptor/channel, is permeable to both Na^+ and Ca^{2+}, but Ca^{2+} provides much smaller conductance than Na^+ does (Nakazawa and Hess, 1993). Ca^{2+} reduces net ionic current through the P2X receptor/channel by its competition with Na^+ at the channel pore. The block observed in the present study may mainly reflect this competitive inhibition. Thus, the size-dependence of the Ca^{2+} block indicates that larger amino acids at the channel pore mouth interferes with the access of Ca^{2+}. For the mutants with a negative charge at the position 333, N333D, but not N333E, exhibited higher sensitivity to Ca^{2+} than the wild type channel. This difference may due to a smaller size of aspartic acid residues than glutamic acid residues.

The selective block of the glycine-substituted mutant was also found for La^{3+}, but not for other multivalent cations tested, suggesting that the size-dependence is not uniform among cation species. One possible explanation for such diversity is the sizes of cations (or those of their hydrated forms). For example, the Shannon and Prett's ionic radius of Ca^{2+} is 1.14 Å at coordination number of 6, and this is larger than that of Mg^{2+} (0.86 Å) or Mn^{2+} (0.81 Å) (Cotton et al., 1995). Similarly, the ionic radius of La^{3+} of 1.06 Å is larger than those of other trivalent cations tested in the present study (Al^{3+} 0.68; Ce^{3+} 1.03; Gd^{3+} 0.94; Nd^{3+} 0.99; in Å). Large multivalent cations may be more readily affected by steric hindrance at the position 333.

In addition to size, negative polarity or charge at the position 333 is also a determinant of the magnitude of the Ca^{2+} block because, when comparing among amino acid residues of similar sizes (Val, Asn and Asp; Chothia, 1975), the sensitivity was increased according to negativity (Val < Asn < Asp; Fig. 1B and C). The sensitivity order of Val < Asn was also found for the block by Mg^{2+}, Mn^{2+} (not shown) and La^{3+} (Fig. 1D and E) and, thus, negative polarity at this position may attract multivalent cations regardless of cation species.

The present findings of the roles of the amino acid residue at the position 333 for multivalent cation block may further supports the importance of this position as the entrance of the channel pore, and may provide useful information about the relationship between the channel structure and functions including ion selectivity.

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References

Arellano, R.O., Woodward, R.M., Miledi, R., 1995. A monovalent cationic conductance that is blocked by extracellular divalent cations in *Xenopus* oocytes. J. Physiol. 484, 593–604.

Brake, A.J., Wagenbach, M.J., Julius, D., 1994. New structural motif for ligand-gated ion channels defined by an ionotropic ATP receptor. Nature 371, 519–523.

Burnstock, G., 1997. The past, present, and future of purine nucleotides as signalling molecules. Neuropharmacology 36, 1127–1139.

Chothia, C., 1975. Structural invariants in protein folding. Nature 254, 304–308.

Cotton, F.A., Wilkinson, G., Gauss, P.L., 1995. Basic Organic Chemistry, 3rd ed. Wiley, New York, NY.

Ding, S., Sachs, F., 1999. Ion permeation and block of P2X(2) purinoceptors: single channel recordings. J. Membr. Biol. 172, 215–223.

Ding, S., Sachs, F., 2000. Inactivation of $P2X_2$ purinoceptors by divalent cations. J. Physiol. 522, 199–214.

Egan, T.M., Haines, W.R., Voigt, M.M., 1998. A domain contributing to the ion channel of ATP-gated $P2X_2$ receptors identified by the substituted cysteine accessibility method. J. Neurosci. 18, 2350–2359.

Haines, W.R., Voigt, M.M., Migita, K., Torres, G.E., Egan, T.M., 2001. On the contribution of the first transmembrane domain to whole-cell current through an ATP-gated ionotropic P2X receptor. J. Neurosci. 21, 5885–5892.

Jiang, L.H., Rassendren, F., Spelta, V., Surprenant, A., North, R.A., 2001. Amino acid residues involved in gating identified in the first membrane-spanning domain of the rat P2X(2) receptor. J. Biol. Chem. 276, 14902–14908.

Khah, B.S., 2001. Molecular physiology of P2X receptors and ATP signalling at synapses. Nat. Rev. 2, 165–174.

Migita, K., Haines, W.R., Voigt, M.M., Egan, T.M., 2001. Polar residues of the second transmembrane domain influence cation permeability of the ATP-gated P2X(2) receptor. J. Biol. Chem. 276, 30934–30941.

Nakazawa, K., Hess, P., 1993. Block by calcium of ATP-activated channels in pheochromocytoma cells. J. Gen. Physiol. 101, 377–392.

Nakazawa, K., Ohno, Y., 1996. Dopamine and 5-hydroxytryptamine selectively potentiate neuronal type ATP receptor channels. Eur. J. Pharmacol. 296, 119–122.

Nakazawa, K., Liu, M., Inoue, K., Ohno, Y., 1997. Potent inhibition by trivalent cations of ATP-gated channels. Eur. J. Pharmacol. 325, 237–243.

Nakazawa, K., Inoue, K., Ohno, Y., 1998a. An aspargine residue regulating conductance through $P2X_2$ receptor/channels. Eur. J. Pharmacol. 347, 141–144.

Nakazawa, K., Ohno, Y., Inoue, K., 1998b. An aspartic acid residue near the second transmembrane segment of ATP receptor/channel regulates agonist sensitivity. Biochem. Biophys. Res. Commun. 244, 599–603.

Negulyaev, Y.A., Markwardt, F., 2000. Block by extracellular Mg^{2+} of single human purinergic $P2X_4$ receptor channels expressed in human embryonic kidney cells. Neurosci. Lett. 279, 165–168.

North, R.A., Surprenant, A., 2000. Pharmacology of cloned P2X receptors. Annu. Rev. Pharmacol. Toxicol. 40, 563–580.

Rassendren, F., Buell, G., Newbolt, A., North, R.A., Surprenant, A., 1997. Identification of amino acid residues contributing to the pore of a P2X receptor. EMBO J. 16, 3446–3454.

Virginio, C., MacKenzie, A., Rassendren, F.A., North, R.A., Surprenant, A., 1999. Pore dilation of neuronal P2X receptor channels. Nat. Neurosci. 2, 315–321.

Zhang, Y., McBride Jr., D.W., Hamill, O.P., 1998. The ion selectivity of a membrane conductance inactivated by extracellular calcium in *Xenopus* oocytes. J. Physiol., 763–776.