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Effects of neuroamines and divalent cations on cloned and mutated ATP-gated channels

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Abstract

Sensitivities to dopamine, 5-hydroxytryptamine, Zn^{2+} and Cd^{2+} were studied in $P2X_1$, $P2X_2$, $P2X_3$ and $P2X_4$ purinoceptors and mutants of $P2X_2$ purinoceptors expressed in *Xenopus* oocytes. Dopamine (10 and 100 μM) and 5-hydroxytryptamine (1 to 100 μM) enhanced the inward current activated by extracellular ATP through $P2X_2$ and $P2X_4$ purinoceptors. Zn^{2+} (1 to 100 μM) and Cd^{2+} (10 μM to 1 mM) enhanced the current through $P2X_2$ purinoceptors. As for $P2X_4$ purinoceptors, the ATP-activated current was, however, enhanced after the washout of Zn^{2+} (100 μM) or Cd^{2+} (1 mM). Three mutants of $P2X_2$ purinoceptors were constructed by substituting negatively charged amino-acid residues. The magnitude of the enhancement by Zn^{2+}, Cd^{2+} and dopamine was attenuated when Asp^{221} was replaced by histidine. The results suggest that dopamine, Zn^{2+} and Cd^{2+} require some common motif for the current enhancement.

Keywords: ATP; Purinoceptor P_{2X}; Ion channel; *Xenopus* oocyte; Site-directed mutagenesis

1. Introduction

Extracellular ATP has been shown to act as a fast neurotransmitter by activating non-selective cation channels (see reviews, Edwards and Gibb, 1993; Surprenant et al., 1995). The ATP-gated channels (P_{2X} purinoceptors) were first cloned from smooth muscle of rat vas deferens ($P2X_1$ purinoceptors; Valera et al., 1994) and rat pheochromocytoma PC12 cells ($P2X_2$ purinoceptors; Brake et al., 1994). Although these and other newly cloned ATP-gated channels appear to be channel-forming receptors (ATP receptor/channels), their structures deduced from amino-acid sequences are quite different from the motif for the so-called 'ligand-gated channel superfamily' including nicotinic acetylcholine receptors and ionotropic glutamate receptors. Thus, ATP receptor/channels have been classified into a novel channel family (Surprenant et al., 1995).

Various compounds including endogenous substances have been reported to modulate the ATP receptor channels in PC12 cells. Dopamine (Inoue et al., 1992; Nakazawa et al., 1993) and 5-hydroxytryptamine (Nakazawa et al., 1994b) facilitate an inward current activated by ATP. Zn^{2+} potentiates the ATP-evoked responses in these cells (Koizumi et al., 1995), as it does in other mammalian peripheral neurons (Clouse et al., 1993; Li et al., 1993). Cd^{2+} also potentiated the ATP-evoked responses in PC12 cells (Ikeda et al., 1996). All these compounds appear to potentiate the responses by increasing the sensitivity to ATP, but not by increasing maximal responses. Such interaction of ATP with these compounds is of interest because several lines of evidence have indicated that dopamine, 5-hydroxytryptamine or Zn^{2+} coexists with ATP or P_{2X} purinoceptors in central or peripheral neuronal regions (for details, see Section 4). The modulation by Cd^{2+} is also interesting because this may relate to toxic effects of heavy metals (Kiss and Osipenko, 1994).

We previously demonstrated that the enhancement by dopamine, 5-hydroxytryptamine, Zn^{2+} or Cd^{2+} also occurs with respect to the ATP-activated current through recombinant $P2X_2$ purinoceptors expressed in *Xenopus* oocytes (Nakazawa and Ohno, 1996). In the present study, we compared the effects of these compounds on four subclasses of P_{2X} purinoceptors ($P2X_1$, $P2X_2$, $P2X_3$ and $P2X_4$). We also employed site-directed mutagenesis to

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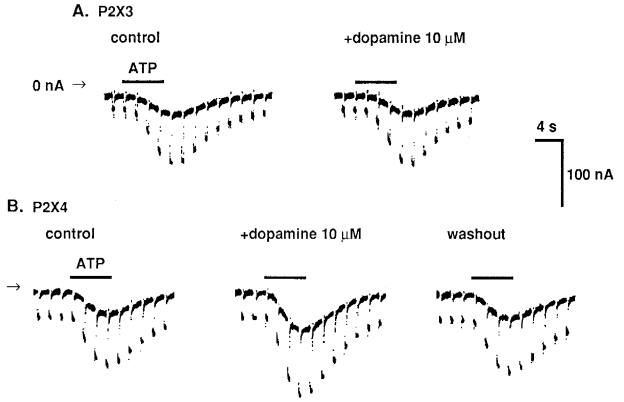

Fig. 1. ATP-activated current in the absence and the presence of 10 μM dopamine in *Xenopus* oocytes expressing $P2X_3$ (A) or $P2X_4$ purinoceptors (B). The oocytes were held at −50 mV, and a 400 ms hyperpolarizing step to −80 mV was applied every 2 s. Current traces upon successive application of ATP (horizontal bars) are shown. Arrows indicate zero current levels. The amplitude of control current at −50 mV was 28 (A) or 32 nA (B), respectively. (A) Lack of influence of dopamine on an inward current through $P2X_3$ purinoceptors evoked by 1 μM ATP. (B) Facilitation by dopamine of an inward current through $P2X_4$ purinoceptors evoked by 10 μM ATP.

examine a possible contribution of negatively charged amino-acid residues to the actions of the compounds.

