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Potentiation by cadmium ion of ATP-evoked dopamine release in rat phaeochromocytoma cells

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1 The effects of cadmium ion (Cd^{2+}) on release of dopamine and on an inward current evoked by extracellular ATP were investigated in rat phaeochromocytoma PC12 cells.

2 Cd^{2+} (100 μM - 3 mM) potentiated the dopamine release evoked by 30 μM ATP from the cells. Cd^{2+} (100 μM) shifted the concentration-response curve of ATP-evoked dopamine release to the left without affecting the maximal response.

3 Suramin (30 μM) completely abolished the dopamine release evoked by 30 μM ATP but only partially inhibited the release evoked by 100 μM ATP consistent with its role as a competitive antagonist. The response evoked by 30 μM ATP in the presence of Cd^{2+} (300 μM) was comparable to that observed with 100 μM ATP alone; however, only the former was almost completely inhibited by suramin.

4 Cd^{2+} (100 μM) potentiated an inward current activated by 30 μM ATP alone. A higher concentration of Cd^{2+} (300 μM) had a smaller effect on amplitude potentiation but significantly prolonged the duration of the current.

5 The time-course of the ATP-evoked dopamine release was investigated using a real-time monitoring system for dopamine release. Although Cd^{2+} (300 μM) had little effect on the time-course of activation the ATP-evoked dopamine release, it produced a long-lasting dopamine release which slowly returned to the baseline.

6 Taken together, these observations suggest that Cd^{2+} enhances ATP-evoked dopamine release by affecting P_2-purinoceptor/channels. The enhancement may be attributed to a Cd^{2+}-dependent increase in sensitivity to ATP.

Keywords: Cadmium ion; ATP; purinergic transmission; dopamine release; ATP-activated current; PC12 cells

Introduction

Adenosine 5'-triphosphate (ATP) has joined the growing list of compounds shown to function as neurotransmitters in various tissues, including smooth muscle (Burnstock & Kennedy, 1985), peripheral neurones (Bean & Friel, 1990; Evans *et al.*, 1992) and the central nervous system (Edwards *et al.*, 1992; Inoue *et al.*, 1992a). Several cDNAs encoding receptors for ATP, P_2-purinoceptors, have been cloned (Lustig *et al.*, 1993; Webb *et al.*, 1993; Brake *et al.*, 1994; Valera *et al.*, 1994). We have previously characterized ion channels activated by ATP in PC12 cells, a cell line derived from a rat phaeochromocytoma (Greene & Tischler, 1976), and demonstrated that these cells are suitable for the study of these channels and of secretion triggered by the channel activation (Inoue *et al.*, 1989; Inoue & Nakazawa, 1992; Nakazawa & Inoue, 1992). The ATP-activated channels are non-selective cation channels coupled with P_2-purinoceptors (P_2-purinoceptor/channel; 'P_{2X}-purinoceptor'). ATP stimulation of the channels leads to a rise in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) and the resultant secretion of catecholamine (Inoue *et al.*, 1989; Inoue & Nakazawa, 1992). Unlike cellular responses evoked by high concentrations of KCl, which are believed to result from activation of voltage-gated Ca^{2+} channels (VGCC), the ATP-evoked responses in PC12 cells are mediated almost exclusively by Ca^{2+}-influx through P_2-purinoceptor/channels, with a negligible contribution by VGCC (Inoue & Nakazawa, 1992; Nakazawa & Inoue, 1992).

