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European Journal of Pharmacology 347 (1998) 141–144

ejp

Short communication

An asparagine residue regulating conductance through $P2X_2$ receptor/channels

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Received 13 February 1998; revised 5 March 1998; accepted 10 March 1998

Abstract

Single channel currents were recorded from *Xenopus* oocytes expressing wild-type and mutated $P2X_2$ receptors. When 100 mM Na^+ was used as the permeant cation, unitary currents of about 80 pS were recorded from the oocyte expressing the wild-type channels. The single channel conductance was roughly halved when Asn^{333} was replaced by Ile (N333I). A similar decrease in single channel currents was also observed when 100 mM Li^+ or Cs^+ was used as the permeant cation. With two other mutants, in which Asp^{315} was replaced by Val (D315V) or Tyr^{330} was replaced by Ile (T333I), single channel conductance was almost the same as that of the wild-type channels. The results suggest that Asn^{333}, which is believed to be involved in the channel pore, plays an essential role in ion transport through $P2X_2$ receptor/channels. © 1998 Elsevier Science B.V.

Keywords: ATP; P2X receptor/channel; Expression; Site-directed mutagenesis; Single channel recording; Ion permeation

1. Introduction

Extracellular ATP acts as a fast neurotransmitter by activating cationic channels in postsynaptic membranes. To date, seven subclasses of ATP-activated channels have been cloned and are termed $P2X_1$–$P2X_7$ receptors (North and Barnard, 1997). The structure of P2X receptor/channels is unique with two transmembrane domains (M1 and M2) and a long extracellular loop, which is quite distinct from the structure of other ligand-gated channels. Recently, Rassendren et al. (1997) demonstrated that 12 amino acid residues in the M2 segment participate in the formation of the channel pore of $P2X_2$ receptor/channels. They did this by substituting amino acid residues in this segment by cysteines and by examining the sensitivity of macroscopic currents to channel pore-blocking agents. In the present study, we recorded single channel currents permeating through $P2X_2$ receptor/channels and mutant receptors in which highly conserved, negatively charged amino acid residues near or within the M2 segment had been neutralized, to examine the contribution of these amino acid residues to ion fluxes through the channel pore.

2. Materials and methods

The cDNA encoding the $P2X_2$ receptor (Brake et al., 1994; the Genbank entry U14414) was supplied by Dr. T. Brake of the University of California, San Francisco. The mutants of the $P2X_2$ receptors were constructed by site-directed mutagenesis. Among five mutants constructed in which negatively charged or polarized amino acids near or in the M2 segment were replaced by neutral amino acids (in single character denotation: D315V, S326I, T330I, N333I and D349V), three mutants (D315V, T330I and N333I) were able to form functional channels. The detailed procedures for the construction of the mutants and the properties of the macroscopic currents through these mutant receptor/channels will be published elsewhere (Nakazawa et al., 1998).

The channels were expressed in oocytes of *Xenopus laevis* according to our previous reports (Nakazawa et al., 1994; Nakazawa and Ohno, 1997). The oocytes were injected with RNA and were incubated at 18°C in ND96

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solution containing (mM) NaCl 96, KCl 2, $CaCl_2$ 1.8, $MgCl_2$ 1, HEPES 5 (pH 7.5 with NaOH) supplemented with 0.01% gentamycin for 5 to 7 days. Single channel currents were recorded by using the cell-attached configuration of the patch clamp technique (Hamill et al., 1991). The oocytes were first placed in a high osmotic solution containing (mM) K-aspartate 200, KCl 10, EGTA 10, HEPES 10 (pH 7.5 with KOH) for 10–15 min to facilitate the mechanical removal of the vitelline envelope (Kullberg et al., 1990). A solution with high K^+ concentration (composition (mM); K-aspartate 100, KCl 10, HEPES 10; pH 7.5 with KOH) was used as an extracellular solution to eliminate the membrane potential outside the patch (Hess et al., 1986). Patch pipettes were coated with Sylgard (Dow Corning, Midland, MI, USA) and fire-polished. The pipettes were filled with a recording solution containing 100 mM Cl-salt of a desired monovalent cation (NaCl, LiCl or CsCl) plus 0.2 mM $CaCl_2$, or a solution containing 70 mM $MgCl_2$ or $CaCl_2$. All the recording solutions were titrated to pH 7.5 with 10 mM HEPES and NaOH. Experiments were performed at room temperature (about 25°C). Electrical signals were recorded with a patch clamp amplifier (Nihon Kohden CEZ-2400, Tokyo Japan), filtered at 1 kHz and stored on a digital data recorder (PC204Ax, Sony, Tokyo, Japan). Off-line analysis of membrane currents was done by using software for patch/whole-cell clamp data (Nihon Kohden QP-120J) on a personal computer (NEC PC9801RA2, Tokyo, Japan). Data were sampled at 0.5 or 1 kHz.