2. Materials and methods

cDNAs encoding the $P2X_1$ purinoceptor (Valera et al., 1994; the EMBL submission X80477), $P2X_3$ purinoceptor (Lewis et al., 1995; the EMBL submission X91167) and $P2X_4$ purinoceptor (Buell et al., 1996; EMBL accession No. X87763) were kindly supplied by Dr. G. Buell of Glaxo Institute for Molecular Biology, and that encoding the $P2X_2$ purinoceptor (Brake et al., 1994; Genbank accession No. U14414) was supplied by Dr. T. Brake of the University of California (San Francisco, CA, USA), respectively. The cDNA encoding the $P2X_1$ purinoceptor has been cloned into pBKCMV (Stratagene, La Jolla, CA, USA). The cDNA encoding the $P2X_2$ purinoceptor, which had originally been cloned into pcDNA1/AMP (Invitrogen, Leek, Netherlands), was subcloned into pBluescript II (SK^-; Stratagene) by ligation at *Eco*RI/*Not*I sites. The

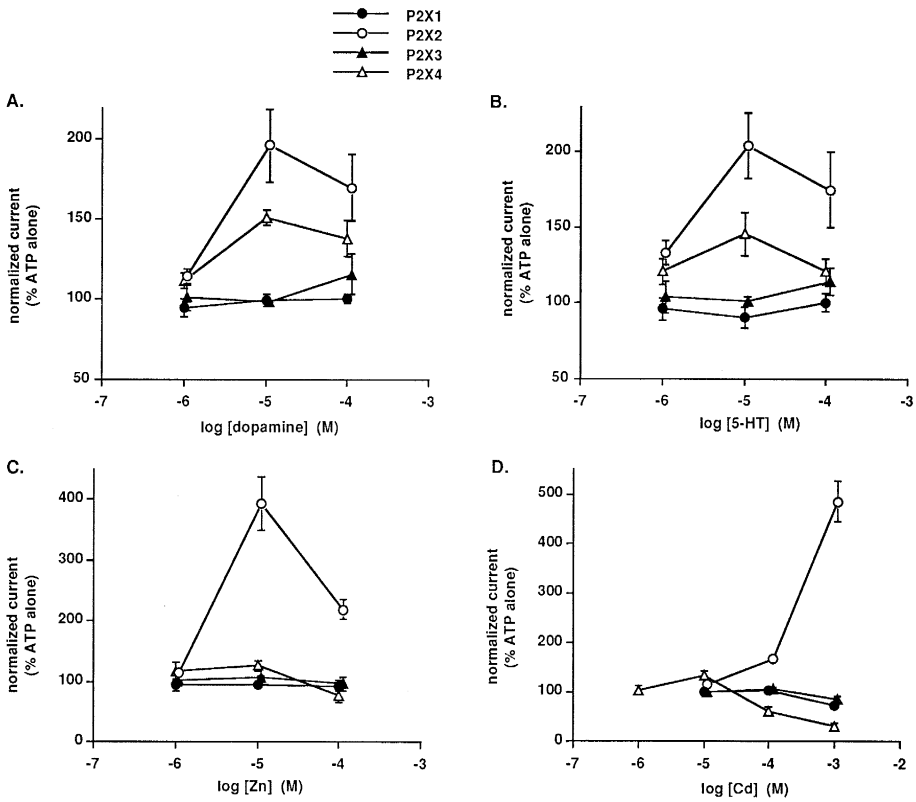

Fig. 2. Summarized data for effects of dopamine (A), 5-hydroxytryptamine (B), Zn^{2+} (C) and Cd^{2+} (D) on $P2X_1$ (filled circles), $P2X_2$ (open circles), $P2X_3$ (filled triangles) and $P2X_4$ purinoceptors (open triangles). Inward current was activated by 100 nM ($P2X_1$), 30 μM ($P2X_2$), 1 μM ($P2X_3$) or 10 μM ($P2X_4$) ATP as shown in Fig. 1. The compounds were present 10 s before and during ATP application. Peak ATP-activated current at −50 mV in the presence of the compounds was normalized to the current before the application of the compounds. For the current through $P2X_4$ purinoceptors in the presence of higher concentrations of Zn^{2+} or Cd^{2+} (Zn^{2+} 100 μM; Cd^{2+}, 100 μM and 1 mM), current amplitude was measured at the end of the ATP application because the current was followed by a sustained fluctuation (see Fig. 3A). Each symbol and bar represents the mean and S.E.M. from 4–7 oocytes tested.

cDNAs encoding the $P2X_3$ and the $P2X_4$ purinoceptor have been cloned into pcDNA3/AMP (Invitrogen). Procedures for expression of the channels and recordings of membrane currents were basically the same as those utilized for nicotinic receptor channels described in our previous report (Nakazawa et al., 1994a). The plasmids were linearized with *Not*I (Toyobo, Osaka, Japan), and sense fragments of RNA were transcribed using T3 ($P2X_1$) or T7 ($P2X_2$, $P2X_3$, $P2X_4$) RNA polymerase (Wako, Osaka, Japan). Defolliculated oocytes, isolated from *Xenopus laevis*, were injected with the synthesized RNA (each oocyte received about 20–40 ng of RNA), and kept at 18°C in ND96 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 3–6 days.

