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European Journal of Pharmacology 495 (2004) 27–33

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Desensitization of $P2X_2$ receptor/channel pore mutants

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Received 8 January 2004; received in revised form 10 May 2004; accepted 12 May 2004

Abstract

Properties of five mutants of $P2X_2$ receptor/channel having amino acid residue-substitution at the pore region were examined by expressing the channels in *Xenopus* oocytes. When the concentration–response relationship for ATP-evoked current was obtained, the current amplitude was increased along with the concentrations of ATP for the wild type channel whereas the amplitude was rather decreased with highest concentrations for four of the five mutants as if an ''inactivation-like'' mechanism occurs to these mutants. Upon a long exposure (30 s) to ATP, time-dependent decay in the ATP-evoked current was observed for three of the five mutants, suggesting that desensitization occurs to these mutants. The time course of the desensitization was well fitted with a single exponential time whereas that of the recovery from the desensitization could be better fitted with multiple exponentials than with a single exponential. The relationship between the desensitization and the ''inactivation-like'' mechanism was discussed.

Keywords: $P2X_2$ receptor; Channel pore mutant; Desensitization; ATP responsiveness

1. Introduction

When appropriate stimulation (change in membrane potential or ligand-binding) is given, voltage- and ligand-gated ion channels, respectively, exhibit transition from closed state to open state. In a number of channels, opened channels gradually shut even if the stimulation continues. This phenomenon is called ''inactivation'' for voltage-gated channels (Hodgkin and Huxley, 1952; Hille, 1992a), and generally called ''desensitization'' for ligand-gated channels (Katz and Thesleff, 1957; Hille, 1992b). The desensitization was also found for ion channels gated by extracellular ATP (P2X receptor/channels; see reviews, Ralevic and Burnstock, 1998; Khakh, 2001; North, 2002). Among P2X receptor/channel subclasses, $P2X_1$ and $P2X_3$ receptor/channels exhibit marked desensitization whereas the $P2X_2$ receptor/channel does not exhibit desensitization when expressed as homomeric channels.

We previously utilized channel pore mutants of $P2X_2$ receptors to determine factors contributing to the potency of multivalent cation block (Nakazawa et al., 2002). In the present study, we studied the dependence of the current permeating through these pore mutants on ATP concentrations, and the kinetics of the current during a long exposure to ATP. We found an ''inactivation-like'' phenomenon in the concentration–response study and desensitization in the kinetic study. The time courses of the desensitization and recovery from the desensitization were analyzed, and the relation between the desensitization and the ''inactivation-like phenomenon was discussed using possible schematic models.

2. Materials and methods

2.1. Ionic current measurement

Mutants of $P2X_2$ receptor constructed from the cloned rat $P2X_2$ receptor (Brake et al., 1994) used in this study were those described by Nakazawa et al. (2002). The substitutions applied to the mutants were Asn^{333} to alanine (N333A), Thr^{336} to alanine (T336A), Leu^{338} to alanine (L338A), Gly^{342} to alanine (G342A) and Asp^{349} to asparagine (D349N). Channels were expressed in *Xenopus* oocytes and ionic currents permeating through them were measured

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doi:10.1016/j.ejphar.2004.05.021

as previously described (Nakazawa and Ohno, 1996; Nakazawa et al., 1998). 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) at room temperature. For the construction of concentration–response curves, ATP (adenosine 5′ -triphosphate disodium salt; Sigma, St. Louis, MO, USA) was applied by superfusion for 7 s with a regular interval of 1 min from the lowest concentration (usually 30 μM). To determine desensitization time course, ATP was applied for 30 s. For the determination of the time course of recovery from desensitization, reference responses to ATP were measured with an interval of 4 min, and trials were made with various intervals.

