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Amino acid substitutions from an indispensable disulfide bond affect $P2X_2$ receptor activation

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Abstract

The roles of six amino acid residues downward from an extracellular disulfide bond involving Cys^{224} in rat $P2X_2$ receptor were examined. When Cys^{224} or Pro^{225} was replaced with alanine, the responsiveness to ATP was lost. When Ile^{226} was replaced with other hydrophobic amino acids, the responsiveness to ATP was reduced or abolished. When Phe^{227} was replaced with leucine or isoleucine, the responsiveness to ATP was abolished. The responsiveness to ATP was moderately decreased with the alanine-substitution for Arg^{228} and it was markedly decreased with the alanine-substitution for Leu^{229}. As for the alanine-substitution for Gly^{230}, the sensitivity was changed, but the maximal response to ATP was not reduced. The results suggested that a precise structure is required for amino acid residues close to the disulfide bond and, in general, the amino acid residues at odd number positions and those closer to the disulfide bond are more influential to the ATP responsiveness.

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

1. Introduction

P2X receptors are ion channel-forming membrane proteins that are activated by extracellular ATP (see reviews, Khakh, 2001; North, 2002). One functional ion channel is presumably formed by three homogenous subunits. Each subunit has two transmembrane regions (TM1 and TM2) and a long extracellular loop (E1) between them. Basic amino acid residues near the outer mouth of the channel pore formed by TM1 and TM2 appear to serve as a part of the binding pocket of ATP molecules (Ennion et al., 2000; Jiang et al., 2000). In addition to these basic residues, amino acid residues in E1 apart from the channel pore have been shown to affect the channel activation by ATP. $P2X_2$ receptor did not respond to ATP when Gly^{247} in E1 was replaced with alanine (Nakazawa and Ohno, 1999). The sensitivity to ATP was reduced when Gly^{248} was replaced with valine and the responsiveness was lost when the residue was replaced with leucine (Nakazawa et al., 2002). The replacement of Trp^{256} with other amino acid residues except for tyrosine also resulted in the loss of responsiveness (Nakazawa et al., 2002). These results suggest that the structure around these positions should be precisely maintained for the channel activation by ATP. Recently, the pairs of cysteines that form disulfide bonds have been identified for $P2X_1$ (Ennion and Evans, 2002) and $P2X_2$ (Clyne et al., 2002) receptors. Among these cysteines, the structure formed by Cys^{224} through a disulfide bond with Cys^{214} appears to be critical because the replacement of this residue with alanine was non-functional (Clyne et al., 2002). Cys^{227} in $P2X_1$, which corresponds to Cys^{224} in $P2X_2$ (see Fig. 1), may also play an indispensable role in the formation of such a structure because its alanine substitution resulted in about 50-fold reduction in the sensitivity to ATP (Ennion and Evans, 2002). In the present study, we examined the roles of amino acid residues following Cys^{224} in $P2X_2$ receptor to understand

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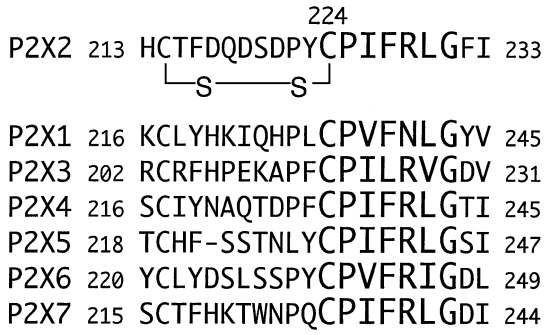

Fig. 1. Amino acid sequence near Cys^{224} in the extracellular loop of $P2X_2$ receptor and corresponding sequences of P2X receptor subclasses. Amino acid residues targeted for site-directed mutagenesis and those in the equal positions were shown as the larger characters. Cys^{224} forms a disulfide bond with Cys^{214}.

structural requirements for the channel activation in this region.