Site-directed mutagenesis was conducted using the Transformer Site-directed mutagenesis kit (Clontech, Palo Alto, CA, USA). cDNA encoding the $P2X_2$ purinoceptor subcloned into pBluescript II was used as a template, and mutants were constructed using mutation primers that contained one mismatch base for the replacement of a target amino-acid residue. A unique *Xho*I site included in the multicloning site of pBluescript II was simultaneously mutated to select transformed plasmids. Achievement of successful transformation was confirmed by sequencing of bases using the CircumVent Phototope DNA sequencing/detection kit (New England Biolabs, Beverly, MA, USA).

Membrane currents were measured with two microelectrode voltage-clamp methods at room temperature (25–27°C). Oocytes were placed in an experimental chamber of about 0.2 ml capacity filled with ND96 solution. ATP and other drugs were applied by superfusion at a constant flow rate of 0.5 ml s^{-1}. ATP was applied for 5 s, every 1 min for $P2X_2$ and $P2X_4$ purinoceptors and every 3 min for $P2X_1$ and $P2X_3$ purinoceptors, because these receptors are readily desensitized (Valera et al., 1994; Lewis et al., 1995). These application intervals, which had been determined in preliminary experiments, were long enough to avoid desensitization to ATP of the concentrations utilized to determine the effects of the compounds on the ATP-activated current. In this determination, the compounds were applied 10 s before and during trials with ATP.

Drugs used were ATP (adenosine 5′-triphosphate disodium salt; Sigma, St. Louis, MO, USA), dopamine hydrochloride (Sigma) and 5-hydroxytryptamine creatine sulfate complex (Sigma). Other chemicals were of reagent grade.

All the data are given as means ± S.E.M.

3. Results

3.1. The ATP-activated current in Xenopus oocytes expressing P_{2X} purinoceptors

In *Xenopus* oocytes injected with RNA encoding any of a single subclass of P_{2X} purinoceptors, an inward current activated by application of extracellular ATP was detected as early as 2 days after the injection (for example, see Fig. 1). The amplitude of the current was increased with the incubation period, and, after 5 days, it reached > 1 μA for $P2X_1$ and $P2X_2$ purinoceptors whereas it reached 50–200 nA for $P2X_3$ and $P2X_4$ purinoceptors at −50 mV with ATP at concentrations that produce maximal responses. We first determined the concentration–response relationship for the ATP-activated current (not shown). The order of the sensitivity of the subclasses to

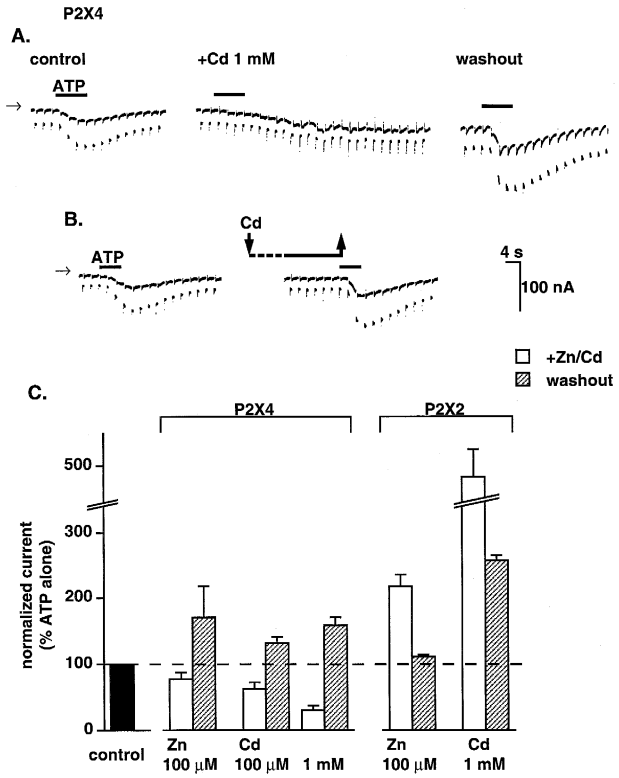

Fig. 3. (A,B) Current responses to 10 μM ATP and influence of 1 mM Cd^{2+} in *Xenopus* oocytes expressing $P2X_4$ purinoceptors. Current traces with successive trials of ATP (horizontal bars) are shown in each panel. The oocytes were held at −50 mV and stepped for 400 ms to −80 mV every 2 s. Horizontal arrows indicate zero current levels. The amplitude of control current at −50 mV was 20 (A) or 22 nA (B), respectively. (A) Effects of Cd^{2+} applied with ATP. When ATP was applied in the presence of Cd^{2+}, a sustained inward shift of membrane current with fluctuation was induced (middle panel). After washout of Cd^{2+}, application of ATP alone elicited an inward current (right panel) greater than the control current (left panel). (B) Facilitation of the ATP-activated current by pretreatment with Cd^{2+}. Cd^{2+} was applied for 20 s until just before the trial with ATP alone in this case (right panel). (C) ATP-activated current during (open columns) and after (hatched columns) application of Zn^{2+} or Cd^{2+} in *Xenopus* oocytes expressing $P2X_4$ (left) or $P2X_2$ purinoceptors (right). The current was normalized to that before application of Zn^{2+} or Cd^{2+}. Each column and bar represents the mean and S.E.M. from 4–6 oocytes tested. For $P2X_4$ purinoceptors, the current was inhibited by 100 μM Zn^{2+} or 1 mM Cd^{2+}, but it became about 1.5-fold greater than the control current after washout of these divalent cations. For $P2X_2$ purinoceptors, the current enhanced by 1 mM Cd^{2+} was not completely restored, and was still 2-fold greater than the control current after washout of Cd^{2+}.

