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pH dependence of facilitation by neurotransmitters and divalent cations of $P2X_2$ purinoceptor/channels

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Abstract

The pH dependence of the facilitation by dopamine (10 μM), 5-hydroxytryptamine (10 μM), adenosine (1 and 100 μM), Zn^{2+} (10 μM) and Cd^{2+} (1 mM) of $P2X_2$ purinoceptor/channels was tested by expressing these channels in *Xenopus* oocytes. In a pH range between 6.0 and 8.5, concentration–response curves for an inward current activated by ATP were shifted toward a lower concentration range at a more acidic pH, indicating that the sensitivity to ATP is pH-dependent. Comparison of the effects of the neurotransmitters and the divalent cations on the ATP-activated current was made using a concentration of ATP which activated 40–50% of the maximal current at each pH value. The current facilitation by dopamine was obvious at pH 7.1 and 7.7, but was not observed at pH 8.5. At pH 6.0, the current was inhibited upon first trials of dopamine, but it was facilitated upon second trials. With 5-hydroxytryptamine and adenosine, the current facilitation was most remarkable at pH 6.0, less remarkable at pH 7.1 and 7.7, and the facilitation was almost abolished at pH 8.5. On the other hand, the current facilitation by Zn^{2+} and Cd^{2+} was more remarkable at alkaline pH values (7.7 and 8.5), and the facilitation was almost abolished at pH 6.0. The results suggest that the facilitation of $P2X_2$ purinoceptors depends on pH, and the pH dependence was different between the neurotransmitters and the divalent cations. © 1997 Elsevier Science B.V.

Keywords: $P2X_2$ purinoceptor; pH dependence; Neuroamine; Adenosine; Zn^{2+}; Cd^{2+}

1. Introduction

Extracellular ATP has been shown to act as a fast neurotransmitter by activating non-selective cation channels ($P2X_2$ purinoceptor/channels: for reviews, see Edwards and Gibb, 1993; Surprenant et al., 1995). Various compounds including endogenous substances have been reported to modulate P2X purinoceptor/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 P2X-purinoceptors in central or peripheral neuronal regions, as discussed in our previous paper (Nakazawa and Ohno, 1997). 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, adenosine, Zn^{2+} or Cd^{2+} also occurs with respect to the ATP-activated current through recombinant $P2X_2$ purinoceptor/channels expressed in *Xenopus* oocytes (Nakazawa and Ohno, 1996, 1997). Recently, King and co-workers reported that acidification increases the sensitivity of $P2X_2$ purinoceptor/channels (King et al., 1996), and, furthermore, suggested that the facilitation by neurotransmitters including 5-hydroxytryptamine and adenosine of these channels is due to acidification of extracellular solutions by these compounds (Wildman et al., 1997). We, however, report here the facilitation by neurotransmitters and divalent cations that is not due to extracellular acidification: dopamine, 5-hydroxytryptamine, adenosine, Zn^{2+} and Cd^{2+} augmented ionic current through $P2X_2$ purinocep-

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tor/channels expressed in *Xenopus* oocytes even after the pH of extracellular solution had been precisely adjusted. We further characterized the pH dependence of the current facilitation, and found the facilitation by neurotransmitters and that by divalent cations exhibited an almost opposite pH dependence.

2. Materials and methods

cDNA encoding $P2X_2$-purinoceptor/channel (Brake et al., 1994; the Genbank entry U14414) was kindly supplied by Dr. T. Brake of the University of California (San Francisco, CA, USA). The cDNA, which had originally been cloned into pcDNA1/AMP (Invitrogen, Leek, UK), was subcloned into pBluescript II (SK^-; Stratagene, La Jolla, CA, USA) by ligation at *Eco*RI/*Not*I sites. 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 T7 RNA polymerase (Wako, Osaka, Japan). Defolliculated oocytes, isolated from *Xenopus laevis*, were injected with the synthesized RNA (each oocyte received about 20 to 40 ng of RNA), and kept at 18°C in a standard ND96 solution containing (mM) NaCl 96, KCl 2, $CaCl_2$ 1.8, $MgCl_2$ 1,2-[4-(2-hydroxyethyl)-1-piperazinyl]ethansulfonic acid (HEPES; Dojin, Kumamato, Japan) 5 (pH 7.5 with NaOH) supplemented with 0.01% gentamycin for 3 to 7 days.