2. Materials and methods

2.1. Oocyte expression and membrane current measurements

The expression of cloned and mutant $P2X_2$ receptor and the recordings of ionic current through the channels were performed according to our previous report (Nakazawa et al., 1998). Briefly, $P2X_2$ receptor mutants were constructed from the cloned $P2X_2$ receptor (Brake et al., 1994) by site-directed mutagenesis. Amino acid residues targeted for mutagenesis were shown in Fig. 1. 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 measurements. 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.

2.2. Immunoblotting analysis

The expression of channel protein was confirmed by immunoblotting analysis. Crude membrane fractions were prepared from oocytes (20 oocytes for the wild-type channel and each mutant) according to Newbolt et al. (1998) with minor modifications. Oocytes were suspended in a 50 × stock of a protease and phosphatase inhibitor cocktail (Sigma, general use; 1 bottle for 100 ml effective solution) diluted in a buffer containing 20 mM Tris–HCl, 2 mM EDTA (disodium salt), 0.5 mM EGTA and 320 mM sucrose by pipetting. The homogenate was horizontally shaken at 100 rpm for 15 min at 4 °C and then centrifuged at 14,000 rpm for 2 min. The supernatants were analyzed by SDS-PAGE gel electrophoresis and immunoblotting. By using $P2X_2$ receptor antibodies (Oncogene, Boston, MA, USA) and anti-rabbit Ig, horseradish peroxidase-linked whole antibody (from donkey; Amersham, Little Chalfont, England), correct channel expression was detected as a 65-kDa band.

2.3. Data analysis

Parameters for ATP sensitivity (EC_{50}, pD_2 and Hill coefficient) were obtained from current responses using the following equation:

$$E = E_{max}A^n/[A^n + (EC_{50})^n], \quad (1)$$

where E is an effect (current response), E_{max} is an maximal response, A is ATP concentration, EC_{50} is concentration required for a half-maximal effect and n is a Hill coefficient (slope factor). When EC_{50} and n were calculated from ATP concentrations used and obtained current responses, Eq. (1) was transformed to:

$$\log[E/(E_{max} - E)] = n(\log A - \log EC_{50}), \quad (2)$$

and linear regression was made using Microsoft® Excel X. pD_2 values were negative logarithm of EC_{50} values. When experimental data were fitted by a two binding-site model, the fraction of one binding-site (f) and that of the other binding-site ($1-f$) were introduced. The effect mediated through each binding-site was calculated from Eq. (1) and the fraction (f or $1-f$), and the sum of these effects was obtained.

3. Results

3.1. Amino acid substitutions and ATP responsiveness

Cys^{224} and neighboring Pro^{225} are completely conserved among seven subclasses of P2X receptors (Fig. 1; Soto et al., 1997). Fig. 2 shows concentration–response relationship for ATP-evoked current recorded from oocytes expressing the wild-type $P2X_2$ receptor and the Cys^{224}- or Pro^{225}-to-alanine-substituted mutants (C224A or P225A, respectively). C224A exhibited no current response to ATP, as has been reported by Clyne et al. (2002). P225A also failed to respond to ATP. To examine the effects of amino acid residues succeeding to Pro^{225}, we first constructed deletion mutants. When three residues next to Pro^{225} (Ile^{226}, Phe^{227} and Arg^{228}) were deleted, the responsiveness to ATP was lost. Deletion of the former two or even Ile^{226} alone also resulted in the loss of the responsiveness. The preliminary results suggest that these amino acid residues also appeared to be essential. Thus, instead of deletion, we introduced amino acid substitutions to these residues in a one-by-one manner.