Modulations by endogenous substances, including neurotransmitters, of the ATP-evoked responses have been reported. Dopamine and its related compounds (Inoue *et al.*, 1992b; Nakazawa *et al.*, 1993), adenosine (Inoue *et al.*, 1994; Koizumi *et al.*, 1994) and 5-hydroxytryptamine (Nakazawa *et al.*, 1994; Koizumi *et al.*, 1995a) enhance an inward current and increase in $[Ca^{2+}]_i$) and dopamine release evoked by ATP in PC12 cells. Various type of divalent cations, including Ca^{2+} and Mg^{2+}, are known to block the ATP-activated current in smooth muscle cells (Honoré *et al.*, 1989) and PC12 cells (Nakazawa *et al.*, 1990; Nakazawa & Hess, 1993). Zn^{2+}, however, exerts a unique effect on ATP-activated channels: it potentiates the ATP-activated current in rat superior cervical ganglion (Cloues *et al.*, 1993), nodose and celiac ganglia neurones (Li *et al.*, 1993) and PC12 cells (Koizumi *et al.*, 1995b). Cd^{2+} is well known to be a potent VGCC blocker (Nowycky *et al.*, 1985), and is often used in various types of tissues or cells including neurones to block VGCCs. However the effects of Cd^{2+} on P_2-purinoceptor/channels or related responses have scarcely been reported except that Cd^{2+} reduces single channel current amplitude of these channels (Nakazawa & Hess, 1993). The present study was designed to investigate the effects of Cd^{2+} on the responses mediated by P_2-purinoceptor/channels in PC12 cells. We demonstrate that Cd^{2+} potentiates the efficacy of P_2-purinoceptor/channels, leading to enhancement of ATP-evoked dopamine release from the cells.

Methods

Cell culture

Culture conditions of PC12 cells were as described previously (Inoue & Kenimer, 1988; Koizumi *et al.*, 1995b). All experiments described in this paper were performed with cells at passage number 53-68. Cells were plated onto collagen-coated 35 mm polystyrene dishes (1×10^6 cells/dish) for 2 days in a humidified atmosphere of 90% air and 10% CO_2 at 37°C.

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Dopamine release

The procedures for the measurement of released dopamine were basically the same as those described by Koizumi *et al.* (1995b). Cells were stimulated by various concentrations of ATP with and without Cd^{2+} dissolved in a balanced salt solution (BSS) with the following composition (mM: NaCl 150, KCl 5.0, $CaCl_2$ 1.8, $MgCl_2$ 1.2, D-glucose 10 and N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid (HEPES) 25 (pH adjusted to 7.4 with NaOH) for 1 min at room temperature. For the Ca^{2+}-depleted experiments, the dishes were washed twice with nominally Ca^{2+}-free BSS for 1 min before ATP application. The amount of dopamine released to superfusate and that remaining in the cells were determined by a high performance liquid chromatography (h.p.l.c.) coupled with an electrochemical detector (e.c.d.) (LC-4B, Bioanalytical systems, West Lafayette, U.S.A.). The percentage of release was calculated by dividing supernatant values by the sum of supernatant and pellet values.

Membrane current measurements

The dishes containing the cultured cells were mounted on an inverted microscope (Olympus IMT-2, Japan) and the cells were superfused with extracellular solution containing (mM): NaCl 140, KCl 5.4, $CaCl_2$ 1.8, $MgCl_2$ 1.0, HEPES 10 and glucose 11.1 (pH was adjusted to 7.4 with NaOH). Current recordings were made with conventional whole-cell configuration of patch-clamp methods (Hamill *et al.*, 1981). Patch pipettes were filled with intracellular solution containing (mM): CsCl 150, HEPES 10, EGTA 5 (pH 7.3 with CsOH). Experiments were performed at room temperature (about 25°C). Data were filtered at 1 kHz and stored on magnetic tape for later analysis.

Real-time monitoring of dopamine release

An analysis of the kinetics of the dopamine release evoked by ATP was performed with a real-time monitoring system of catecholamine release as previously described (Kumakura *et al.*, 1986; Koizumi *et al.*, 1990; 1992). The monitoring system is funadmentally composed of a cell perfusion component and an e.c.d. monitoring component. In brief, PC12 cells (2×10^7 cells) sandwiched between membrane filters (pore size, 3 μm, Nuclepore, CA, U.S.A.) were placed in a chamber (250 μl inner volume) and perfused with BSS at a flow rate of 1 ml min^{-1} at room temperature (about 20°C). The perfusate from the cells was directly introduced into the ECD monitoring component (LC-4B, Bioanalytical systems), using an electrode potential setting of +0.45 V versus Ag/AgCl reference electrode to detect oxidizable substances released from the cells. The cells were first perfused with BSS for 30 min and then stimulated with a 30 s pulse of 250 μl of ATP in BSS. They were then perfused for an additional 20 min with BSS followed by a second 30 s pulse of ATP solution. The response evoked by the first and the second ATP application are defined in the text as S1 and S2, respectively. Various concentrations of Cd^{2+} were applied to the cells during the second ATP application. In several experiments, the perfusate from the cells was collected and the released dopamine was determined by h.p.l.c.-e.c.d. Using these values for calibration, the concentration of dopamine was calculated from the current-time curves obtained from the e.c.d.- monitoring component.