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. The pH of ND96 solution was adjusted with HEPES-NaOH between 7.1 and 7.7. ATP and other drugs were applied by superfusion at a constant flow rate of 0.5 ml/s. ATP was applied for 10 s, every 1 min. Before testing the effects of various compounds, ATP alone was repeatedly applied to confirm the stability of the current response to ATP. Test compounds were applied 20 s before and during trials with ATP. When pH was adjusted to 6.0 or 8.5, HEPES was replaced with equimolar 2-[*N*-morpholino]ethanesulfonic acid (MES; Sigma, St. Louis, MO, USA) or *N*-tris[hydroxymethyl]methyl-3-aminopropanesulfonic acid (TAPS; Sigma), respectively. In these cases, oocytes was bathed in a standard ND96 solution (pH 7.5), and the solution was switched to solutions of desired pH values upon the application of test compounds (for control responses to ATP, the switching was made 20 s before the ATP application).

Drugs used were ATP (adenosine 5′-triphosphate disodium salt; Sigma), dopamine hydrochloride (Sigma), 5-hydroxytryptamine creatine sulfate complex (Sigma), adenosine (Sigma). Other chemicals were of reagent grade. After addition of drugs to extracellular solutions, pH was re-adjusted with NaOH. With the test compounds, slight acidification was observed in extracellular solutions. Among the test compounds (not including ATP), the largest change was observed with 100 μM adenosine (0.04 pH unit or smaller). None of other test compounds produced changes larger than 0.03 pH unit.

All the data are given as mean ± S.E.

3. Results

3.1. The pH-dependence of inward current activated by ATP

King et al. (1996) have demonstrated that the ATP sensitivity of $P2X_2$ purinoceptor/channels expressed in *Xenopus* oocytes increases with acidification. This finding was also confirmed in our study (Fig. 1). As neurotransmitters and divalent cations facilitate the ATP-activated current without affecting maximal current responses (see Section 1), appropriate concentrations of ATP should be selected to observe the current facilitation, in other words, to avoid current saturation.

Examples of such current saturation are illustrated in Fig. 2. In these preliminary experiments, the concentrations of these compounds (10 μM dopamine, 10 μM 5-hydroxytryptamine, 100 μM adenosine, 10 μM, Zn^{2+} and 1 mM Cd^{2+}) were selected such that maximal current facilitation was obtained (Nakazawa and Ohno, 1996, 1997). A lower concentration of adenosine (1 μM) was also tested because the compound exhibited opposite modulations on cloned $P2X_2$ purinoceptor/channels (facilitation; Nakazawa and Ohno, 1996) and channels expressing

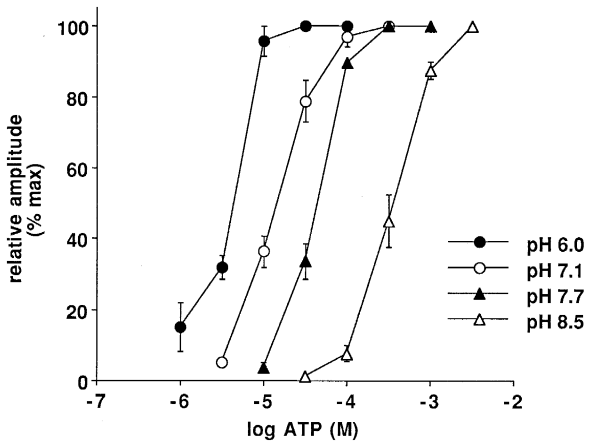

Fig. 1. Concentration–response relationship for ionic current activated by ATP at different pH values (6.0, 7.1, 7.7 and 8.5) in *Xenopus* oocytes expressing $P2X_2$ receptor/channels. The recording conditions were similar to those shown in Fig. 2A. The amplitude of currents activated by various concentrations of ATP was normalized to maximal current amplitude. Each symbol represent the mean from 3 to 5 oocytes tested at pH 6.0 (filled circles), pH 7.1 (open circles), pH 7.7 (filled triangles) and pH 8.5 (open triangles). Bars are S.E.