2.2. Data analysis

All the data were given as mean ± S.E. Curve fittings to data were made using Microsoft' Excel X. For "activation" of channels (see Fig. 2), curves were calculated from the following equation (Tallarida and Jacob, 1979):

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

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). As for "inactivation" curves, the following equation was used:

$$E = E_{max} \cdot (1 - A^n / [A^n + (EC_{50})^n]). \quad (2)$$

Curve fittings to the recovery from desensitization (Fig. 5) were made with an assumption of the following Hodgkin–Huxley type gating mechanism (Hille, 1992a):

$$I = m^k \cdot I_{max} \quad (3)$$

where I is current activated by ATP, I_{max} is its maximal value, k is the number of gates, and m is a gate variable given by:

$$m = 1 - \exp(-t/\tau) \quad (4)$$

where τ is a time constant.

3. Results

3.1. Concentration–response relationship

Fig. 1 shows concentration–response relationships for ATP-activated current permeating through the wild type and pore mutant $P2X_2$ receptor/channels. For the pore mutants, alanine-substituted ones were used except for D349N because D349A was an non-expressing mutant. Compared to the wild type channel, N333A and D349N exhibited higher sensitivities to ATP. Unlike the wild type channel, current responses to ATP were rather reduced at highest concentrations (300 μM, 1 mM or 3 mM) for N333A, T336A, L338A and D349N. To analyze these properties quantitatively, we plotted the relationships separately, and curve fittings were made (Fig. 2). Modeling after the gating theory for voltage-gated channels (Hodgkin and Huxley, 1952; Hille, 1992a), "activation" and "inactivation" curves were fitted to the data as described in Materials and methods. All the curves were well fitted to the data when assuming a common Hill coefficient of 2. For "activation", EC_{50} values for N333A (50 μM) and D349N (60 μM) were smaller than that for the wild type receptor (100 μM). On the other hand, a larger EC_{50} value (180 μM) was necessary for L338A activation fitting. As for "inactivation", the decease of the current responses to higher concentrations of ATP could be fitted with curves with EC_{50} values of millimolar for N333A, T336A, L338A and D349N. The results imply that the decrease can be explained if assuming that higher concentrations of ATP shut an "inactivation" gate.

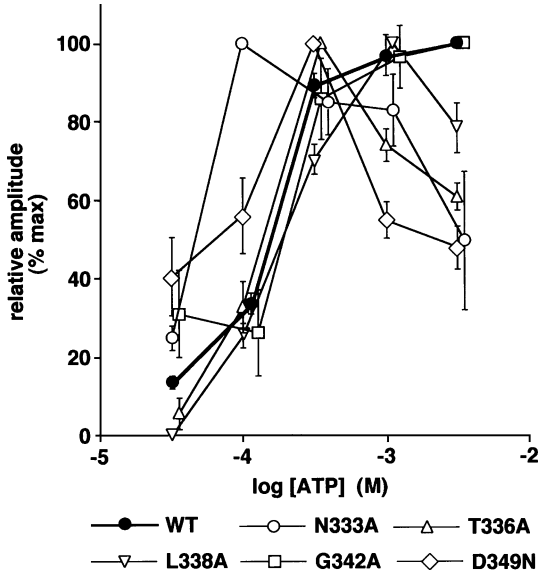


Fig. 1. Concentration–response relationships for ionic current activated by ATP permeating through the wild type (WT) and five pore mutant (N333A, T336A, L338A, G342A and D349N) channels. A current response to each concentration of ATP at − 50 mV was normalized to the maximal response in each oocyte. Each symbol represents the mean obtained four to six oocytes tested. Bars are S.E.

3.2. Desensitization time course

Fig. 3 compares time course of inward current activated by ATP for 30 s. The current permeating through the wild type channel sustained during a 30-s exposure to 1 mM ATP (Fig. 3A) whereas that through D349N markedly decayed (Fig. 3B). The results indicate that desensitization occur to D349N but not to the wild type channel. The current remaining at the end of the 30-s exposure to 100 μM or 1 mM ATP was plotted in Fig. 3C. Marked desensitization was observed with the current permeating through N333A and G342A as well as D349N. The extent of