Drugs

Drugs and chemicals were obtained from the following sources: adenosine 5'-triphosphate disodium salt (ATP) and cadmium chloride were from Yamasa Co. (Choshi, Japan) and Sigma (MO, U.S.A.), respectively. HEPES was from Dojin, (Kumamoto, Japan). Suramin monosodium salt was from Bayer (Leverkusen, Germany). Other drugs were purchased from Wako Pure Chemical (Osaka, Japan).

Statistics

All the data were given as mean ± s.e.mean. Statistical differences in values for dopamine release and inward currents were determined by analysis of variance and Dunnett's test for multiple comparisons.

Results

Figure 1 shows the effect of various concentrations of Cd^{2+} on the release of dopamine evoked by 30 μM ATP. Cd^{2+} alone had no effect on the spontaneous dopamine release from PC12 cells; however, it potentiated the ATP-induced dopamine release in a concentration-dependent manner from 100 μM to 3 mM. The ATP-evoked response in the presence of 3 mM Cd^{2+} reached about 400% of the response seen with ATP alone.

Figure 2 shows the effect of Cd^{2+} (100 μM) on the ATP concentration-dependence curve of ATP-evoked dopamine release from the cells. Cd^{2+} (100 μM) shifted the ATP response curve to the left but did not change the maximal response to ATP.

The ATP-evoked dopamine release, both in the presence and absence of Cd^{2+}, was abolished when extracellular Ca^{2+} was removed (Figure 3). Suramin (30 μM) also almost completely abolished the dopamine release evoked by 30 μM ATP both in the presence and absence of Cd^{2+}, however it only partially attenuated the response to 100 μM ATP alone (Figure 3).

Figure 4 shows the effect of Cd^{2+} on the inward current activated by ATP. Cd^{2+} (100 or 300 μM) potentiated the current induced by 30 μM ATP (Figure 4a).

The magnitude of the potentiation of the peak current amplitude was smaller with 300 μM Cd^{2+} than with 100 μM Cd^{2+} (Figure 4b); however, with 300 μM Cd^{2+}, the duration of the current was prolonged. The current trace obtained in the presence of 30 μM ATP without Cd^{2+} was expanded and superimposed on that observed with 300 μM Cd^{2+} (Figure 4c). Cd^{2+} accelerated the activation of the current upon the application of ATP and delayed its deactivation upon removal of ATP.

We also investigated the effects of Cd^{2+} on ATP-evoked dopamine release using a real-time monitoring system for dopamine release. We collected the superfusates corresponding to the current-time curves obtained by this system and measured the amount of dopamine by h.p.l.c.-e.c.d. We found high correlation between the area of the current-time curve and the

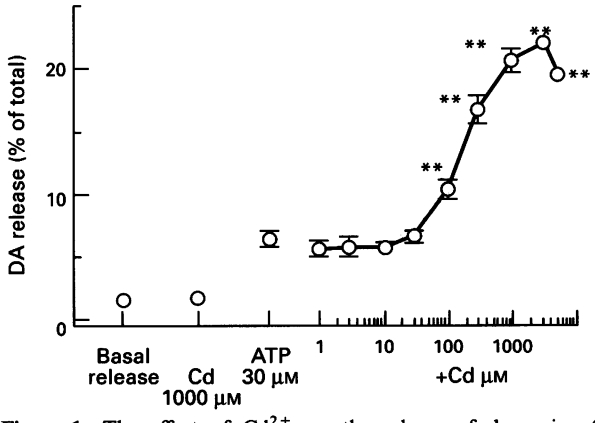

Figure 1 The effect of Cd^{2+} on the release of dopamine (DA) evoked by 30 μM ATP from PC12 cells. The amount of released dopamine was expressed as a percentage of total cellular content. These are results from a typical experiment with each data point being mean ± s.e.mean of triplicate measurements. At least two such experiments were performed. Asterisks show a significant difference from the response evoked by 30 μM ATP alone (**$P<0.01$).