ATP (adenosine 5′-triphosphate disodium salt) was purchased from Sigma (St. Louis, MO, USA). The concentrations of ATP were selected such that current traces with no or only slight overlap of channel openings were recorded. For the wild-type $P2X_2$ receptor/channel and the mutants T330I and N333I, appropriate concentrations of ATP were 10 and 30 μM, which are lower than the EC_{50} for the macroscopic current permeating through $P2X_2$ receptor/channels (Nakazawa and Ohno, 1997), with the recording solutions containing monovalent cations as a major charge carrier. For the mutant D315V, a higher concentration of ATP (100–300 μM) was used because this mutant exhibits a low sensitivity to ATP (Nakazawa et al., 1998). When recording solutions containing divalent cations as a major charge carrier were used, the concentration of ATP had to be increased to 100–300 μM (wild-type, T330I and N333I) or 3 mM (D315V) to overcome the divalent cation-induced reduction in free ATP concentrations (Honoré et al., 1989; Nakazawa, 1994). With these concentrations, channel activity was observed in nearly all the patches formed on 5- to 7-day-incubated oocytes injected with RNAs, and stable currents could be recorded for at least 5 min (up to 30 min). In contrast, no channel activity was found in the patches formed on uninjected oocytes with ATP-containing recording solutions, or on RNA-injected oocytes with recording solutions that did not contain ATP. All the data shown in this report were representative of at least four experiments performed under similar conditions.

3. Results

Fig. 1A illustrates single channel currents recorded from *Xenopus* oocytes expressing wild-type $P2X_2$ receptor/channels (left) and the mutant N333I (middle) and D315V (right) receptors with Na^+ as the major charge carrier. With the wild-type channels and D315V, single channel currents of about −4 pA at −60 mV were recorded, whereas single channel currents through N333I channels were only half as large as these channel currents. The current–voltage relationships for single channel currents shown in Fig. 1B indicates that the wild-type channel, D315V and T330I possessed a similar single channel conductance of about 80 pS, which was estimated from the current amplitude at potentials more negative than −20 mV. N333I exhibited a smaller single channel conductance of about 40 pS.

This decrease in single channel currents through N333I channels was also obvious when Li^+ was used as a major

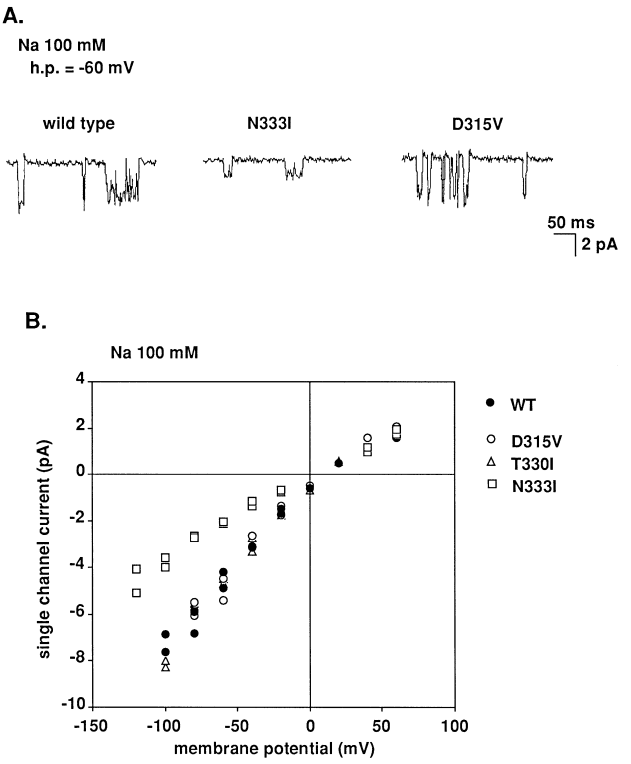

Fig. 1. Single channel currents recorded from *Xenopus* oocytes expressing wild-type $P2X_2$ receptors and their mutants. Na^+ (100 mM) was used as a major charge carrier. (A) Currents permeating through the wild-type $P2X_2$ receptor/channel (left), the mutant N333I (middle) and the mutant D315V (right). Holding potential was −60 mV. (B) Current–voltage relationship for single channel currents. Each symbol represent the amplitude of single channel currents recorded from an oocyte expressing the wild-type channel (filled circles), D315V (open circles), T330I (open triangles) or N333I (open squares), respectively.

charge carrier, as compared with currents through the wild-type channels, D315V and T330I in Fig. 2A. When Cs^+ was used as the major charge carrier, the wild-type channel exhibited two conduction levels, both of them being frequently observed (Fig. 2B). Two conduction levels were also frequently observed with N333I channels, but the levels were different from those of the wild-type channels; the larger conduction level through N333I channels was comparable to the smaller conduction level through the wild-type channels (Fig. 2B).