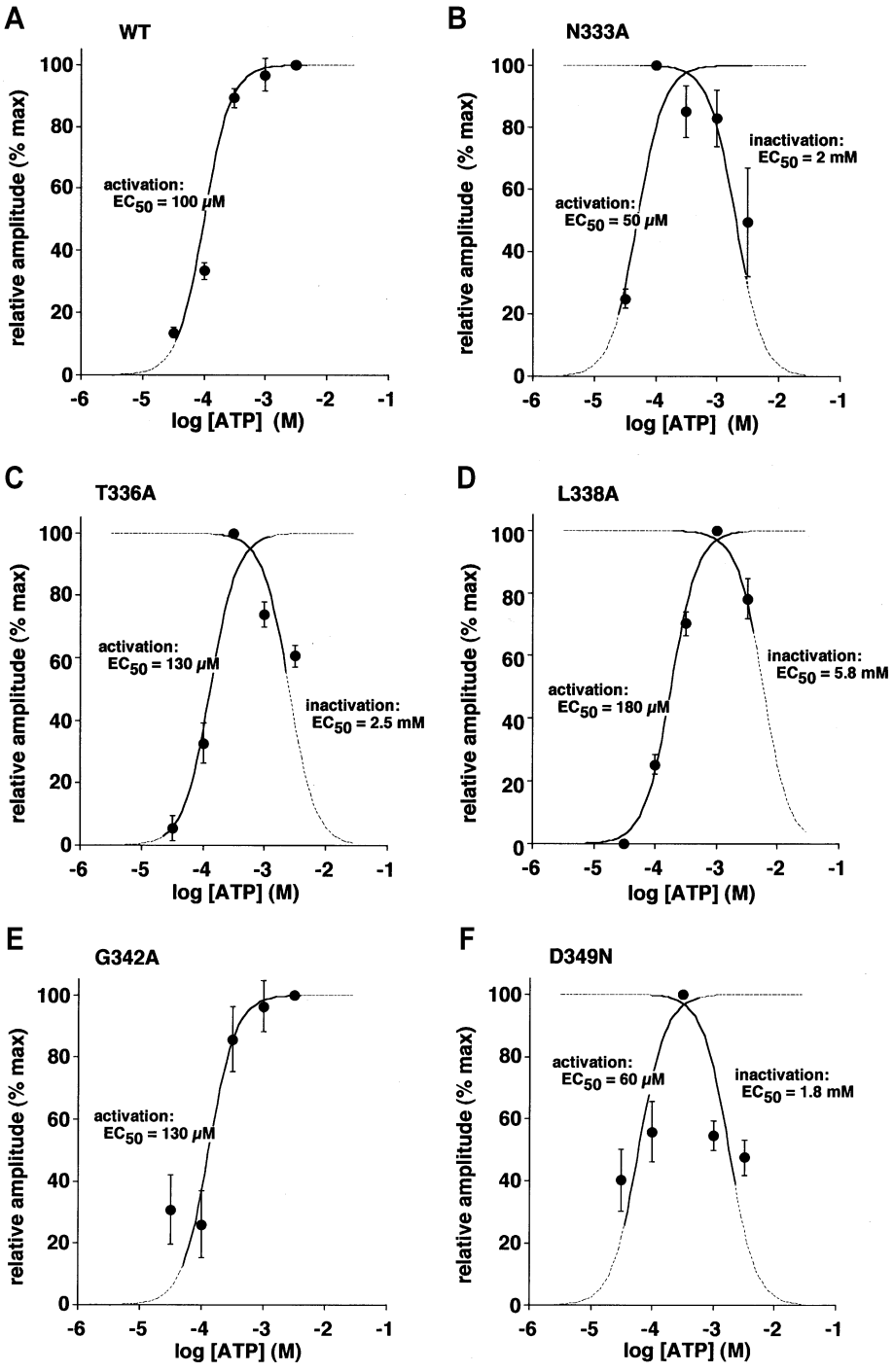


Fig. 2. Curve-fittings to concentration–response data for the ATP-activated current. "Activation" and "inactivation" curves were fitted to the data shown in Fig. 1 assuming a Hill coefficient of 2 and EC_{50} values shown in panels A to F.

desensitization was not different between the current activated by 100 μM ATP and that by 1 mM ATP for each mutant when the remaining current amplitude was compared (Fig. 3C). The current activated by a lower concentration (30 μM) of ATP was not desensitized up to 1 min for D349N (not shown).