in PC12 cells (inhibition; Inoue et al., 1992), from which the $P2X_2$ purinoceptor/channel was cloned (Brake et al., 1994). In Fig. 2A, the facilitation by dopamine of the current activated by the same concentration of ATP (30 μM) was compared at different pH values. The ATP-activated current was more remarkably facilitated at pH 7.7 than at pH 7.1. For other compounds tested (5-hydroxytryptamine, adenosine, Zn^{2+} and Cd^{2+}), the current facilitation examined with 30 μM ATP was also accentuated at more alkaline pH values (7.4 and 7.7), and markedly reduced at pH 7.1 (Fig. 2B). This phenomenon is most likely attributed to the fact that 30 μM ATP alone is able to activate a current fraction as large as 80% of the maximal current at pH 7.1, but the current fraction activated is smaller at pH 7.4 and 7.7 (Fig. 1). Thus, to compare the current at different pH values, the usage of the concentrations of ATP which activate a similar current fraction is desirable. For this purpose, we adopted 3, 10, 30, and 300 μM ATP for pH 6.0, 7.1, 7.7 and 8.5, respectively, in the following experiments. These concentrations activated a fraction of 40–50% of the maximal current at each pH value (Fig. 1).

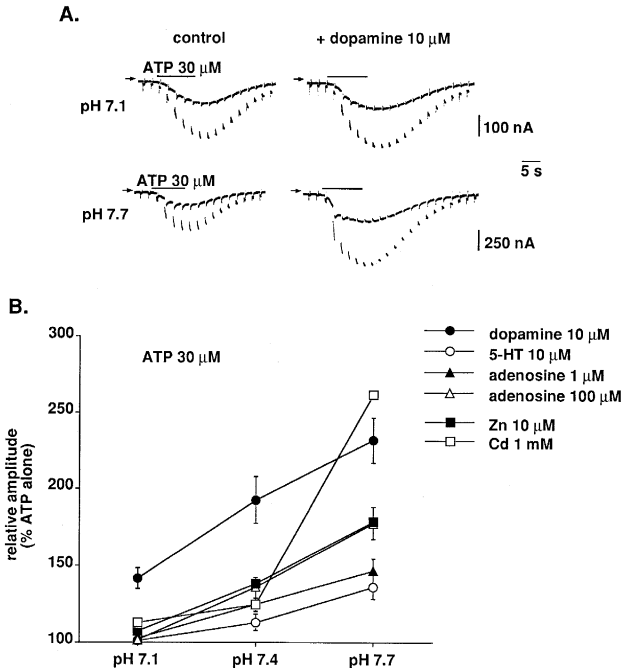

Fig. 2. (A) Effects of dopamine (10 μM; A) on ionic current activated by 30 μM ATP at pH 7.1 (upper panels) and pH 7.7 (lower panels). The oocytes were held at −50 mV, and a 400 ms hyperpolarizing step to −80 mV was applied every 2 s. The ATP-activated current just before (control; left) and during the application of dopamine (right) are compared. Arrows indicate zero current levels. (B) Increase in the magnitude of the current facilitation at more alkaline pH values when currents were activated by a single concentration (30 μM) of ATP. The current amplitude normalized to that just before the application of various compounds is plotted against pH values. Each symbol and bar represent mean and S.E. from 5 to 10 oocytes tested with dopamine (10 μM, filled circles), 5-hydroxytryptamine (5-HT, 10 μM, open circles), adenosine (1 μM, filled triangles; 10 μM open triangles), Zn^{2+} (10 μM, filled squares) or Cd^{2+} (1 mM, open squares).

