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European Journal of Pharmacology 474 (2003) 205–208
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Short communication

Intracellular disulfide bond that affects ATP responsiveness of $P2X_2$ receptor/channel

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Received 20 March 2003; received in revised form 20 June 2003; accepted 27 June 2003

Abstract

The role of intracellular cysteine residues in $P2X_2$ receptor/channel was investigated. When dithiothreitol was intracellularly applied, both the maximal response and the sensitivity of the wild-type channel to ATP were decreased. On the other hand, Cu^{2+} phenanthroline did not affect the responsiveness. When two intracellular cysteine residues (Cys^9 and Cys^{430}) were replaced with alanine, both the maximal response and the sensitivity was decreased with the replacement at Cys^9, whereas no such decrease was observed with the replacement at Cys^{430}. These results suggest that an intracellular disulfide bond involving Cys^9 regulates the responsiveness of $P2X_2$ receptor/channel to ATP.

Keywords: ATP; P2X receptor; Site-directed mutagenesis; Cysteine; *Xenopus* oocyte

1. Introduction

P2X receptors are ion-channel-forming membrane proteins that are activated by extracellular ATP (see reviews of Ralevic and Burnstock, 1998; Khah, 2001; North, 2002). One functional ion channel is presumably formed by three homogeneous subunits. Each subunit has two transmembrane regions, a long extracellular loop between them, a short intracellular N-terminal region and a relatively long intracellular C-terminal region. Intracellular cysteine residues involved in cyclic nucleotide-gated ion channels have been shown to modulate the responsiveness to cGMP and cAMP (Gordon et al., 1997; Rosenbaum and Gordon, 2002). $P2X_2$ receptor contains two intracellular cysteine residues: one is in the N-terminal region (Cys^9) and the other is in the C-terminal region (Cys^{430}). In the present study, we investigated the roles of these intracellular cysteine residues in the responsiveness of $P2X_2$ receptor to ATP.

2. Material and methods

The expression of $P2X_2$ receptor and its mutants and the recordings of ionic current through the channels were performed according to our previous reports (Nakazawa et al., 1998, 1999). Briefly, $P2X_2$ receptor mutants were constructed from the cloned $P2X_2$ receptor (Brake et al., 1994) by site-directed mutagenesis. The wild-type and the mutant channels were expressed in *Xenopus* oocytes for a 4-day incubation at 18 °C, and the oocytes were served for membrane current recordings. 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. Intracellular application of dithiothreitrol or other agents was made by injection 30 min prior to current recordings. About a one-hundredth of oocyte volume (50 nl) of 100 × solutions were injected. Cu^{2+} phenanthroline (Kobayashi, 1968) was prepared from cupric sulfate and phenathroline according to Rosenbaum and Gordon (2002). Cu^{2+} phenanthroline and iodine was first prepared in ethanol as 1000 × solutions, diluted to 100 × solutions with distilled water. Dithiothreitrol was dissolved in distilled water. The expected final concentrations are 2

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doi:10.1016/S0014-2999(03)02038-7

mM (dithiothreitrol), 1.5 μM (cupric sulfate), 5 μM (phenanthroline) and 300 μm (iodine), respectively. Statistical analysis was made by two-tailed Student's *t*-test with Welch's correction or the analysis of variance (ANOVA) followed by Tukey's test. Statistical significance was judged when $P < 0.05$.