amount of released dopamine either in the absence and presence of Cd^{2+} ($n=7$; $r^2=0.994$). Thus, we regarded the current-time curves as the time-courses of dopamine release. Typical current-time curves upon stimulation by ATP with and without Cd^{2+} are shown in Figure 5. ATP was applied to the cells twice for 30 s, with the second application 20 min after the first, with and without Cd^{2+}. Figure 5a shows the current-time curves of dopamine release evoked by ATP alone. With 30 μM ATP a slightly smaller response was observed on the second application whereas 100 μM ATP induced an obviously larger second response (Figure 5a, upper traces). Cd^{2+} sig-

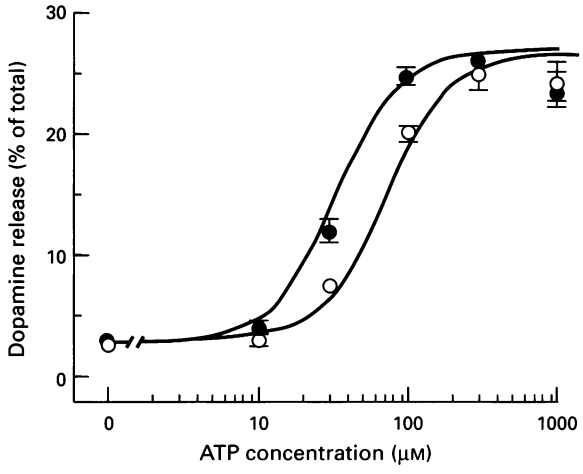

Figure 2 The effect of Cd^{2+} (100 μM) on the concentration-response curve of ATP-evoked dopamine release. The amount of released dopamine was expressed as a percentage of total cellular content. These are results from a typical experiment with each data point being mean±s.e.mean of triplicate measurements. Two such experiments were performed: ● and (○) show the response evoked by ATP with and without Cd^{2+}, respectively.

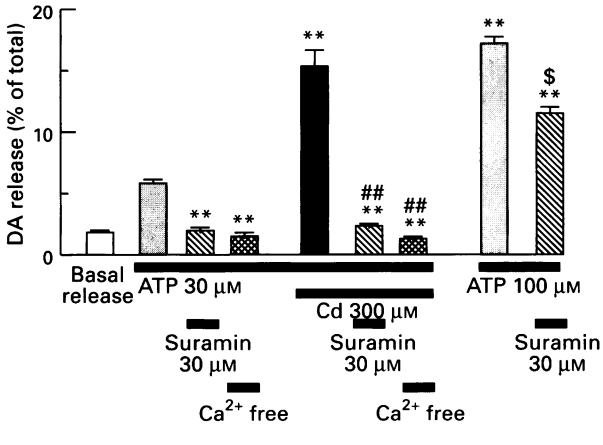

Figure 3 The effects of suramin (30 μM) on the ATP-evoked dopamine (DA) release with and without Cd^{2+} (300 μM). The amount of dopamine was expressed as a percentage of total cellular content. The open column shows spontaneous dopamine release. Dotted and closed columns show the dopamine release evoked by ATP alone (30 and 100 μM) and the release evoked by 30 μM ATP with 300 μM Cd^{2+}, respectively. Hatched and cross-hatched columns show the evoked responses with 30 μM suramin and those under Ca^{2+} -depleted conditions. Data are mean±s.e.mean ($n=3$). Symbols show a significant difference from the response evoked by 30 μM ATP alone (**$P<0.01$), that from the response evoked by 30 μM ATP with 300 μM Cd^{2+} (##$P<0.01$) and that from the response evoked by 100 μM ATP alone ($P<0.05$), respectively.