The current amplitude remaining at the end of the 30-s exposure does not necessarily reflect desensitization time course because this value may be affected by other factors such as activation kinetics or multiple components of desenstization. Thus, the time course was further analyzed by measuring time constants. The oocytes were periodically stepped to −80 from −50 mV to obtain large current amplitude and to confirm clamp conditions of oocytes. For the current mediated through D349N shown in Fig. 3B, the decay could be fitted by a single

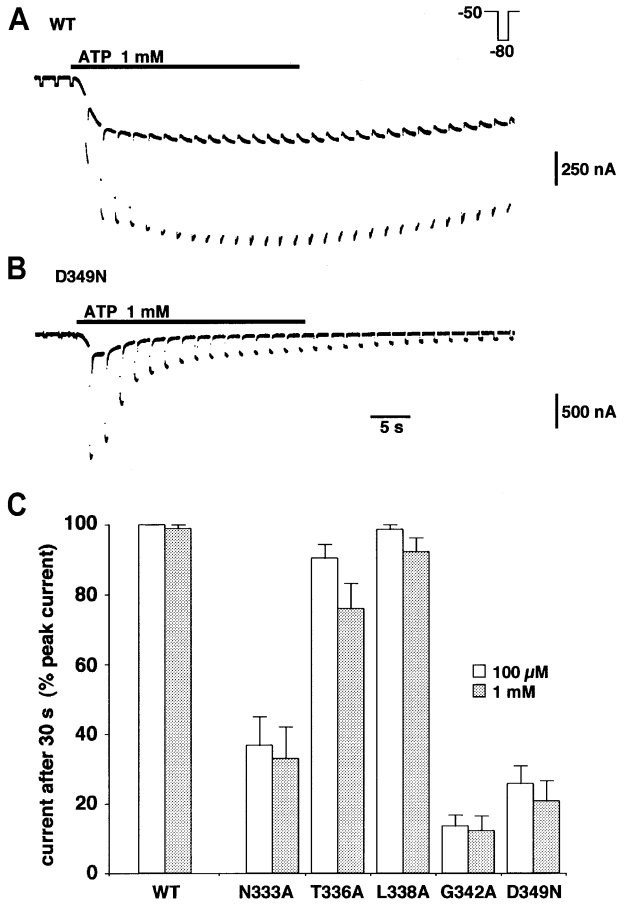


Fig. 3. Desensitization of ATP-activated current. (A, B) Comparison of time courses of ATP-activated current permeating through the wild type (WT; A) and D349N mutant (B) channels. ATP (1 mM) was applied for 30 s. The oocytes were held at − 50 mV and stepped to − 80 mV for 400 ms every 2 s. Note that the current through the wild type channel (A) was not desensitized whereas that through D349N mutant channel (B) was desensitized. (C) Comparison of the extent of desensitization. The current remaining at the end of 30 s application of 100 μM or 1 mM ATP was normalized to the peak amplitude, and plotted for the wild type (WT) and five pore mutant channels. Each column represents the mean obtained from five to seven oocytes tested. Bars are S.E.

exponential time course with a time constant of 5 s (Fig. 4A). Time constants obtained in this manner were compared in Fig. 4B. Like the current amplitude remaining at the end of the ATP exposure (Fig. 3C), the time constants were not different between the currents activated by 100 μM and 1 mM ATP for N333A and D349N. With G342A, the time constants for the current activated by 1 mM ATP were, however, smaller than that by 100 μM ATP.

3.3. Recovery from desensitization

Once desensitized, the current was not readily restored. After a 2-min washout period, the current permeating through D349N was recovered to about a half of the initial level (Fig. 5A). Fig. 5B and C shows the time course of recovery from desensitization of the currents through N333A and D349N. For both mutants, the currents were recovered to about initial levels after a 4-min washout period. For G342A, the current was recovered only to 36.1 ± 11.6% of the initial level after 4 min.