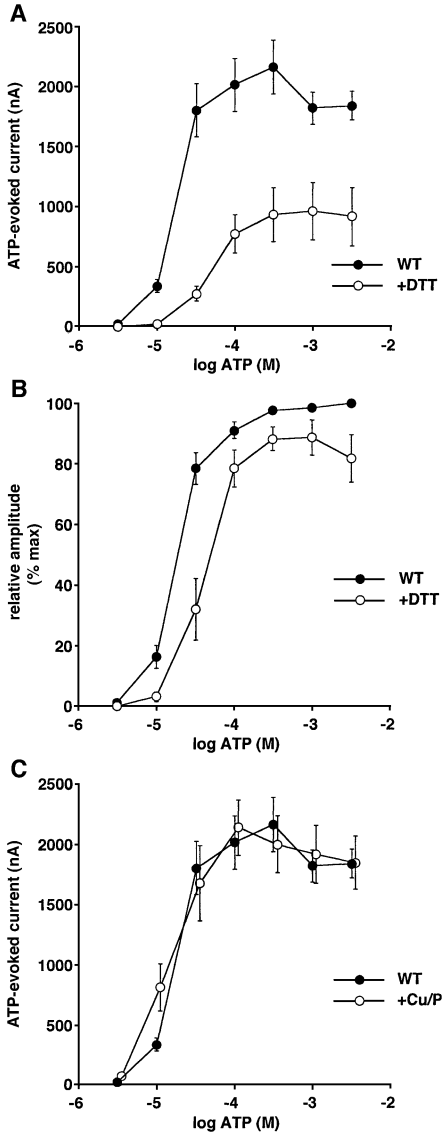

Fig. 1. Effects of dithiothreitrol and Cu^{2+} phenanthroline on ionic current evoked by ATP mediated through the wild-type $P2X_2$ receptor/channel expressed in *Xenopus* oocytes. Oocytes were held at −50 mV. Each symbol and bar represents the mean and S.E. obtained from five to seven oocytes tested. (A) Concentration–response relationship for the ATP-evoked current through the wild-type $P2X_2$ receptor/channel. The data from dithiothreitrol-injected oocytes (+DTT) were compared with those from untreated oocytes (WT). (B) Normalized current responses plotted against ATP concentrations. The data shown in (A) were normalized to maximal responses to ATP in individual oocytes. (C) Concentration–response relationship obtained from Cu^{2+} phenanthroline-injected oocytes (+Cu/P) compared with that from uninjected oocytes (WT). Absolute current responses were plotted against ATP concentrations.

3. Results

Fig. 1A compares the concentration–response relationships for ionic current through the wild-type $P2X_2$ receptor/channel activated by ATP at −50 mV in dithiothreitrol-injected oocytes with that in uninjected oocytes. In the dithiothreitrol-injected oocytes, the maximal amplitude of ATP-evoked current was about a half as large as that in the uninjected oocytes. The current amplitude was significantly smaller in the dithiothreitrol-injected oocytes than in the uninjected oocytes at ATP concentrations of 10 μM and higher (Welch's test). The sensitivity to ATP was compared by plotting normalized current amplitude against ATP concentrations (Fig. 1B). The concentration–response relationship was about threefold shifted to right by the dithiothreitrol injection. The normalized current amplitude was significantly smaller in the dithiothreitrol-injected oocytes than in the uninjected oocytes at ATP concentrations of 10 and 30 μM (Welch's test). The current response obtained from Cu^{2+} phenanthroline-injected oocytes was not different from that from uninjected oocytes (Fig. 1C). The current response was also unaffected by iodine injection (not shown).

Fig. 2 compares the concentration–response relationship for the ATP-activated current in oocytes expressing the wild-type channel and those expressing cysteine-to-alanine substituted mutants (substitution at Cys^9 alone, Cys^{430} alone, or both Cys^9 and Cys^{430}). With the substitution at Cys^{430}, the maximal response (Fig. 2A) and the sensitivity to ATP (Fig. 2B) were not changed. On the other hand, remarkable changes were observed with the substitution at Cys^9: the maximal response was decreased by 60% (Fig. 2A) and the sensitivity was lowered by about threefold (Fig. 2B). Significant difference was found for the absolute current amplitude (Fig. 2A) at ATP concentrations at 10 μM and higher, and it was found for the normalized current amplitude (Fig. 2B) at ATP concentrations from 10 to 100 μM (Tukey's test). Introduction of the second mutation at Cys^9 did not add further decrease in the responsiveness to ATP (Fig. 2A and B). Effects of dithiothreitrol and Cu^{2+} phenanthroline on the Cys^9-to-alanine substituted channel were tested. Dithiothreitrol did not decrease the maximal current response through the Cys^9-to-alanine substituted channel (Fig. 2C), in contrast to a remarkable decrease in the maximal current response through the wild-type channel (Fig. 1A). Dithiothreitrol did not change the sensitivity of the Cys^9-to-alanine substituted channel to ATP (Fig. 2D). Cu^{2+} phenanthroline affected neither the maximal current response nor the sensitivity to ATP (Fig. 2C and D).