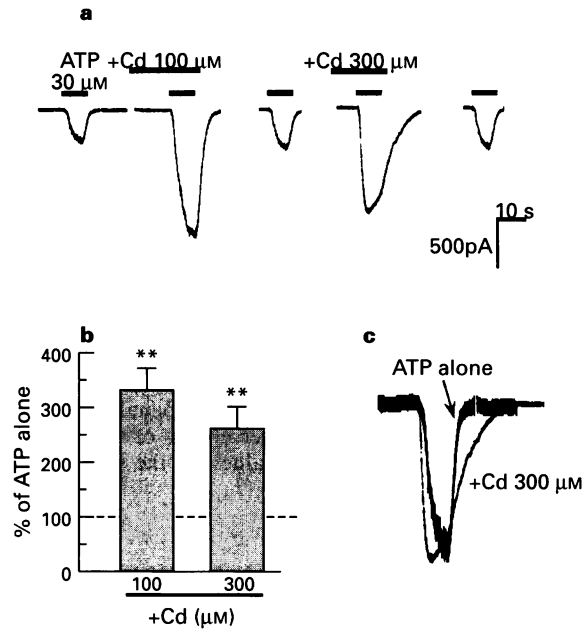

Figure 4 Potentiation by Cd^{2+} of the ATP-activated current. (a) Current responses obtained from a PC12 cell. Holding potential was −60 mV. The currents show the responses evoked by the 30 μM ATP with and without Cd^{2+} (100 and 300 μM). (b) Comparison of the ATP (30 μM)-evoked currents augmented by 100 μM and 300 μM Cd^{2+}. Data were obtained from 5 cells tested. Symbols show a significant difference from the response activated by 30 μM ATP alone (**$P<0.01$). (c) Time-course of the ATP (30 μM)-activated current in the absence and presence of 300 μM Cd^{2+}. The current traces obtained from (a) were expanded and superimposed.

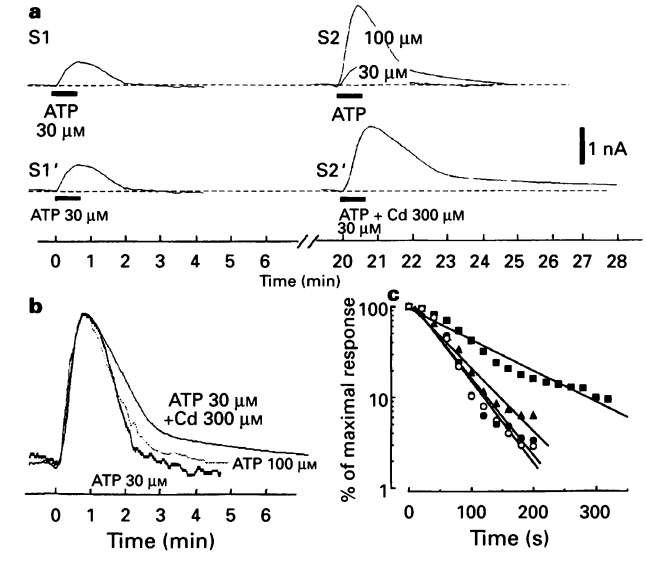

Figure 5 The time-course of ATP-evoked dopamine release obtained with a real-time monitoring system, and the effects of Cd^{2+} on it. Data shown are representative of at least three experiments. (a) The upper traces show the representative time-course of dopamine release evoked by ATP (30 and 100 μM). The lower traces show the time-course of the release evoked by 30 μM ATP with (right) and without (left) 300 μM Cd^{2+}. The cells were exposed to the stimuli twice and each stimulation was separated by 20 min. Horizontal bars show ATP-application (30 s). (b) Comparison of kinetics of ATP-evoked dopamine release with or without 300 μM Cd^{2+}. The traces obtained from (a) were expanded and superimposed. (c) Logarithmic plot of decay time-course of the dopamine release evoked by 30 μM ATP (○ and ●), 100 μM ATP (▲) and 30 μM ATP with 300 μM Cd^{2+} (■). Closed symbols show the responses evoked by the second ATP-application (S2) in various conditions 20 min after the first ATP-application (30 μM); (○) show the response evoked by the first ATP-stimulation (S1). The data were obtained as shown in (a) and (b).