Curve fittings were made for time courses of the recovery from desensitization using the Eqs. (3) and (4) in Materials and methods. When a single "recovery" gate is assumed, the current amplitude after short washout periods (1 and 2 min) was poorly fitted by curves adjusted to fit to the current amplitude after a 4-min washout period (Fig. 5B and C; $k = 1$). This means that the initial recovery was slower than that expected from this single gate model. The fittings became better when multiple "recovery" gates are assumed (Fig. 5B and C;

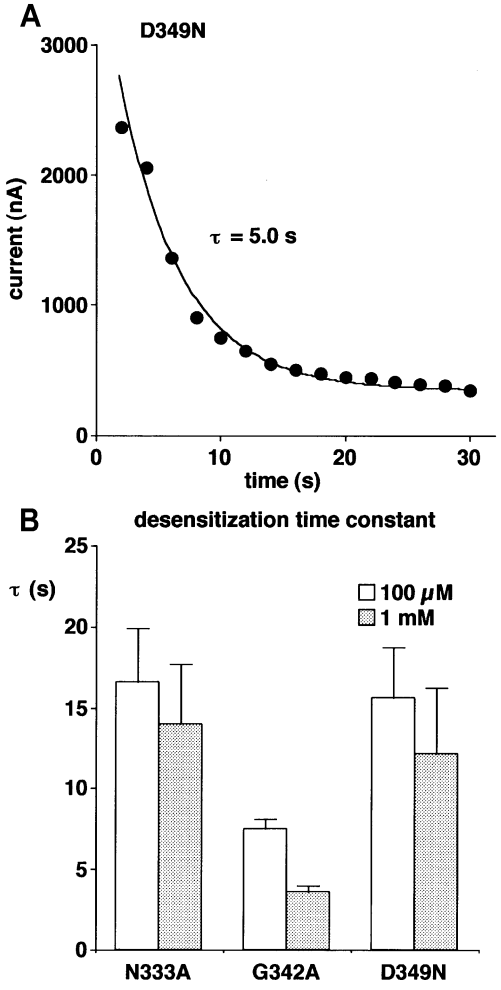


Fig. 4. Desensitization time constants. (A) A curve-fitting to the desensitization time course of ATP-activated current permeating through D349 mutant channel. The data were obtained from the current trace shown in Fig. 3B. The current amplitude at − 80 mV was plotted against the time after the beginning of the ATP-application. The data were well fitted with a single exponential time course with a time constant of 5.0 s. (B) Desensitization time constants for three pore mutants. The time constants were obtained as illustrated in A. Each column represents the mean obtained from five to seven oocytes tested. Bars are S.E.