4. Discussion

The responsiveness of $P2X_2$ receptor was decreased by a reduction agent dithiothreitrol but not by oxidation agents Cu^{2+} phenanthroline or iodine. When cysteine-to-alanine substitution was made, a similar decrease of the

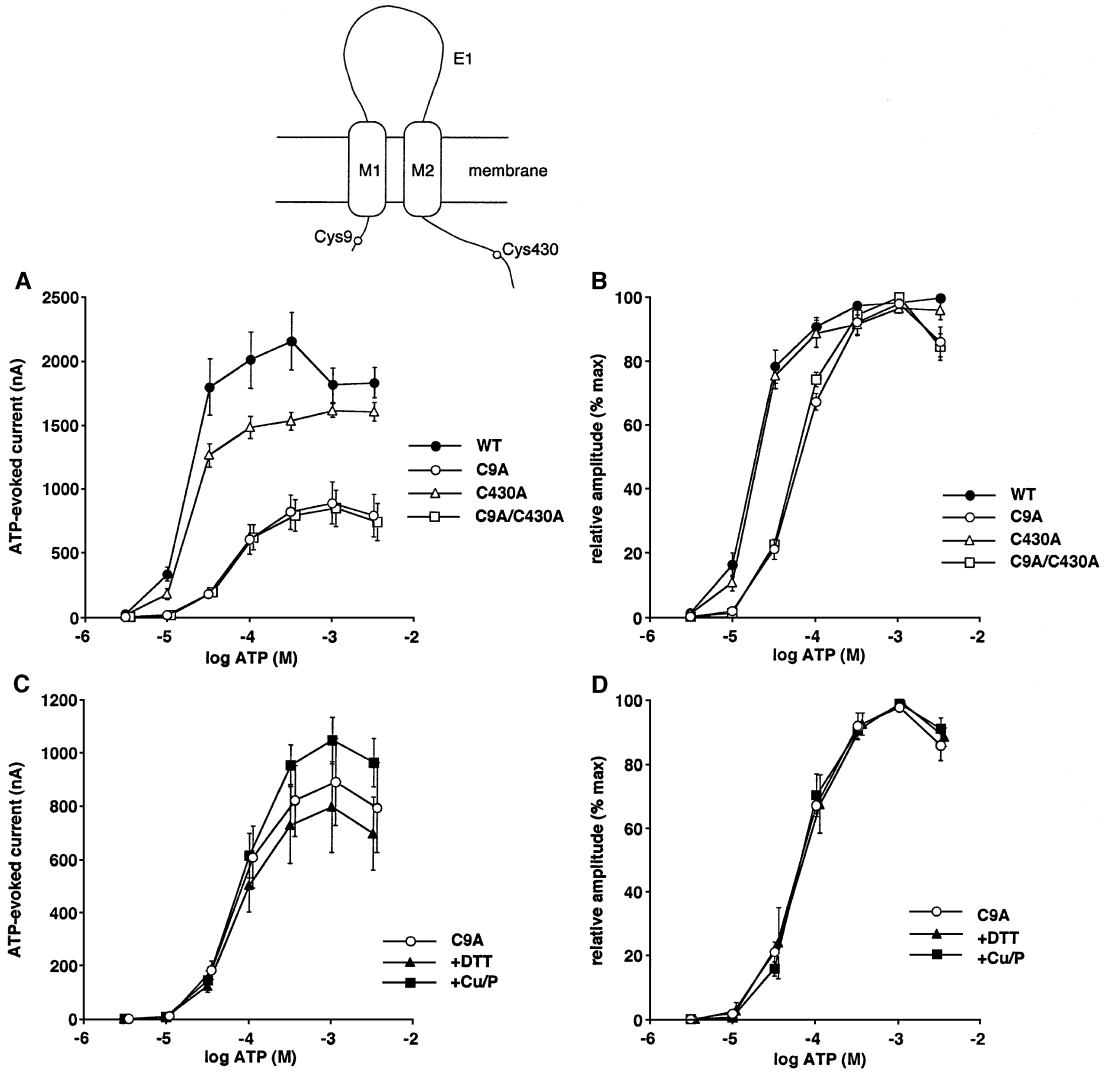

Fig. 2. Responses of cysteine-to-alanine mutants to ATP. Oocytes were held at − 50 mV. Each symbol and bar represents the mean and S.E. obtained from five to seven oocytes tested. (A) Concentration–response relationship for ATP-evoked current mediated through the wild-type $P2X_2$ receptor/channel (WT) and three cysteine-to-alanine mutants (substitution at Cys^9 alone, Cys^{430} alone or both Cys^9 and Cys^{430}; C9A, C430A and C9A/C430A, respectively). (B) Normalized current responses. The data shown in (A) were normalized to maximal responses to ATP in individual oocytes. (C) ATP-evoked current mediated through the Cys^9-to-alanine substituted mutant channel. Concentration–response relationship obtained from dithiothreitrol- (+DTT) and Cu^{2+} phenanthroline-injected oocytes (+Cu/P) was compared with that from uninjected oocytes (C9A). (D) Normalized current responses. The data shown in (C) were normalized to maximal responses to ATP in individual oocytes.

responsiveness to ATP was observed in the Cys^9-to-alanine mutant channel, and dithiothreitrol did not produce a further decrease in the responsiveness in this mutant. These results suggest that an intracellular disulfide bond is necessary for the physiological responsiveness of $P2X_2$ receptor to ATP, and that Cys^9 participates in this disulfide bond. In rat $P2X_1$ receptor, multimerization of homomeric receptor subunits has been shown to be decomposed by dithiothreitrol (Nicke et al., 1998). A larger part of this multimerization seems to be achieved by non-disulfide bonds because the decomposition to monomers occurs even without reduction agents. A smaller part of the multimerization, however, appears to be achieved by disulfide bonds because $P2X_1$ oligomers up to hexamers do not completely disappear in the absence of dithiothreitrol. Cysteine residues are not found in the intracellular N- or C-terminal region of rat $P2X_1$ receptor (Soto et al., 1997). Our finding of the modification by an intracellular