ATP was (roughly estimated EC_{50} values are given in parentheses) $P2X_1$ (300 nM) > $P2X_3$ (3 μM) > $P2X_4$ (10 μM) > $P2X_2$ (50 μM). For subsequent experiments, we selected concentrations at which ATP produced 25–50% of maximal currents (3 μM for $P2X_1$, 30 μM for $P2X_2$ and $P2X_4$, and 10 μM for $P2X_3$), so that we could readily detect the current facilitation without saturation.

3.2. Effects of dopamine, 5-hydroxytryptamine, Zn^{2+} and Cd^{2+} on ATP-activated current through subclasses of P_{2X} purinoceptors

The effects of dopamine, 5-hydroxytryptamine, Zn^{2+} and Cd^{2+} on the ATP-activated current through four subclasses of P_{2X} purinoceptors are summarized in Fig. 2. Some of the data concerning $P2X_1$ and $P2X_2$ purinoceptors have already been presented in our previous report (Nakazawa and Ohno, 1996). At 10 μM, dopamine or 5-hydroxytryptamine enhanced the ATP-activated current in *Xenopus* oocytes expressing $P2X_2$ purinoceptors, but not that in oocytes expressing $P2X_1$ purinoceptors (Fig. 2A and B). The magnitude of the enhancement of the current through $P2X_2$ purinoceptors decreased when the concentration of dopamine or 5-hydroxytryptamine was increased from 10 to 100 μM. Dopamine or 5-hydroxytryptamine did not affect the current through $P2X_1$ purinoceptors. The current through $P2X_4$ purinoceptors was, like that in $P2X_2$ purinoceptors, enhanced by dopamine or 5-hydroxytryptamine whereas the current through $P2X_3$ purinoceptors was, as in $P2X_1$ purinoceptors, insensitive to these compounds (Figs. 1 and 2). The current facilitation by dopamine or 5-hydroxytryptamine on $P2X_2$ or $P2X_4$ purinoceptors was reversible: the ATP-activated current was readily returned to the control level in subsequent trials with ATP alone (Fig. 1B).

Fig. 2C and D illustrate the effects of Zn^{2+} and Cd^{2+} on ionic currents through the subclasses of P_{2X} purinoceptors. Zn^{2+} (1–100 μM) and Cd^{2+} (10–1000 μM) facilitated the ATP-activated current through the $P2X_2$ purinoceptor. The current enhanced by Zn^{2+} and 100 μM Cd^{2+} readily returned to the control level on washout, as in the case of dopamine or 5-hydroxytryptamine, but the current enhanced by 1 mM Cd^{2+} was not readily restored (see below and Fig. 5A). The current enhancement by 1 mM Cd^{2+} was not completely reversible in some oocytes, and the current amplitude remained about 1.5-fold as large as the control current even after several trials with ATP alone.

Zn^{2+} or Cd^{2+} did not enhance the current through $P2X_1$ or $P2X_3$ purinoceptors (Fig. 2C and D). As for $P2X_4$ purinoceptors, the effects of these divalent cations were rather complicated. When ATP was applied in the presence of Zn^{2+} (100 μM) or Cd^{2+} (100 μM and 1 mM), an inward shift of membrane current with irregular fluctuation was observed (Fig. 3A, middle panel). The next washout and a retrial with ATP in the absence of Zn^{2+} or Cd^{2+}, however, elicited an inward current greater than the current before the application of Zn^{2+} or Cd^{2+} (Fig. 3A, left panel). Current enhancement was also observed when Zn^{2+} or Cd^{2+} was added as pretreatment until just before the application of ATP alone (Fig. 3B): the current was increased to $138.3 \pm 7.2\%$ (Zn^{2+} 100 μM, $n = 3$) or $167.5 \pm 18.7\%$ of the control (Cd^{2+} 1 mM, $n = 3$), respectively. We summarized the amplitude of the ATP-activated