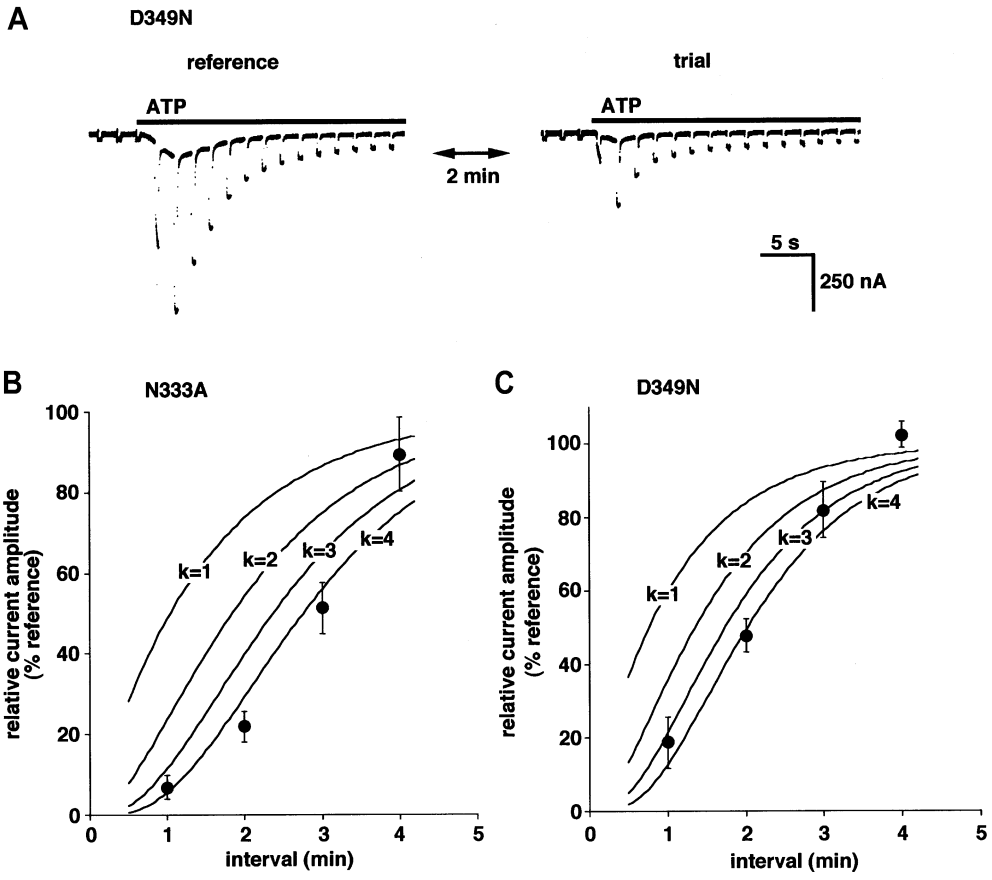


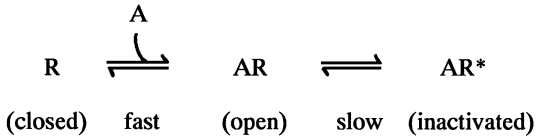
Fig. 5. Recovery from desensitization. (A) Current responses to two sequential 30-s applications of 1 mM ATP with an interval of 2 min in a D349N expressing oocyte. The response to the second application (trial) was about 40% of that to the first application (reference) in this case. (B) Time courses of recovery from desensitization of the current permeating through N333A (B) and D349N (C). Each symbol represents the mean from six oocytes tested. Bars are S.E.M. Three curves in each panel are curve-fittings with assumption of one, two, three and four homogeneous gates with a time constant of 1.5 min (N333A) or 1.1 min (D349N), respectively.

$k=2$, 3 and 4) because these multiple gate models allow slower initial recovery.

4. Discussion

In the present study, we first analyzed the "inactivation-like" property observed in the concentration–response relationship for the mutants of $P2X_2$ receptor/channel, and then analyzed the desensitization process of the mutants to compare the desensitization with the "inactivation". The mutants used in the present study possess amino acid substitutions at TM2 region of $P2X_2$ receptor/channel. This region contributes to the forming of the channel pore, and N333, T336, L338, G342 and D349 face the aqueous phase in the pore (Rassendren et al., 1997; Egan et al., 1998). The binding-site for ATP molecules are believed to be somewhere in the extracellular region of P2X receptor involving basic residues near the outer mouth of the channel pore (Ennion et al., 2000; Jiang et al., 2000). Thus, the changes in sensitivity to ATP (Fig. 1) or kinetics (Fig. 3) may not be due to direct influence by the amino acid substitutions of ATP binding, but due to some allosteric influence.