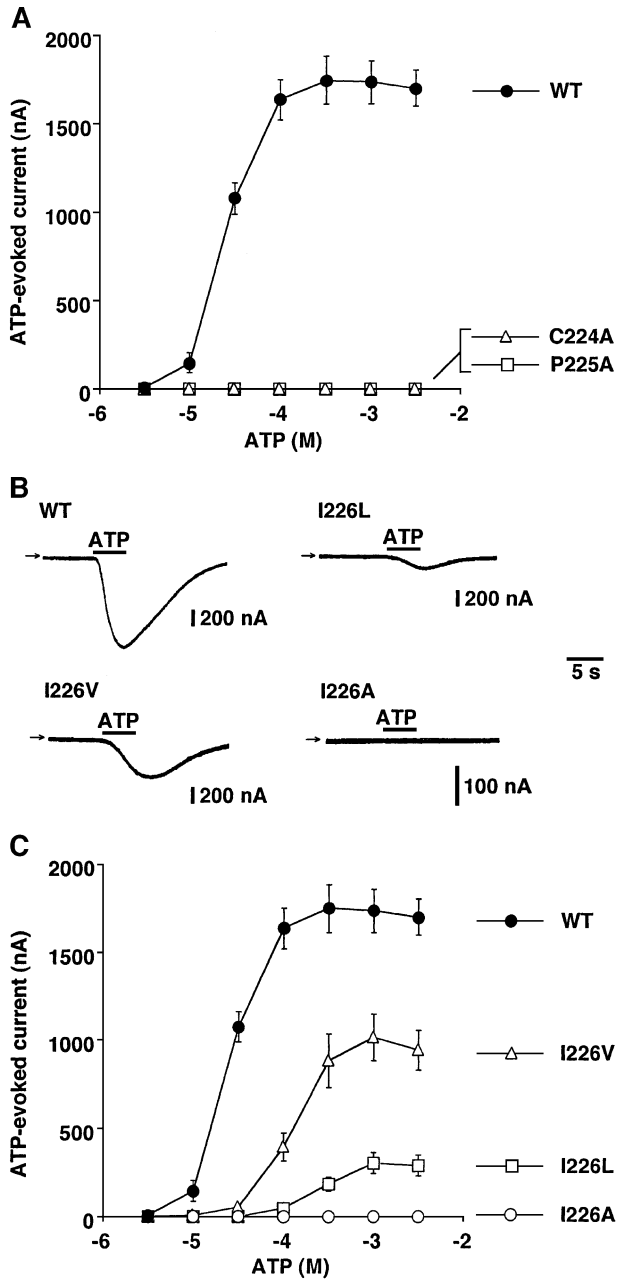

Fig. 2. (A) Disappearance of current responses to ATP by the replacement of Cys^{224} or Pro^{225} with alanine (C224A or P225A). Peak amplitude of currents activated by ATP at −50 mV was plotted against ATP concentrations. Each symbol and bar represent mean and S.E. obtained from five to eight oocytes. In contrast to current responses in oocytes expressing the wild-type channel (WT), no response was observed in those expressing the mutant channels. (B) Currents activated by 30 μM ATP in oocytes expressing the wild-type (WT) and Ile^{226}-to-leucine (I226L), -valine (I226V) and -alanine (I226A)-substituted mutant channels. The oocytes were held at −50 mV. Arrows indicate zero current levels. (C) Concentration–response relationship for the wild-type and Ile^{226}-replaced channels. The current responses were obtained as shown in B. The concentration–response relationship for the wild-type channel (WT) was also shown for comparison. Each symbol and bar represent mean and S.E. obtained from five to eight oocytes.

Ile^{226} was replaced with hydrophobic amino acid residues because this position was occupied by isoleucine or valine in seven P2X receptor subclasses (Fig. 1). Fig. 2B shows current responses to 100 μM ATP in oocytes expressing the wild-type and the mutant channels. When substituted with leucine, which has a volume similar to that of isoleucine (Chothia, 1975), the ATP-evoked current was markedly reduced (Fig. 2B,C; I226L). When substituted with valine, a smaller hydrophobic residue, the reduction of the current