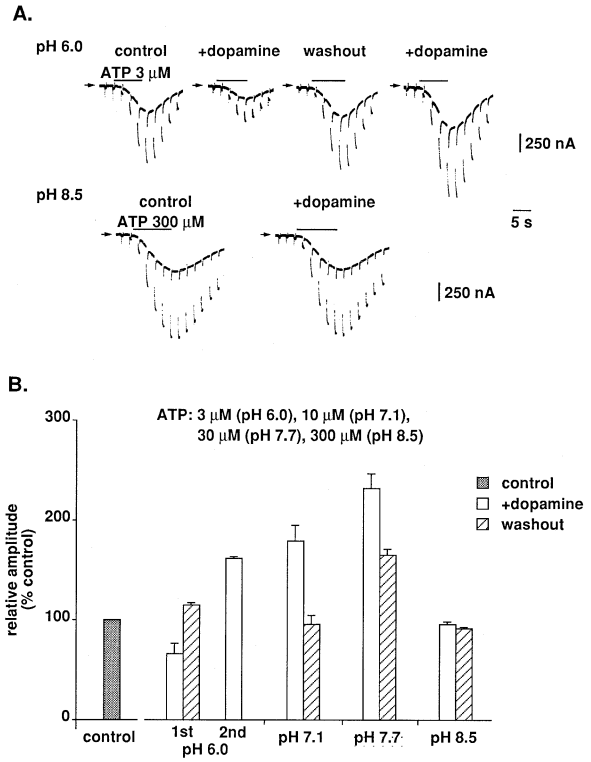

Fig. 3. (A) Effects of dopamine (10 μM) on the ATP-activated current at different pH values. The current was activated by 3 μM ATP at pH 6.0 (upper panels) or by 300 μM ATP at pH 8.5 (lower panels). The recording conditions were the same as in Fig. 2A. (B) Summarized data for the enhancement by dopamine of the ATP-activated current at pH 6.0–8.5. The current was activated by 3 (pH 6.0), 10 (pH 7.1), 30 (pH 7.7) and 300 (pH 8.5), respectively. The normalized current amplitude during the application of dopamine, and that after the washout of these compounds are shown. The data were the mean and S.E. from 5 to 8 oocytes tested.

3.2. The pH-dependence of the current facilitation by neurotransmitters

Fig. 3 compares the effects of 10 μM dopamine on the ATP-activated current at various pH values. Dopamine did not facilitate the current at pH 8.5 (Fig. 3A, lower panel). The effects of dopamine on the current at pH 6.0 were complicated: the current was inhibited by the first application of dopamine (Fig. 3A, upper panel, the second trace), but the current was enhanced by the second application of dopamine (the last trace). It is noted that such inversion of the effect (inhibition to facilitation) was not observed under any other experimental conditions utilized in the present study: inhibition was never observed upon the first trials of the test compounds, and when the current enhancement was obvious upon the first trials of the test compounds, the second trials produced the enhancement comparable to or somewhat smaller (by up to 30%) than

the enhancement by the first trials. Dopamine markedly facilitated the ATP activated current at two neutral pH values (7.1 and 7.7; Fig. 3B).

5-Hydroxytryptamine (10 μM) and adenosine (100 μM), like dopamine, did not facilitate the current at pH 8.5 (Fig. 4). These compounds enhanced the current at other less alkaline pH values tested, and the enhancement was largest at pH 6.0 (Fig. 4).

The current enhancement was less readily restored to the control level when the magnitude of the enhancement was increased, judging from the amplitude of the current activated by successive applications of ATP alone (washout; Figs. 3 and 4). The current, which had been largely enhanced, however, returned to the control level normally within several trials with ATP alone.