N333A, T336A, L338A and D349N exhibited reduced responses to higher concentrations of ATP (Fig. 2). These "downward limbs" could be fitted with "inactivation" curves with EC_{50} values of millimolar order. This fact may not imply the appearance of a second low-affinity binding-site on receptor subunits with these mutations. Rather, this fact may imply that the induction of this "inactivation-like" site requires higher energy than that necessary for the open state, and, thus, larger occupation by ATP molecules of the receptors is necessary. This is explained by the following simple sequential scheme (Tallarida and Jacob, 1979): where R is a receptor and A is an

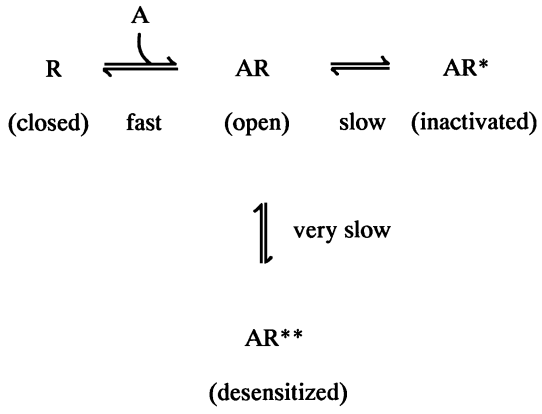


Scheme 1.

agonist (ATP). In this scheme, only one ATP molecule and one receptor subunit are shown for simplicity. The number of inactivated receptors (AR*) is negligible when the concentration of ATP is low. The number of AR* becomes considerable only when the concentration of ATP is high and as, a result, the number of AR is large.

If the process toward the inactivated state in Scheme 1 is slow enough, the current amplitude does not readily reach its steady-state, and this process will be observed as a current decay during a long exposure to ATP. In fact, the currents permeating through N333A and D349N, which exhibited ‘‘inactivation’’ in the concentration–response relationships (Fig. 2B and F), decayed during a 30-s exposure to high concentrations of ATP (Fig. 3B, C) whereas the current through the wild type channel did not exhibit such decay (Fig. 3A, C). However, this is not the case with the remaining pore mutants, T336A, L338A and G342A. T336A and L338A exhibited ‘‘inactivation’’ in the concentration–response relationships (Fig. 2C and D), but the current permeating through these mutants did not remarkably decay (Fig. 3C). As for G342A, the ‘‘inactivation’’ was not observed in the concentration–response relationship (Fig. 2E), the current through this mutant markedly decayed (Fig. 2C). The discrepancy may be explained if another ‘‘desensitized’’ state is added to the Scheme 1: (Scheme 2).

According to this scheme, ‘‘inactivation’’ and ‘‘desensitization’’ are independent processes. The transition to the inactivated state (AR*) is slow but faster than the transition to the desensitized state (AR**). AR and AR* readily reach their equilibrium, and the portion of the inactivated channels is observed as a reduction in peak amplitude, but not as a decay in current amplitude with time. In contrast, AR and



Scheme 2.

AR** do not readily reach their equilibrium, and the transition to AR** is observed mainly as a current decay with time after peak current. The transition to AR** may be slower enough for the current decay to be independent of agonist concentrations (Fig. 3). N333A and D349N can shift into both AR* and AR**, T336A and L338A can shift mainly into AR*, G342A can shift only into AR**, and the wild type channel can shift into neither AR* nor AR**. Further study will be necessary whether or not the proposed two states can be defined as molecular conformations of the receptor.

The desensitization time course of N333A, G342A and D349N could be fitted by a single exponential (Fig. 4). This suggests that the closing of a single gate is sufficient to induce the desensitization. On the other hand, the time course of the recovery from the desensitization was not well fitted by a single exponential, and the introduction of multiple exponentials resulted in better fittings to the data