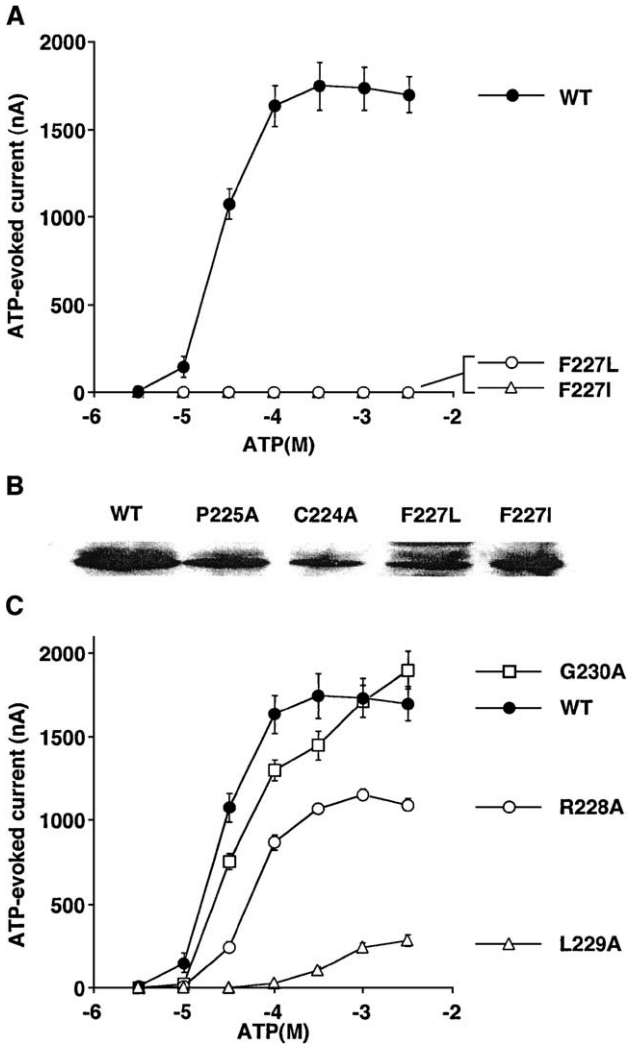

Fig. 3. (A) Disappearance of current responses to ATP by the replacement of Phe^{227} with leucine (F227L) or isoleucine (F227I). Data obtained from five to eight oocytes were shown as in Fig. 2A. (B) Immunoblotting analysis using anti-$P2X_2$ receptor antibody. A section corresponding to $P2X_2$ receptor protein (about 65 kDa) was shown to compare the expression in membrane fractions prepared from the oocytes injected with the wild-type channel (WT) and four non-ATP responsive mutants shown in Fig. 2A (C224A and P225A) and A of this figure (F227L and F227I). (C) Concentration–response relationship for the wild-type and Arg^{228}-(R228A), Leu^{229}- (L229A) and Gly^{230}-to-alanine-substituted mutants. The concentration–response relationship for the wild-type channel (WT) was also shown for comparison. Data obtained from five to eight oocytes were shown as in Fig. 2A.

was less remarkable than in the case of the leucine-substitution (Fig. 2B,C; I226V). When substituted with alanine, a hydrophobic residue smaller than valine, the current responsiveness to ATP was abolished (Fig. 2B,C; I226A).

Phe^{227}, a residue succeeding to Ile^{226}, is conserved among six of seven P2X receptor subclasses (Fig. 1). In the remaining subclass ($P2X_3$), leucine is present instead of phenylalanine. When Phe^{227} was replaced with leucine, the current response to ATP disappeared (Fig. 3A; F227L). The current response has also disappeared when replaced with isoleucine (Fig. 3A; F227I). The disappearance of the current response may not be due to non-expression of receptor protein because the protein expression of F227L or F227I was confirmed by immunoblotting analysis (Fig. 3B). The protein expression was also confirmed for the C224A, P225A (Fig. 3B) or I226A (not shown).

Three residues succeeding to Phe^{227} were alanine-substituted (Fig. 3C). The current response to ATP was reduced to about 60% when Arg^{228} was replaced with alanine (R228A). The current response was, however, much reduced (to about 20%) when Leu^{229} was replaced with alanine (L229A). As for the replacement of Gly^{230}, the maximal current response was comparable to that with the wild-type channel (G230A).