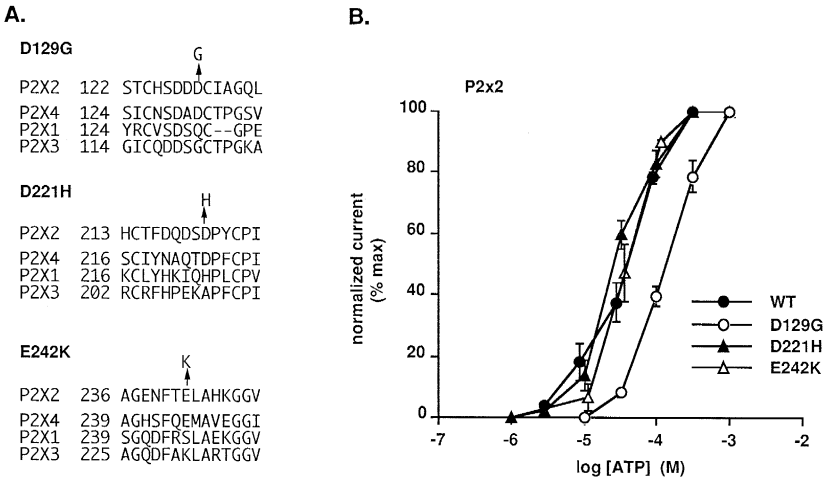

Fig. 4. (A) Experimental design for substitution of negatively charged amino-acid residues in $P2X_2$ purinoceptors. Amino-acid sequences of four subclasses of P_{2X} purinoceptors are partly shown using single characters. Asp^{129}, Asp^{221} or Glu^{242} of $P2X_2$ purinoceptors, which also occurs in $P2X_4$ purinoceptors at each corresponding position, was replaced by glycine (D129G), histidine (D221H) or lysine (E242K), respectively, which occurs in $P2X_1$ or $P2X_3$ purinoceptors at each corresponding position. (B) Concentration–response relationship for the ATP-activated current through wild-type $P2X_2$ purinoceptors (WT, filled circles) and three mutants of these receptors (D129G, open circles; D221H, filled triangles; E242K, open triangles) expressed in *Xenopus* oocytes. Oocytes were repeatedly exposed to ATP, the concentrations of which were successively increased, and the peak amplitude of the ATP-activated current at −50 mV was normalized to the maximal current in individual oocytes. Each symbol represents the mean from 4–6 oocytes. Vertical bars are S.E.M.

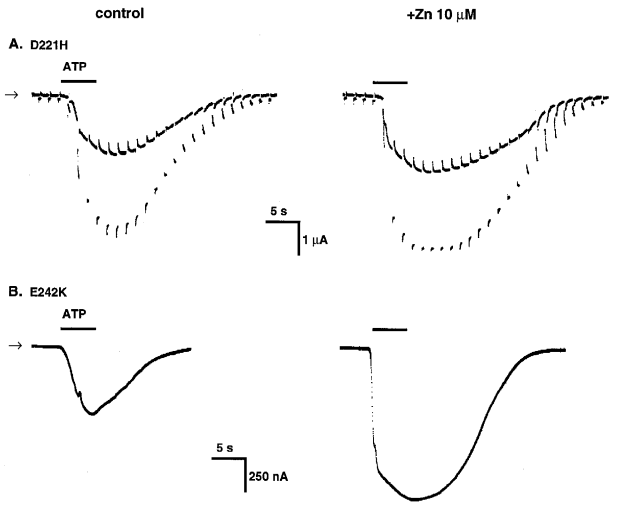

Fig. 5. Effects of Zn^{2+} (10 μM) on currents activated by 30 μM ATP in oocytes expressing mutants of $P2X_2$ purinoceptors (A, D221H; B, E242K). The oocytes were held at −50 mV. In A, a 400 ms hyperpolarizing step was applied every 2 s. Arrows indicate zero current levels. The amplitude of control current at −50 mV was 1.8 μA (A) or 530 nA (B), respectively. Note that the ATP-activated current was increased only about 1.3-fold for D221H (A) whereas it increased as much as 2.3-fold for E242K (B).

current through $P2X_4$ purinoceptors during the application of Zn^{2+} and Cd^{2+}, and that after the removal of these cations in Fig. 3C. At lower concentrations (Zn^{2+}, 1 and 10 μM; Cd^{2+}, 10 μM), these divalent cations did not affect the ATP-activated current either when they were present or after their removal. For comparison, we also show the data with $P2X_2$ purinoceptors in Fig. 3C. The facilitation by 1 mM Cd^{2+} of the current through $P2X_2$ purinoceptors was incompletely restored, and the current remained greater than the control level after the removal of Cd^{2+}.

3.3. Effects of dopamine, 5-hydroxytryptamine, Zn^{2+} and Cd^{2+} on ATP-activated current through mutated $P2X_2$ purinoceptors

Dopamine and 5-hydroxytryptamine selectively enhanced the ATP-activated current through $P2X_2$ and $P2X_4$ purinoceptors, but not that through $P2X_1$ or $P2X_3$ purinoceptors. In addition, although Zn^{2+} or Cd^{2+} facilitated the ATP-activated current through $P2X_2$ purinoceptors, but not through $P2X_4$ purinoceptors, the ATP-