disulfide bond may not be applicable to other P2X receptor subclasses including rat $P2X_1$, $P2X_5$ and $P2X_6$ receptors that lack corresponding cysteine residues. Unlike intracellular cysteine residues, extracellular cysteine residues are highly conserved among P2X subclasses. The pairs of cysteines that form disulfide bonds have been identified for $P2X_1$ (Ennion and Evans, 2002) and $P2X_2$ receptors (Clyne et al., 2002), recently.

In cyclic nucleotide-gated channels, disulfide bonds also modulate the sensitivity to agonists, namely, intracellular

cGMP and cAMP (Gordon et al., 1997; Rosenbaum and Gordon, 2002). The formation of the disulfide bond between Cys^{35} of the N-terminal region and Cys^{481} of the C liker region increases the responsiveness to cGMP and cAMP. These Cys^{35} and Cys^{481} are normally reduced, and oxidation agents (Cu^{2+} phenanthroline and iodine) enhance the channel activation. In contrast to the cysteine residues in the cyclic nucleotide-gated channel, Cys^{9} in $P2X_2$ receptor is normally oxidized and the reduction appears to result in decrease in the responsiveness to ATP. The counterpart of the disulfide bond may not be the other intracellular cysteine residue Cys^{430} because the replacement of Cys^{430} did not change the responsiveness to ATP (Fig. 2A and B). Because P2X receptors are believed to form trimers, a disulfide bond may be made intermolecularly between subunits. If this is the case, one disulfide bond may be formed between two of three subunits in one homomeric receptor. The remaining subunit may stay unbound or bind to some protein intrinsically expressed in or beneath cell membrane in *Xenopus* oocytes. Other possibilities including binding of individual subunits to domain proteins through three disulfide bonds cannot be excluded.

Acknowledgements

This work was partly supported by Health and Labour Science Research Grants for Research on Advanced Medical Technology and Research on Environmental Health from the Ministry of Health, Labour and Welfare, Japan, awarded to Y.O. and K.N., and a grant-in-aid for scientific research from the Ministry of Education, Science, Sports and Culture, Japan (KAKENHI 13672319), awarded to K.N.

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