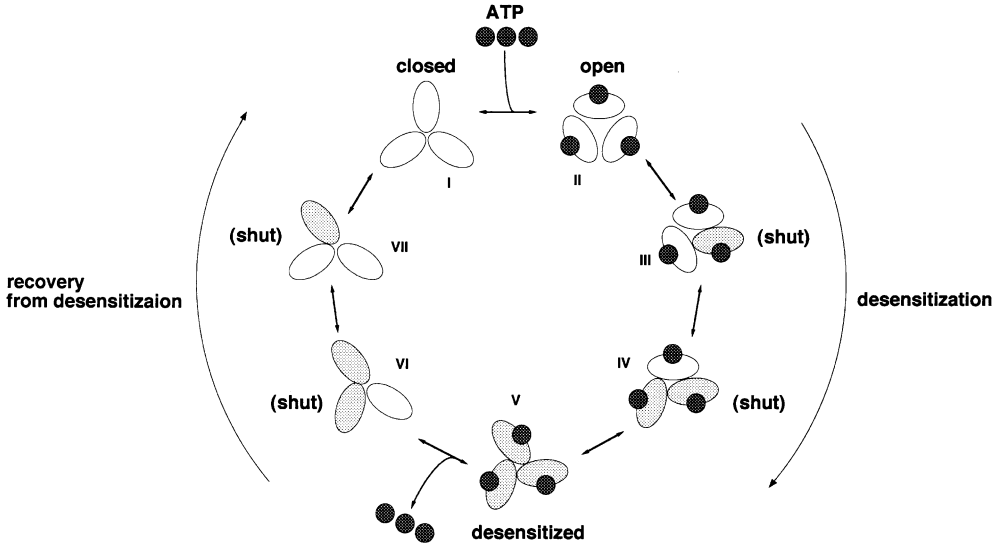


Fig. 6. A schematic model for desensitization and the recovery from desensitization of $P2X_2$ receptor pore mutants. One channel consists of three homogenous subunits. A closed channel (I) is open when ATP molecules bind (II). The open channel is shut when one of the three subunits is shifted to its ‘‘desensitized’’ conformation (III). As for the recovery from desensitization, the desensitized channel (V) is shut until all the subunit is shifted from the ‘‘desensitized’’ conformation (I; closed state).

(Fig. 5). The requirement of multiple exponentials implies that more than one gate should be "on" before the opening of the channel. Based on the trimeric composition of P2X receptors (Nicke et al., 1998; Stoop et al., 1999), these gating mechanisms can schematically explained as shown in Fig. 6. A trimeric $P2X_2$ receptor/channel opens upon the binding of ATP molecules (from state I to state II). The open channel then slowly shifts to the desensitized state. In this process, three subunits independently change their conformations (states III, IV and V), and the channel is shut when one of the subunits has changed its conformation (state III). Thus, the desensitization progresses in the first order kinetics. As for the recovery from the desensitization, the subunits also independently change their conformations (states VI, VII and I), but the channel cannot open until all the subunits have changed the conformations (state I). Thus, the recovery process follows multiple order kinetics.

Although the model shown in Fig. 6 is one of possible explanations, the recovery from the desensitization may, in any case, involves some very slow (in minutes) mechanisms. In rat superior cervical ganglia, $P2X_1$ receptors disappear from cell surface within 1 min after stimulation by agonists, and they are redistributed in the order of minutes at room temperature (Li et al., 2000). Similar redistribution of $P2X_2$ receptors has also been reported in rat hippocampal neurons, and this phenomenon occurs faster (5–10 s) at 32–34 °C (Khakh et al., 2001). The latter appears to require protein kinase C because the phenomenon was not observed when the phosphorylation site was blocked by mutagenesis (T18A). Interestingly, the current permeating through T18A also decays (Khakh et al., 2001) because of its inability to shift to the "second" open state, which appears with the phosphorylated wild type channel during a long exposure to ATP. The relationship of this current decay in T18A and the desensitization of the pore mutants observed in the present study remains to be clarified.

Neither desensitization nor the "inactivation-like" phenomenon was observed with the wild type $P2X_2$ receptor. This implies that the channel pore of the wild type receptor is elaborate enough to escape from these shutting mechanisms. Alternatively, native desensitizing P2X subclasses, such as $P2X_1$ or $P2X_3$, may possess pore structures suitable for desensitization. This view may be favored by the finding that desensitization of chimeric channels constructed from $P2X_1$ and $P2X_2$ are determined by pore forming regions (Werner et al., 1996).

Acknowledgements

We are grateful to H. Sawa for the participation in a part of experiments. This work was partly supported by a Health and Labour Science Research Grant for Research on Advanced Medical Technology from the Ministry of Health, Labour and Welfare, Japan, 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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