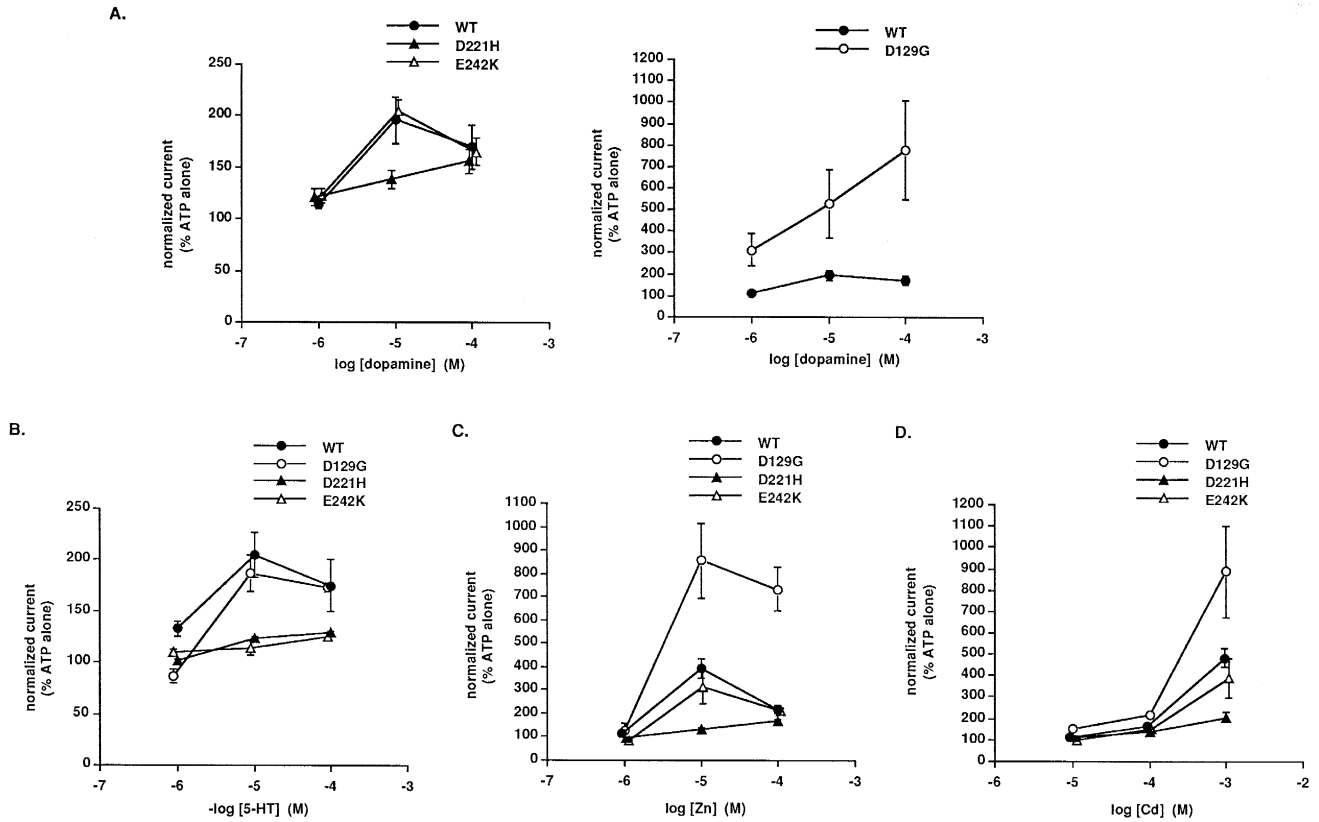

Fig. 6. Summarized data for effects of dopamine (A), 5-hydroxytryptamine (B), Zn^{2+} (C) and Cd^{2+} (D) on wild-type $P2X_2$ purinoceptors (WT, filled circles) and three mutants of the receptors (D129G, open circles; D221H, filled triangles; E242, open triangles). Inward current was activated by 30 μM ATP as shown in Fig. 5. Peak ATP-activated current at −50 mV in the presence of the compounds was normalized to the current with ATP alone. Each symbol and bar represents the mean and S.E.M. from 4–7 oocytes tested. For dopamine, the results obtained with D129G are shown separately in the right panel of A because the current facilitation was markedly greater with this mutant than with the remaining channels.

activated current after the removal of these divalent cations was greater than the control current in both $P2X_2$ and $P2X_4$ purinoceptors. The results suggest that some common molecular motifs responsible for these facilitatory current modulations are present in $P2X_2$ and $P2X_4$ purinoceptors, but not in the remaining two subclasses. Such motifs may involve negatively charged or polarized amino-acid residues responsible for the binding of these modulators, because Zn^{2+} and Cd^{2+} are positively charged, and dopamine and 5-hydroxytryptamine can also interact with these residues via amine groups in their structures. According to the comparison of primary structures of subclasses of P_{2X} purinoceptors by Collo et al. (1996), three negatively charged amino-acid residues (Asp^{129}, Asp^{221} and Glu^{242}) are present in corresponding positions of $P2X_2$ and $P2X_4$ purinoceptors, but not in those of $P2X_1$ or $P2X_3$ purinoceptors (Fig. 4A). Thus, we constructed three mutants of $P2X_2$ purinoceptors in which each of the three negatively charged amino-acid residues in common with $P2X_4$ purinoceptors was replaced by an uncharged or positively charged residue. The replacement of the amino-acid residues was designed so that they were changed into residues of $P2X_1$ or $P2X_3$ purinoceptors in the corresponding positions, and the mutants were termed D129G, D221H and E242K, respectively (Fig. 4A). It is noted that the amino-acid residues selected for substitution are included in a proposed large extracellular loop between two transmembrane regions of P_{2X} purinoceptors (Surprenant et al., 1995; Collo et al., 1996).