3.3. The pH-dependence of the current facilitation by divalent cations

Fig. 5A illustrates the effects of Cd^{2+} on the ATP-activated current at pH 6.0 and 8.5. In contrast to the current facilitation by the neurotransmitters in the previous section (Figs. 3 and 4), Cd^{2+} markedly enhanced the current at pH 8.5, but did not affect the current at pH 6.0 (Fig. 5A). Like Cd^{2+}, the current enhancement by Zn^{2+} was obvious at pH 8.5 and abolished at pH 6.0 (Fig. 5B). At neutral and alkaline pH values (7.1, 7.7 and 8.5), both Zn^{2+} and Cd^{2+} facilitated the ATP-activated current, and the facilitation was most remarkable at pH 8.5 (Fig. 5B and C).

The current enhancement by the divalent cations was less readily restored to the control level when the magnitude of the enhancement was increased, but even the

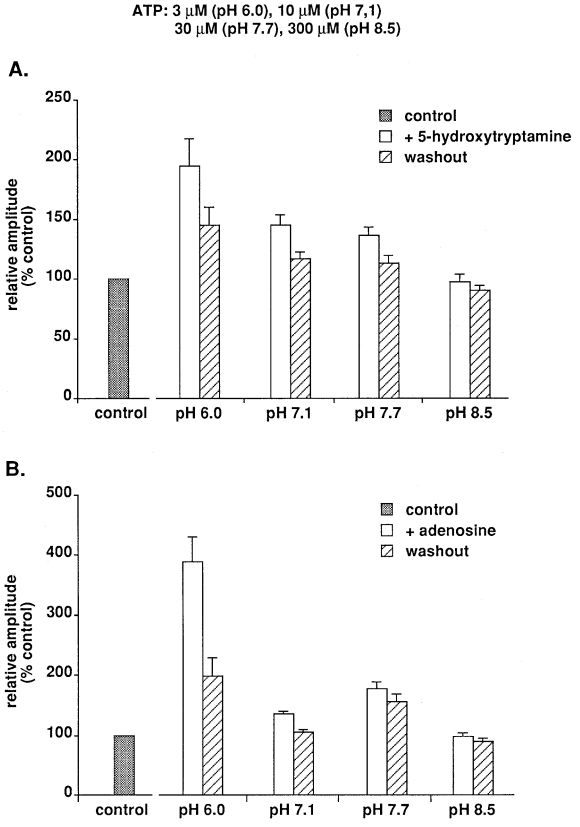

Fig. 4. (A, B) Comparison of the enhancement by 5-hydroxytryptamine (10 μM; A) and adenosine (100 μM; B) of the ATP-activated current at pH 6.0–8.5. The current recording was made and the data are shown as in Fig. 3. The data were the mean and S.E. from 5 to 8 oocytes tested.