3.2. Sensitivities to ATP

To compare the sensitivity to ATP, the concentration–response data were normalized to the maximal response (Fig. 4). Theoretical curves were fitted to the normalized data (see Section 2), and EC_{50} values and Hill coefficients were determined. For the wild-type channel, the EC_{50} value was 29 μM and the Hill coefficient was 2.3. Ile^{226}-, Arg^{228}- and Leu^{229}-substituted mutants exhibited lower sensitivities to ATP than the wild-type channel did (Fig. 4A–C), and the order of the sensitivities was R228A>I226V ≈ I226L>L229A. Hill coefficients for these mutants (varying from 1.7 to 2.6) were similar to that for the wild-type channel. When the curve fitting was applied to the data with G230A, the Hill coefficient as well as the ATP-sensitivity was lower than those for the wild-type channel (Fig. 4D, solid curve). The data points in Fig. 4D were, however, not well fitted to the solid curve. In fact, a regression coefficient of the fitting for the G230A

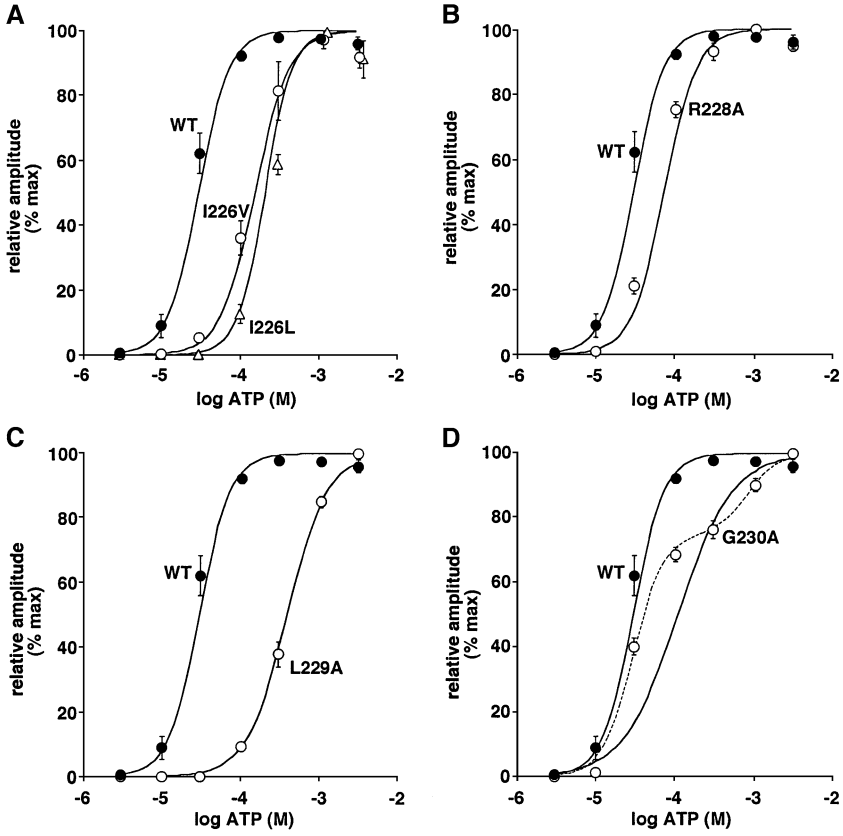

Fig. 4. (A–C) Curve-fittings to concentration–response data for the wild-type and the mutant channels. Current responses to ATP were normalized to maximal responses in individual oocytes and mean values were plotted against ATP concentrations. A curve was fitted to data assuming a homogenous binding-site for each channel type as shown in Section 2. Bars are S.E. Parameters calculated and used for fittings are: EC_{50} (in μM); 29 (WT), 151 (I226V), 202 (I226L), 72 (R228A), 379 (L229A), 108 (G230A); Hill coefficient; 2.3 (WT), 2.0 (I226V), 2.6 (I226L), 2.1 (R228A), 1.7 (L229A), 1.3 (G230A). (D) Curve-fittings to concentration–response data for G230A mutants. The data and were shown and the solid curve with an EC_{50} value of 108 μM and a Hill coefficient of 1.3 was fitted to the data as in A–C. For the broken curve, it was constructed assuming 75% of binding sites are equivalent to the wild-type receptor and the remaining 25% of binding sites have a lower affinity (EC_{50}; 800 μM) and the same slope (Hill coefficient; 2.3).

