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Potentiation by adenosine of ATP-evoked dopamine release via a pertussis toxin-sensitive mechanism in rat phaeochromocytoma PC12 cells

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1 The effects of adenosine on adenosine 5'-triphosphate (ATP)-evoked dopamine release from rat phaeochromocytoma PC12 cells was investigated to determine whether adenosine exerts a regulatory effect on the ATP-evoked response. Adenosine potentiated ATP (30 μM)-evoked dopamine release in a concentration-dependent manner over a concentration-range of 1 to 100 μM. Adenosine (100 μM) shifted the concentration-dependence of the ATP-evoked response to the left without affecting the maximal response.

2 Aminophylline, a non-selective adenosine receptor antagonist, and CP66713, a selective antagonist at the A_2 subclass of adenosine receptors, abolished the adenosine-induced potentiation. Furthermore, 8-cyclopentyltheophylline, a selective antagonist at the adenosine A_1 receptor partially inhibited the adenosine-evoked potentiation. CGS22492, a selective A_2 receptor agonist, potentiated ATP-evoked dopamine release whereas N^6-cyclohexyladenosine (CHA), a selective A_1 receptor agonist, had no effect.

3 Pertussis toxin (PTX), a bacterial exotoxin which catalyzes the ADP-ribosylation of guanosine 5'-triphosphate (GTP)-binding proteins (G-proteins), inhibited the adenosine-induced potentiation of dopamine release. Dibutyryl cyclic AMP (db cyclic AMP), an analogue of cyclic AMP, had no effect on the release on the ATP-evoked response.

4 Adenosine potentiated the ATP-evoked rise in intracellular Ca^{2+} concentration ($[Ca]_i$) in PC12 cells. This potentiation was also observed with CGS 22492 but not with CHA. PTX completely inhibited the adenosine-induced potentiation of the rise in $[Ca]_i$.

5 On the basis of these findings, we suggest that the adenosine-induced potentiation of ATP-evoked dopamine release was due to an increase in $[Ca]_i$ in the cells. Although the potentiation is most likely mediated by a subclass of A_2 receptors, the subclass may be different from those previously reported since the potentiation was sensitive to PTX and was not reproduced by db cyclic AMP.

Keywords: ATP; adenosine; PC12 cells; dopamine release; intracellular Ca^{2+} concentration; pertussis toxin

Introduction

Adenosine 5'-triphosphate (ATP) has a functional role as a neurotransmitter or modulator in smooth muscle (Burnstock & Kennedy, 1985) and in neural tissues (Bean, 1990; Evans, 1992), including the central nervous system (Inoue & Nakazawa, 1992; Edwards *et al.*, 1992; Edwards & Gibb, 1993). ATP activates non-selective cation channels (Bean, 1992), leading to muscle contraction (Bean, 1992) and catecholamine secretion (Inoue *et al.*, 1989; Nakazawa & Inoue, 1992). We previously characterized the ATP-activated channels in PC12 cells, a cell line derived from a rat phaeochromocytoma (Green & Tischler, 1975), and demonstrated that these cells are suitable for the study of the channels and secretion triggered by channel activation (Inoue *et al.*, 1989; Inoue & Nakazawa, 1992; Nakazawa & Inoue, 1992).

Few previous reports have dealt with the modulatory effects of endogenous substances on the ATP-evoked effects. Agents acting on dopamine receptors were shown to potentiate ATP-evoked currents in PC12 cells (Inoue & Nakazawa, 1992; Nakazawa *et al.*, 1993). ATP-activated phosphatidyl inositide breakdown and arachidonate release are reported to be affected by adenosine, a product of ATP metabolism, in rat thyroid FRTL-5 cells (Okajima *et al.*, 1989; Nazarea *et al.*, 1991). ATP and adenosine act synergistically to mobilize intracellular Ca^{2+} via the formation of inositol 1,4,5-trisphosphate in a smooth muscle cell line (Gerwins & Fredholm, 1992). Adenosine has also been proposed as a neuromodulator or neurotransmitter in various neuronal cells or tissues, with mainly inhibitory action (Phillis *et al.*, 1975; Okada & Kuroda, 1980). PC12 cells have been reported to have functional adenosine receptors (Hide *et al.*, 1991), raising the possibility that adenosine can modulate ATP-evoked responses in PC12 cells. We have therefore examined the effects of adenosine on ATP-evoked dopamine release and the increase in intracellular Ca^{2+} concentration ($[Ca]_i$) in the cells.

In the present study, we demonstrate that adenosine increases ATP-evoked dopamine release by potentiating the $[Ca]_i$ rise in the cells. This potentiation is achieved via a pertussis toxin-sensitive and adenosine 3':5'-cyclic monophosphate (cyclic AMP)-independent mechanism.

Methods

Cell culture

PC12 cells were the kind gift of Dr Terry Rogers (Johns Hopkins University School of Medicine, Baltimore, MD, U.S.A.). Culture conditions were as described previously (Inoue & Kenimer, 1988). In brief, the cells were received in our laboratory at passage 46 and were expanded, and a seed lot was frozen at passage 47 and stored in liquid nitrogen. All experiments described in this manuscript were performed with cells at passage number between 53 and 68. Cells were cultured in 75 cm^2 flask in Dulbecco's Modified Eagle's Medium containing 7% foetal bovine serum (GIBCO, NY, U.S.A.), 7% heat-inactivated (56°C, 40 min) horse serum (Cell culture Laboratories, Ohio, U.S.A.), 2 mM L-glutamine (M.A. Bioproducts, MD, U.S.A.), and 50 μg ml^{-1} gentamicin