Channel activity was also recorded when divalent cations (Mg^{2+} and Ca^{2+}) were used as a charge carrier (Fig. 2C). With 70 mM Mg^{2+}, the single channel conductance was about 10 pS when estimated from the current amplitude between −40 and −160 mV for the wild-type channels and all three mutants. As the single channel currents were small and the open time of the currents was brief (up to several tens of ms) with Mg^{2+} (Fig. 2C) or Ca^{2+}, a distinct smaller conduction level, even if it existed, was not detected with N333I or other types of channels.

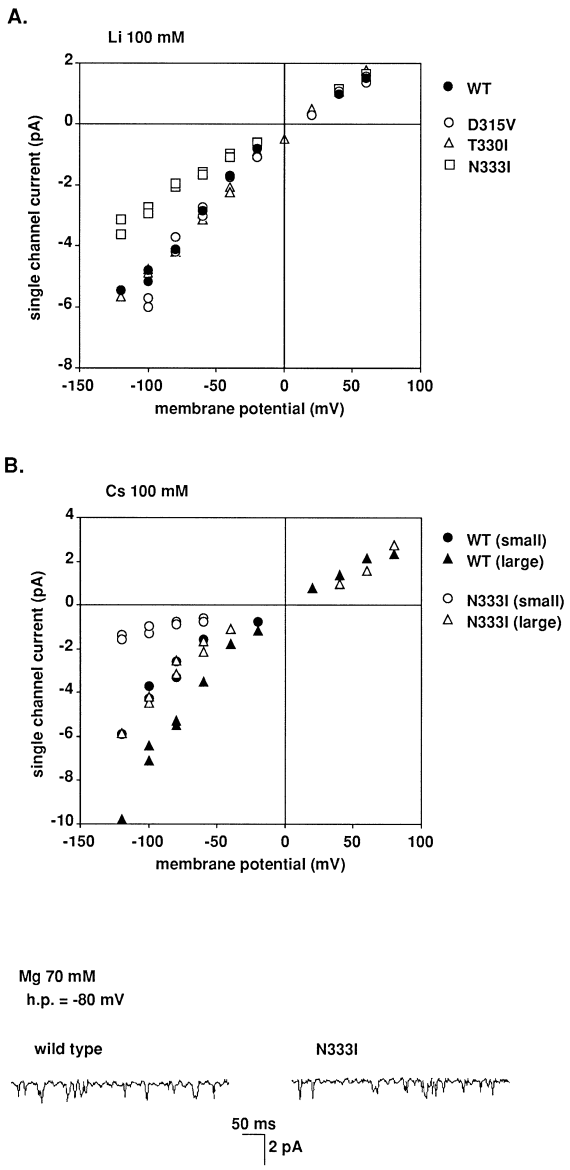

Fig. 2. Single channel currents recorded with various permeant cations. (A). Current–voltage relationship for single channel currents obtained with 100 mM Li^+ as the charge carrier. Each symbol represents the amplitude of single channel currents recorded from an oocyte expressing wild-type channels (filled circles), D315V (open circles), T330I (open triangles) or N333I (open squares), respectively. (B) A small (circles) and a large (triangles) conduction level observed with 100 mM Cs^+ as the charge carrier. Each symbol represents the amplitude of single channel currents recorded from an oocyte expressing the wild-type channels (circles) or N333I (triangles), respectively. (C) Brief opening of channels when Mg^{2+} (70 mM) was used as the charge carrier. Current traces were obtained from oocytes expressing the wild-type channels (left) and N333I (right), respectively. Holding potential was −80 mV.

4. Discussion

The neutralization of Asn^{333}, but not of Asp^{315} or Tyr^{330}, resulted in a decrease in single channel currents through $P2X_2$ receptor/channels. This finding suggests that Asn^{333} play an essential role in maintaining the conductance through the wild-type channel. A similar decrease in single channel currents after the substitution of negatively charged or polarized amino acid residues has also been reported for nicotinic acetylcholine receptor/channels (Imoto et al., 1988).

As Asn^{333} is involved in the pore-forming region (Rassendren et al., 1997), the neutralization of this residue may directly affect its interaction with permeant ions, rather than indirectly promote a conformational change. $P2X_2$ receptor/channels are cation-selective with poor selectivity among small cation species (Evans et al., 1996). Thus, it is possible that Asn^{333} may interact with permeant cations through its negative polarity. However, this negatively polarized residue may not serve as a binding-site for permeant cations because, if this were the case, the neutralization of this residue would accelerate the passage of cations through the channel pore, which would increase the single channel currents. Instead, the decrease in single channel currents through N333I receptors may be due to the reduction of the local concentration of permeant cations in the channel pore (Hille, 1992). Because of electrostatic energy, a negative charge in the channel pore is able to concentrate cations and dilute anions, and, if this charge is removed, as in the case of N333I, such effects are lost and the local concentration of permeant cations is reduced. Another possibility is that Asn^{333} determines the size of the channel pore. As P2X receptors appear to form multimeric channels (Lewis et al., 1995), Asn^{333} residues in multiple subunits may repel each other to enlarge the channel pore, and the neutralization of these residues may result in a decrease in the pore size, leading to the decrease in ion permeation.

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