($r = 0.91$) was markedly smaller than those for the remaining channels (0.98–1.00). The poor fitting suggests that this simple theoretical fitting was not available for G230A. Thus, a fitting was made assuming two independent binding sites (Fig. 4D, broken curve). A curve could be fitted with the data when assuming that 75% of the current response is mediated through a binding site having the same EC_{50} and Hill coefficient as the wild-type channel does, and the remaining 25% is mediated through another binding site having the same Hill coefficient but a larger EC_{50} value (800 μM).

3.3. ATP responsiveness and amino acid positions

In Fig. 4, we used the mean values for the curve fittings. Similar curve fittings were applied to data obtained from individual oocytes, and sensitivities to ATP were determined. pD_2 values obtained in this manner as well as the maximal current responses were plotted for the wild-type and the mutant channels in Fig. 5. For the channels with which the current responses to ATP were observed, a clear correlation was found between the maximal current amplitude and the ATP sensitivity; the channels having exhibited smaller maximal current amplitude exhibited lower sensitivity to ATP. In the amino acid sequence beginning from Ile^{226} to Gly^{230}, the substitution of an amino acid residue at an odd number position resulted in larger changes than that at an even number position. For example, the substitution of Phe^{227} resulted in the loss of ATP responsiveness, whereas the substitution of Ile^{226} or Arg^{228} did not lose the responsiveness in most cases. Similarly, the substitution of Leu^{229} resulted in large decreases in both the maximal amplitude and the ATP sensitivity, but the substitution of Arg^{228} or Gly^{230} resulted in relatively small decreases in these indexes. Within the odd or even number positions, the substitution of an amino acid residue closer to the disulfide bond involving Cys^{224} resulted in larger changes in the ATP responsiveness. The substitutions of Phe^{227} resulted in the loss of the

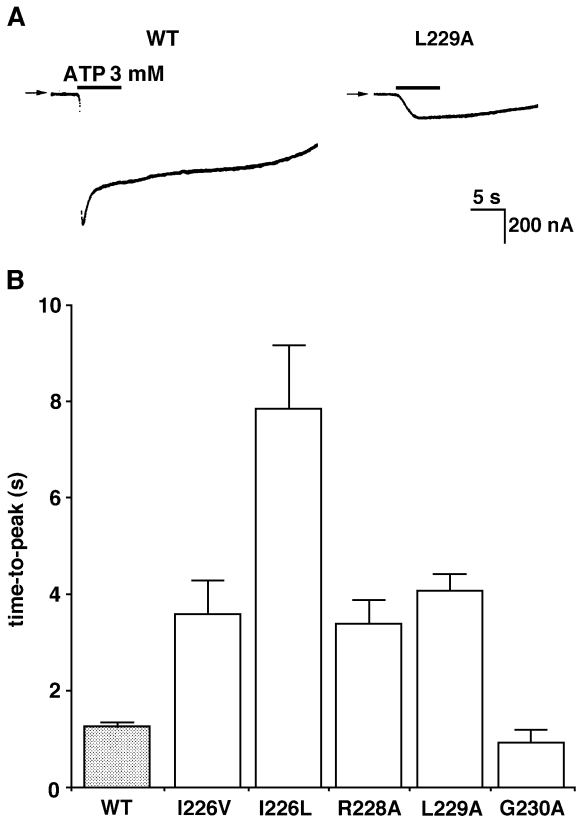

Fig. 6. Activation time course of ATP-evoked current through the wild-type and mutant channels. (A) Currents activated by 3 mM ATP in oocytes expressing the wild-type (WT) and L229A mutant channels. The oocytes were held at − 50 mV. Arrows indicate zero current levels. (B) Comparison of time required for peak current evoked by 3 mM ATP ("time-to-peak"). Each column and bar represent mean and S.E. obtained from five to eight oocytes.