nificantly potentiated dopamine release evoked by 30 μM ATP (Figure 5a, lower traces). When the concentration of ATP was raised to 100 μM, Cd^{2+} only slightly augmented the dopamine release (not shown). Figure 5b shows the comparison of kinetics of ATP-evoked dopamine release with and without 300 μM Cd^{2+}. The traces of current-time curves evoked by 30 μM ATP with and without 300 μM Cd^{2+} or 100 μM ATP alone were superimposed. The time-course of the dopamine release evoked by 30 μM ATP was similar to that evoked by 100 μM ATP. The time-course of the response with Cd^{2+}, however, was different from that without Cd^{2+}. Although the activation time-course was not affected by Cd^{2+}, it did produce a long-lasting response which slowly decreased to the baseline. Figure 5c shows a logarithmic plot of the deactivation time-course of dopamine release. The time constant with 30 μM ATP was increased by 300 μM Cd^{2+} from 48.3 to 126.7 s.

Discussion

We have demonstrated here that Cd^{2+} dramatically potentiated the release of dopamine evoked by ATP in PC12 cells. This enhancement may result from facilitation of the ATP-gated channels because Cd^{2+} also enhanced ATP-activated currents in the cells (Figure 4). Cd^{2+} by itself stimulates inositol 1,4,5-trisphosphate (IP_3) formation and Ca^{2+} mobilization in human skin fibroblasts (Smith *et al.*, 1989), which raises a possibility that Cd^{2+} causes IP_3 generation followed by Ca^{2+} mobilization and leads to dopamine release, resulting in apparent enhancement of the ATP-evoked dopamine release. However, stimulation by ATP plus 300 μM Cd^{2+} failed to evoke dopamine release in the absence of extracellular Ca^{2+} (Figure 2), suggesting that the dopamine release totally depends on external Ca^{2+}. Thus, even if Cd^{2+} causes Ca^{2+} mobilization, it may not contribute to the release.

Cd^{2+}, as well as other divalent cations such as Zn^{2+}, Ni^{2+} and Co^{2+}, is known to inhibit various types of channels, namely, voltage-gated sodium, potassium and calcium channels, and GABA-, N-methyl-D-aspartate- and 5-hydroxytryptamine-gated channels (see Kiss & Osipenko, 1994 for reviews). The enhancement by Cd^{2+} may be characteristic of the ATP-gated channels. The effects of some other divalent cations on the P_2-purinoceptor/channels have already been reported. Ca^{2+} and Mg^{2+} block the ATP-activated current in smooth muscle cells (Honoré *et al.*, 1989) and PC12 cells (Nakazawa *et al.*, 1990). In contrast, Zn^{2+} potentiates the ATP-activated current in rat superior cervical ganglion (Cloues *et al.*, 1993), nodose and coeliac ganglia neurones (Li *et al.*, 1993) and PC12 cells (Koizumi *et al.*, 1995b). Ni^{2+} also enhanced the ATP-activated current in rat superior cervical ganglion (Cloues *et al.*, 1993). Thus, Cd^{2+} is similar to Zn^{2+} and Ni^{2+} with respect to the facilitatory action on the P_2-purinoceptor/channels, and the action may arise from the same mechanisms. Cd^{2+} may decrease the threshold for activation of the P_2-purinoceptor/channels and enhance their activity, as suggested for the enhancement by Zn^{2+} (Cloues *et al.*, 1993; Li *et al.*, 1993). The finding that Cd^{2+} shifted the concentration-response curve of the ATP-evoked dopamine release to the left without affecting the maximal response to ATP supports this idea (Figures 1, 2). Cd^{2+} may facilitate the binding of ATP molecules to the P_2-purinoceptor/channels, but may not increase the number of functional P_2-purinoceptor/channels. The affinity of suramin, a competitive antagonist at P_2-purinoceptors, may also be increased by Cd^{2+}. Suramin abolished the dopamine release evoked by 30 μM ATP with 300 μM Cd^{2+} whereas it only partially inhibited the release evoked by 100 μM ATP alone, in spite of the fact that the dopamine release in the absence of suramin was comparable under these two conditions (Figure 3). The result implies that Cd^{2+} does not selectively increase the affinity of the binding-site for ATP without increasing the affinity for suramin because, if this is the case, the fraction inhibited by suramin should have been the same in the two cases. Cd^{2+} may facilitate association of both agonists and antagonists to P_2-purinoceptors, as has already been suggested for the potentiation by Zn^{2+} of ATP-evoked responses in PC12 cells (Koizumi *et al.*, 1995b).