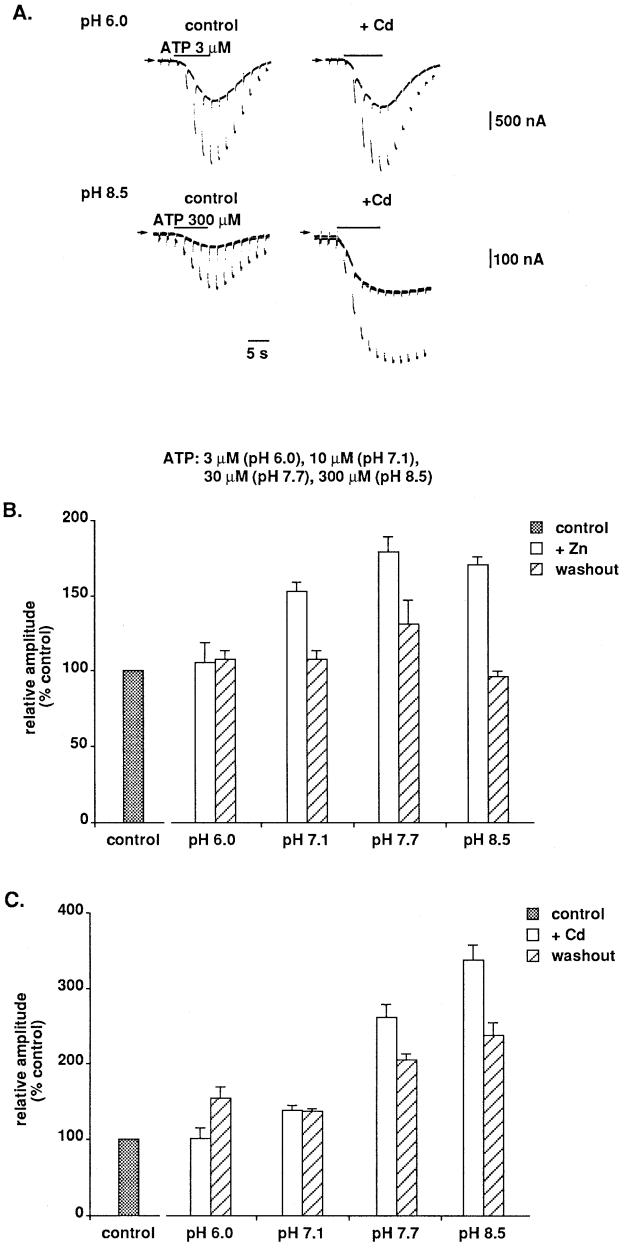

Fig. 5. Effects of Zn^{2+} (10 μM; A) and Cd^{2+} (1 mM; B) on ionic current activated by ATP at different pH values. The current recording was made and the data are shown as in Fig. 3. (A) Effects of Cd^{2+} (1 mM) on the ATP-activated current. The current was activated by 3 μM ATP at pH 6.0 (upper panels) or by 300 μM ATP at pH 8.5 (lower panels). (B, C) Comparison of the enhancement by Zn^{2+} (10 μM; A) and Cd^{2+} (1 mM; B) of the ATP-activated current at pH 6.0–8.5. The data were the mean and S.E. from 5 to 7 oocytes tested.

current which had been largely enhanced returned to the control level normally within several trials with ATP alone, as was the case of the neurotransmitters in the previous section. However, the strong enhancement by 1 mM Cd^{2+} at pH 7.7 and 8.5 remained high even after these trials. It is also noted that the ATP-activated current at pH 6.0 after the washout of Cd^{2+} was larger than the control current or the current in the presence of Cd^{2+} (Fig. 5C).

4. Discussion

We have investigated the pH dependence of the facilitation of $P2X_2$ purinoceptor/channels expressed in *Xenopus* oocytes. Neurotransmitters (dopamine, 5-hydroxytryptamine and adenosine) and divalent cations (Zn^{2+} and Cd^{2+}) enhanced ionic current permeating through these channels at neutral pH when the concentration of ATP was such that it activated a smaller fraction of the channels (Fig. 2). Wildman et al. (1997) reported that dopamine and adenosine enhanced the ionic current through $P2X_2$ purinoceptors expressed in *Xenopus* oocytes, but that this facilitation was due to acidification of extracellular solutions because it disappeared after re-adjustment of pH of the solutions containing these compounds. They also reported that dopamine failed to facilitate the current whether pH was re-adjusted or not. In the present study, we also carefully re-adjusted the pH of extracellular solutions, but the facilitation remained with these three neurotransmitters. There are several small differences between our experimental condition and theirs (for example, they used a lower concentration of ATP (3 μM) and a higher concentration of 5-hydroxytryptamine (100 μM)), but the differences do not seem substantial. We cannot explain this rather puzzling discrepancy at present.