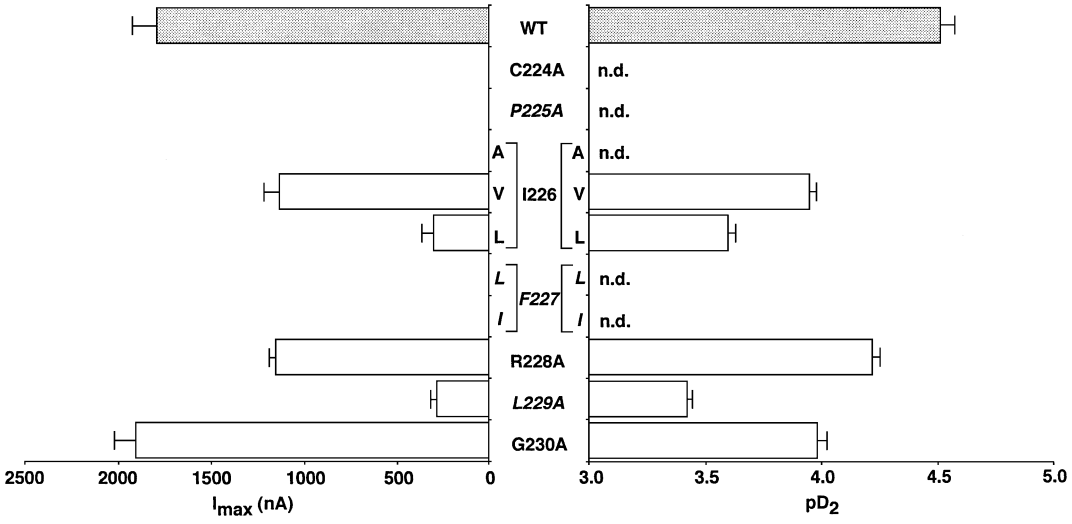

Fig. 5. Comparison of the maximal current amplitude (I_{max}) and the sensitivity to ATP (pD_2) among the wild-type and the mutant channels. The parameters were calculated from each oocytes and mean values were shown for each channel type. Bars are S.E. n.d.: not determined because of the loss of the ATP responsiveness.

responsiveness, whereas the substitution of Leu^{229} resulted in large decreases in the indexes for the responsiveness, but not in the loss of responsiveness. As for the amino acid substitutions at the even number positions, the order of the maximal current responses was Gly^{230}>Arg^{228}>Ile^{226} though the ATP sensitivities were comparable between Arg^{228} and Gly^{230}.

3.4. Activation kinetics

When ATP was applied, activation kinetics was generally faster for the wild-type channel than that for the low responsiveness mutants such as I226V or L229A. To compare the activation kinetics quantitatively, we measured the time required for peak current amplitude when activated by 3 mM ATP (''time-to-peak''; Fig. 6). The time-to-peak was <2 s for the wild-type channel, whereas it was >3 s for the low responsiveness mutants (I226V, I226L, R228A and L229A). The time-to-peak for G230V was similar to that for the wild-type channel.

4. Discussion

We have examined the effects of amino acid substitutions downward from Cys^{224} in the extracellular loop of $P2X_2$ receptor. When Cys^{224} was replaced with alanine, the responsiveness to ATP was lost (Fig. 2A), suggesting that the disulfide bond involving this residue is indispensable for the responsiveness as has been reported by Clyne et al. (2002). The alanine-substitution of Pro^{225} also resulted in the loss of the ATP responsiveness (Fig. 2A). Proline residues are able to disturb the formation of rigid structures such as α-helixes or β-sheets. This ability of Pro^{225} may provide distortion necessary for Cys^{224} to contribute to the formation of the disulfide bond.

As for the substitutions of Ile^{226}, the current response to ATP largely remained when substituted with valine (Fig. 2B,C). In contrast, the substitution with leucine resulted in a marked decrease in the current response (Fig. 2B,C). These results are puzzling because, among hydrophobic residues, leucine is similar to isoleucine in size but valine is smaller than these residues (Chothia, 1975). Interestingly, five of seven P2X receptor subclasses possess an isoleucine residue in this position, and the remaining two subclasses possess a valine residue (Fig. 1). Some common property between isoleucine and valine is necessary to maintain structures proper for the channel activation.