In addition to its augmentation of the amplitude of the ATP-activated current, Cd^{2+} (300 μM) accelerated the activation of the current upon the ATP-application and delayed deactivation of the current upon the removal of ATP (Figure 4). In addition, the peak amplitude of ATP-activated current with 300 μM Cd^{2+} was smaller than that with 100 μM Cd^{2+}. Cloues *et al.* (1993) have already reported similar modulations by Zn^{2+} of an ATP-activated current in rat superior cervical ganglion: lower concentrations of Zn^{2+} enhance the amplitude of the current activated by ATP whereas higher concentrations of Zn^{2+} inhibit its amplitude and concomitantly increase the duration of the current. It is likely that Cd^{2+} at higher concentrations inhibits the channels by a secondary mechanism. Reduction by Cd^{2+} of single channel conductance of the ATP-activated channels (Nakazawa & Hess, 1993) may account for this secondary inhibition of the macroscopic current. As for the changes in kinetics, increased affinity for ATP may account for them, but other possibilities including modulations of open/close behaviours of the channels cannot be excluded. Although Cd^{2+} potentiated the ATP-evoked dopamine release in a concentration-dependent manner over a concentration-range from 100 μM to 3 mM, the amplitude of ATP-activated current with 300 μM Cd^{2+} was smaller than that with 100 μM Cd^{2+} (see Figures 1 and 4). This discrepancy may be attributed to a sustained current with 300 μM Cd^{2+} compared with that with 100 μM Cd^{2+}. The sustained current in the presence of 300 μM Cd^{2+} may result in an increase of total Ca^{2+}-influx during stimulation, leading to further enhancement of the ATP-evoked release.

The time-course of the dopamine release evoked by 30 μM ATP, determined by a real-time monitoring system for dopamine release, was similar to that seen with 100 μM ATP though the latter was larger than the former with respect to their maximal responses (Figure 5). The maximal amplitude of dopamine release evoked by 100 μM ATP alone was roughly the same as that evoked by 30 μM ATP with 300 μM Cd^{2+}, but the time-course was obviously different: Cd^{2+} significantly delayed decay kinetics of the dopamine release evoked by ATP alone (Figure 5b, c). The delayed decay may have arisen from the delayed deactivation of the ATP-activated current (Figure 4).

Cadmium is one of the most toxic metals and has been shown to induce a variety of toxic effects (Waalkes *et al.*, 1992). The cadmium-induced intoxication in man, is generally thought to be a result of long-term oral or inhalation exposure due to cadmium in the drinking water, food or the occupational environment (Elinder & Nordberg, 1985). Although the concentration of cadmium is high in kidney and liver in human subjects (Chaube *et al.*, 1973), orally administered cadmium has been reported to be highly accumulated in the brain in rat (Murphy *et al.*, 1992). It is well-known that Cd^{2+} competes with Ca^{2+} both at VGCCs and at intracellular various Ca^{2+} binding proteins (Tsien, 1983; Miller, 1987; Nelson, 1986). If the concentration of Cd^{2+} reaches the level necessary to potentiate the ATP-evoked responses as shown in this study, it is possible that, the enhancement of ATP-evoked responses may also contribute to its toxicity though a more definitive conclusion must await further investigations.

Taken together, we have demonstrated here that, unlike other channels, the P_2-purinoceptor/channels in PC12 cells are potently facilitated by Cd^{2+}. This observation calls our attention to the use of Cd^{2+} as a pharmacological tool to discriminate the ATP-evoked responses from other responses.

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