The current facilitation by Zn^{2+} and Cd^{2+} was largely attenuated at acidic pH: the facilitation was smaller at pH 7.1 than at pH 7.7, and was almost abolished at pH 6.0 (Fig. 5). Thus, the facilitation may require deprotonation of $P2X_2$ purinoceptor/channels. Similar attenuation of the facilitation by Zn^{2+} of the ATP-activated current at acidic pH was recently reported in rat nodose ganglion neurons (Li et al., 1996). Among amino acid residues, histidine is the only residue that has a pK_a value between 6.0 and 7.7 (pK_a 6.2 at imidazole ring in polypeptide chain; Creighton, 1984). Some histidine residue may play a key role in the current facilitation by controlling conformational changes in channel protein. In contrast, the facilitation by dopamine, 5-hydroxytryptamine or adenosine was abolished at pH 8.5 (Figs. 3 and 4). No amino acid residue has a pK_a between neutral pH and pH 8.5, but pH 8.5 is rather close to pK_a for the hydroxyl group of cysteine (pK_a 9.1–9.5) or tyrosine (pK_a 9.7) and the amino group side chain of lysine (pK_a 10.4) (Creighton, 1984). Deprotonation may occur at pH values more acidic than the pK_a vlaues of these residues if multiple hydroxyl or amino groups are closely located one another, and these residues, thereby, may contribute to the conformational control responsible for the facilitation by the neurotransmitters. Another possibility is that alkalization resulted in changes in protonation/deprotonation states of the neurotransmitters. The pK_a of alkylamine is about 10 (Ege, 1989), and that of 5-hydroxytryptamine is reported to be 9.8 (Windholz et al., 1976), suggesting that 5-hydroxytryptamine and, presumably, dopamine is protonated and positively charged at neural pH. On the other hand, the pK_a of phenylamine is 4.6 (Ege, 1989), and, thus, the amino group of adenosine may be deprotonated and the compound may be uncharged at neutral pH. These pK_a values indicate that the alkalization adopted in the present study (pH 8.5) may not affect the protonation/deprotonation states of the neurotransmitters as long as special local interactions between the neurotransmitter molecules and the structure of the channel protein surrounding the binding-site are not introduced. Finally, we cannot exclude a possibility of the contribution of amino acid residues with acidic pK_a values, such as histidine, to the attenuation of the current facilitation at alkaline pH because the apparent pK_a of these amino acid residues can be more alkaline if another acidic amino acid residue cooperates with them, as suggested by Prod'hom et al. (1989) for the pH-dependent modulation of L-type Ca^{2+} channels.

We previously reported that neuroamines and divalent cations facilitate ionic current permeating through $P2X_2$ purinoceptor/channels, but not that through $P2X_1$ purinoceptor/channels, and that the current facilitation is largely attenuated when an aspartic acid residue (Asp^{221}) of the $P2X_2$ receptor/channel is replaced by histidine, the amino acid residue that is present in the corresponding position of the $P2X_1$ purinoceptor/channel (Nakazawa and Ohno, 1996, 1997). The attenuation of the current facilitation in the present study at acidic or alkaline pH may not be attributed to direct modulation by pH of Asp^{221} because aspartic acid residues are completely deprotonated in the pH range adopted in the present study (Creighton, 1984). It is, however, possible that Asp^{221} may contribute to the pH-dependent modulation of the current facilitation by cooperating with other amino acid residues as suggested above.

At pH 6.0, dopamine rather inhibited the ATP-activated current upon the first trials of this compound, but facilitated the current upon its second trials (Fig. 3). The results suggest that dopamine exhibits an inhibitory action on $P2X_2$ purinoceptor/channels in addition to the facilitation at acidic pH. As the extracellular pH in tissues can fall markedly (less than 6.0) during ischemia (see Steen et al., 1992), this inhibition by dopamine may counteract the increased sensitivity to ATP in such pathological conditions. Similarly, the disappearance of the facilitation by Zn^{2+}, an endogenously occurring divalent cation in the nervous system (Frederickson, 1989), at acidic pH (Fig.

5B), may be relevant when the sensitivity of $P2X_2$ purinoceptor/channels to ATP is increased.

In conclusion, the present study clearly demonstrated that dopamine, 5-hydroxytryptamine, adenosine, Zn^{2+} and Cd^{2+} enhance ionic current permeating through $P2X_2$ purinoceptor/channels under the condition where the slight acidification of external solutions induced by these compounds was compensated. The current facilitation by the neurotransmitters and that by the divalent cations exhibited rather opposite pH-dependent profiles, which suggests that different amino acid residues determine appropriate conformation for these two types of the channel facilitation.

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