The substitutions of Phe^{227} with hydrophobic amino acid residues (leucine and isoleucine) resulted in the loss of the ATP responsiveness (Fig. 2A). Among P2X receptor subclasses, $P2X_3$ alone has leucine and all the remaining six subclasses have phenylalanine in this position. When Phe^{227} is replaced with leucine, five sequential amino acid residues beginning from Cys^{224} in $P2X_2$ receptor (cysteine–proline–isoleucine–leucine–arginine) completely accord with the residues at corresponding positions in $P2X_3$ receptors (Fig. 1). In spite of this fact, the replacement resulted in the loss of the channel function.

The results obtained from the alanine-substitution of Arg^{228}, Leu^{229} and Gly^{230} showed that a distinct reduction in the ATP responsiveness was found for L229A, but not for R228A or G230A (Fig. 3C). By combining these results and the results obtained from the substitutions of Ile^{226} and Phe^{227}, it appears that the replacement of the residues at odd number positions more dramatically reduces the ATP responsiveness than that at even number positions (Fig. 5). Freist et al. (1998) have pointed out that a sequence stretch of the positions 170–330 in the extracellular loop of P2X receptor proteins exhibits similarities with the catalytic domains of class II aminoacyl-tRNA synthetases as shown by secondary structure predictions and sequence alignments. In their prediction, the region involving the above-mentioned amino acid residues participates in the formation of β-sheets. If these residues are involved in the β-sheets, the residues aligning in one side (Phe^{227} and Leu^{229}) may be more influential on the channel function of P2X receptor than those in the other side (Ile^{226}, Arg^{228} and Gly^{230}).

The Hill coefficient of the wild-type $P2X_2$ receptor was about 2 (Fig. 4), which is in common with those in other reports (e.g., Nakazawa et al., 1991; Nakazawa, 1994). The value of 2 may indicate that the channel activation requires two ATP molecules (Tallarida and Jacob, 1979) though Bean (1990) has shown that the activation requires three ATP molecules at lower concentrations. The Hill coefficient of about 2 was not affected by the substitutions of the amino acid residues tested except for the alanine-substitution of Gly^{230} (Fig. 4). G230A exhibited a Hill coefficient of 1.3 when assuming homogenous binding sites (Fig. 4D, solid curve), but a better fitting could be obtained when the second binding sites of a lower affinity (Fig. 4D, broken curve). For the latter fitting, a Hill coefficient of about 2 was adopted for both the higher and the lower affinity sites. Thus, the introduction of alanine into the position 230 may not affect the number of ATP molecules for the channel activation, but may affect some process involved in the channel activation.

The present study and our previous studies (Nakazawa and Ohno, 1999; Nakazawa et al., 2002) have shown that small changes in a sequence from about the position 220 to about the position 260 in the extracellular loop can result in the loss of the ATP responsiveness. Jiang et al. (2000) also reported that alanine-substitution of Asn^{202} or Asp^{261} resulted in the loss of the ATP responsiveness. In addition, Buell et al. (1996) reported that $P2X_4$ receptor, which is insensitive to two-subclass selective purinoceptor antagonists (pyridoxal-5-phosphate-6-azophenyl-2′,4′-disulfonic acid and pyridoxal 5-phosphate), restored sensitivities to these antagonists when Glu^{249} was replaced with lysine. These results suggest that this extracellular amino acid sequence may directly contribute to some indispensable process between the recognition of ATP molecules and the

channel opening. Ennion et al. (2000) suggested that basic amino acid residues close to the channel pore (Lys^{68}, Lys^{70}, Arg^{292} and Arg^{309}) serve to recognize ATP molecules in $P2X_1$ receptor and, thus, the ATP binding pocket may form close to the outer mouth of the channel pore. Similar results were also obtained for $P2X_2$ receptor (Jiang et al., 2000). The extracellular amino acid sequence described above (the positions 224–230) is not located between this possible binding pocket and the channel pore. If this sequence contributes to some indispensable process between the recognition of ATP molecules and the channel opening, it is desired that the sequence is spatially positioned close to the "activation" link between the binding pocket and the channel pore. This view may be supported by the slower activation process observed in the low responsiveness mutants (Fig. 6). It is possible that the extracellular loop is "packed" densely enough for the sequence to reach the "activation" link.

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