ATP elicited inward currents in oocytes injected with RNA encoding any of these mutants, suggesting that each mutant is able to form functional homomeric channels. Fig. 4B illustrates the concentration–response relationship for the ATP-activated current through the mutant channels. D221H and E242K exhibited a sensitivity to ATP similar to that of wild-type $P2X_2$ purinoceptor/channels while D129G showed a several fold lower sensitivity than these channels when compared on the basis of EC_{50} values roughly estimated from Fig. 4B.

Dopamine, Zn^{2+} and Cd^{2+} enhanced the inward current activated by 30 μM ATP through the mutant channels at concentrations that enhanced the current through the wild-type channels, though the magnitude of the enhancement was different among the mutants (Fig. 5 and Fig. 6A,C,D): the rank order of the magnitude of the enhancement was D129G > E242KM ≅ wild type > D221H for all these three compounds. 5-Hydroxytryptamine enhanced the current activated by 30 μM ATP permeating through D129G, as it did the current through the wild-type channels, but it did not significantly affect the current through D221H or E242K (Fig. 6B).

It has been shown that the current enhancement by these four compounds is greater when the current is activated by a lower concentration of ATP: the enhancement appears to saturate with higher concentrations of ATP because of the limitation of available channels (Inoue et al., 1992; Clouse et al., 1993; Li et al., 1993; Nakazawa et al., 1993, 1994b; Brake et al., 1994; Koizumi et al., 1995; Ikeda et al., 1996). Thus, it is possible that the greater enhancement of the current through D129G is due to the lower sensitivity of this mutant to ATP (Fig. 4B). To examine this possibility, we studied the effects of the compounds on inward currents activated by a higher concentration (100 μM) of ATP, at which the fraction of activated channels in D129G may be equivalent to that in other mutant channels or the wild-type channels at 30 μM ATP. Fig. 7 shows the comparison of the enhancement under these conditions. The magnitude of the current enhancement in D129G became comparable to that in the wild type for dopamine, Zn^{2+} and Cd^{2+}. 5-Hydroxytryptamine failed to enhance the current through D129G activated by 100 μM ATP.

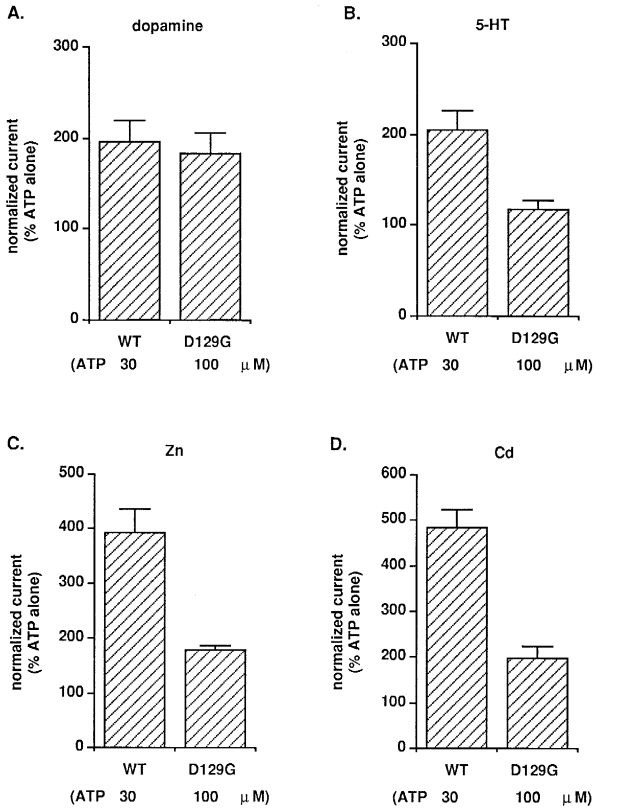

Fig. 7. Comparison of current facilitation in wild-type (WT) channels and D129G mutant at ATP concentrations of similar potency. Based on the concentration-response relations shown in Fig. 5, 100 μM was selected for D129G, and current facilitation was examined. The data with 10 μM dopamine (A), 10 μM 5-hydroxytryptamine (B), 10 μM Zn^{2+} (C) or 1 mM Cd^{2+} were compared with those obtained from the wild-type $P2X_2$ purinoceptor. Each column and bar represents the mean and S.E.M. from 4–7 oocytes.

4. Discussion

We have demonstrated, using recombinant channels expressed in *Xenopus* oocytes, that dopamine, 5-hydroxy-

tryptamine, Zn^{2+} and Cd^{2+} affect differently four subclasses of P_{2X} purinoceptors, and that substitution of negatively charged amino-acid residues results in changes in the sensitivity of $P2X_2$ purinoceptors to these compounds.

P_{2X} purinoceptors present in various tissues are known to exhibit different sensitivities to agonists. Molecular cloning and expression of the cloned subclasses of P_{2X} purinoceptors have demonstrated that such different sensitivities are attributable to the properties of individual subclasses. For example, $P2X_2$ purinoceptors cloned from PC12 cells exhibit low sensitivity to ATP, and do not respond to α,β-methylene ATP (Brake et al., 1994), whereas $P2X_1$ purinoceptors cloned from rat vas deferens exhibit high sensitivity to ATP, and respond to α,β-methylene ATP (Valera et al., 1994). The present study has shown that modulations by the neuroamines and the divalent cations of $P2X_2$ purinoceptors, which have been reported for the purinoceptors in primary neurons or PC12 cells (Inoue et al., 1992; Nakazawa et al., 1993, 1994b; Clouse et al., 1993; Li et al., 1993; Koizumi et al., 1995; Ikeda et al., 1996), are reproducible in recombinant channels, and that the modulations depend exclusively on the types of subclasses (Figs. 1 and 2). These findings suggest that the modulations are attributable to P_{2X} purinoceptors themselves, but not to some cellular component particularly expressed in individual tissues.

We constructed three mutants of $P2X_2$ purinoceptors in which single negatively charged amino-acid residues (Asp^{129}, Asp^{221}, Glu^{242}) were substituted (Fig. 4A). The results obtained with the mutants (Figs. 6 and 7) suggest that none of these negatively charged amino-acid residues accounts solely for the facilitation by dopamine, Zn^{2+} or Cd^{2+}, because the facilitation was attenuated, but not abolished, with any single mutations examined. The facilitation by 5-hydroxytryptamine may not be attributable to any single residue, because the facilitation was similarly attenuated in both D221H and E242K. As for D129G, the magnitude of the facilitation by dopamine, Zn^{2+} and Cd^{2+}, but not that by 5-hydroxytryptamine, was greater than in wild-type channels when the current was activated by 30 μM ATP (Fig. 6). However, this may have been due to a lower sensitivity to ATP of this mutant (Fig. 4B) because the facilitation was comparable to or even smaller than in the wild-type channels when the comparison was made with equipotent concentrations of ATP for each type of channel (Fig. 7).

The attenuation of the current facilitation observed on substitution of an amino-acid residue may indicate that this residue participates in the formation of the binding sites of the modulators for the facilitation. However, another possibility, namely that the substitution produces some change in the conformation of the channels and that this change secondarily influences the current facilitation, cannot be excluded. Regardless of direct or indirect influence, the changes observed on substitution may provide information for the relation among the binding sites for the modulators. The facilitation by Zn^{2+} and that by Cd^{2+} exhibited common properties: both enhanced $P2X_2$ purinoceptors (Fig. 2), and the replacement of Asp^{221} by histidine (D221H) selectively reduced the enhancement (Fig. 6). Thus, it is probable that Zn^{2+} and Cd^{2+} bind to a common motif of the channels. Dopamine may bind to this motif because the enhancement of the current through $P2X_2$ purinoceptors was also selectively attenuated in D221H (Fig. 6). The binding site for 5-hydroxytryptamine may also share the same motif, as shown by the reduction of the enhancement in D221H, but may contain another particular motif because the replacement of Glu^{242} by lysine also attenuated the current enhancement (Figs. 6 and 7).

Although the substitution for Asp^{221} in $P2X_2$ purinoceptors resulted in the attenuation of the enhancement by Zn^{2+} or Cd^{2+}, these divalent cations failed to facilitate $P2X_4$ purinoceptors that possess Asp at an equivalent position (Fig. 2). Instead, the ATP-activated current through $P2X_4$ purinoceptors was enhanced after the removal of these divalent cations (Fig. 3). One possible explanation is that Zn^{2+} and Cd^{2+} exert a sustained facilitatory effect on $P2X_4$ purinoceptors through the binding site involving this aspartic acid residue, but that this facilitation is masked by an inhibitory effect that is occurring simultaneously. Divalent cations, including Cd^{2+}, are known to block ion permeation through ATP-gated channels in PC12 cells in single-channel current measurements (Nakazawa and Hess, 1993), though a macroscopic current activated by ATP is enhanced by Cd^{2+} (Ikeda et al., 1996). Such a blocking action may occur preferentially at $P2X_4$ purinoceptors. Persisting enhancement of the ATP-activated current through $P2X_2$ purinoceptors after the removal of Zn^{2+} or Cd^{2+} (Fig. 3C) may support this idea of sustained facilitation.

Several lines of evidence indicate a physiological significance of the current facilitation reported upon here though they may not be conclusive at present. Zn^{2+} is abundant in hippocampal nerve endings (Frederickson, 1989) and RNA for $P2X_4$ purinoceptors is highly expressed in this region (Collo et al., 1996). Endogenous or exogenous ATP activates cationic currents in neurons in autonomic ganglia (Evans et al., 1992; Clouse et al., 1993; Li et al., 1993; Nakazawa, 1994), and dopamine is stored in specific cells in sympathetic ganglia (Eränkö, 1978). 5-Hydroxytryptamine induces inward currents in populations of central and peripheral neurons where ATP also elicits inward currents (Edwards et al., 1992; Yang et al., 1992; Nakazawa, 1994).

To summarize and conclude, we have investigated sensitivities to dopamine, 5-hydroxytryptamine, Zn^{2+} and Cd^{2+} in different subclasses of P_{2X} purinoceptors. We also constructed mutants of $P2X_2$ purinoceptors. From the results with the mutants, combined with results with the subclasses, we conclude that dopamine, Zn^{2+} and Cd^{2+} share some common motif in their binding sites. Recombinant channels and their mutants, as reported here, may be useful to clarify structural requirements for allosteric mod-

ulations of channels or other channel functions in $P2X_2$ purinoceptors as well as other ligand-gated channels.

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