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sulphate (Boehringer Mannheim GmbH, Germany) in a humidified atmosphere of 90% air and 10% CO_2 at 37°C. Cells were removed from flasks for subculture and for plating into assay dishes using a Ca^{2+}/Mg^{2+} -free solution containing (mM): NaCl 172, KCl 5.4, NaH_2PO_4 1 and glucose 5.6, pH 7.4. Afer about 5 min in this solution, the cells were detached by gently tapping the side of the flask. The cells were removed, plated onto collagen-coated 35 mm polystyrene dishes (1×10^6 cells/dish) and used 2 days later. For measurement of $[Ca]_i$ in single cells, cells were plated onto poly-L-lysine (Sigma, MO, U.S.A.)-coated glass coverslips ($24 \times 60 \times 0.15$ mm, Flexiperm, W.C. Haraeus GmbH, Hanau, Germany) at a density of 2.5×10^5 cells per well (8×11 mm), and cultured for an additional 2 days.

Dopamine release

Released dopamine was measured as described by Ohara-Imaizumi *et al.* (1991). All the procedures including incubation, washing, and drug application, were made using 1 ml/dish of a balanced salt solution (BSS) with the following composition (mM): NaCl 150, KCl 5.0, $CaCl_2$ 1.2, $MgCl_2$ 1.2, NaH_2PO_4 1.2, D-glucose 10, ethylenediaminetetraacetic acid (EDTA) 0.1 and N-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid (HEPES) 25, pH adjusted to 7.4 with NaOH. Dishes were washed twice and incubated with BSS for 1 h at room temperature. After washing once with BSS, ATP was added to the dishes and incubated for 1 min. Agonists and antagonists to adenosine receptors were added to the medium 1 min before and during ATP application. At the end of the incubation period, the solutions were transferred immediately to sample cups containing 0.25 ml of 1 N $HClO_4$ for measurement of dopamine released into the solution. The cellular dopamine was extracted with 0.2 N $HClO_4$ by sonication in the dish. After centrifugation (at 4°C for 2 min, 1000 *g*), supernatants of both the incubation solutions and the sonicated cellular solutions were collected for measurement of dopamine content. Dopamine content was determined with a high performance liquid chromatography-electrochemical detection system (Bioanalytical systems, West Lafayette, IN, U.S.A.). The percentage of release was calculated using the values obtained for the dopamine content in the incubation solution (A) and the dopamine content remaining in the cells (B) using the following equation: % of total dopamine = $100 \times A/(A + B)$.

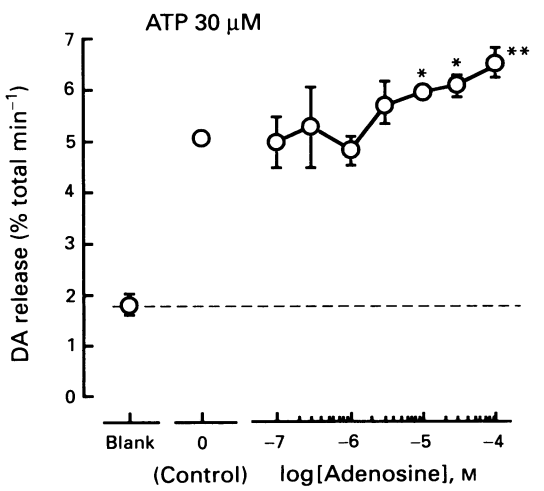

Figure 1 Effect of adenosine on ATP (30 μM)-evoked dopamine (DA) release from PC12 cells. Adenosine was present 1 min before and during ATP application. Values represent the released dopamine (% of total content per min) of 2 separate experiments ($n = 6$). Broken line indicates spontaneous release (1.8 ± 0.11% of total content per min). Data are mean ± s.e.mean. Asterisks indicate significant differences from the response by ATP alone (control): $*P < 0.05$; $**P < 0.01$.

$[Ca]_i$ concentration

The increase in $[Ca]_i$ in single cells was measured by the fura-2 method as described by Grynkiewicz *et al.* (1985) with minor modifications. All the procedures including incubation, washing, and drug application, were made using balanced salt solution (BSS). The cells were washed with BSS and incubated with 10 μM fura-2 acetoxymethylester at 37°C in BSS. After 30 min incubation, the cells were washed with 0.2 mM of BSS. The coverslips were mounted on an Olympus IMT-2 inverted epifluorescence microscope equipped with a 75 W xenon-lamp and band-pass filters of 340 nm wavelength, for measurement of the Ca^{2+}-dependent signal, and 360 nm wavelength, for measurement of the Ca^{2+}-independent signal. Image data, recorded by a high-sensitivity silicon intensifier target camera (C-2741-08, Hamamatsu Photonics, Co., Hamamatsu, Japan) were processed by a personal computer. Fluorescence ratio images were obtained and calibrated as described elsewhere (Miyakawa *et al.*, 1989). The cells were treated twice with ATP, and the second treatment was made with drugs. The ratio of the second response over the first response (S2/S1) was calculated and adopted as an index to compare the data among different cells.

Pretreatment with PTX

Pretreatment of PC12 cells with PTX was performed as described previously (Inoue & Kenimer, 1988). PTX was added to the conditioned medium at a final concentration of 2 ng ml^{-1}, and the cells were incubated for 20 h at 37°C. With this procedure, a 41-kDa PTX-sensitive G-protein, was almost completely ADP-ribosylated in PC12 cells (Inoue & Kenimer, 1988).

Drugs

Drugs and chemicals were obtained from the following sources: ATP (Yamasa Co., Choshi, Japan), adenosine and N^6-cyclohexyladenosine (CHA; A_1 agonist) were from Sigma (MO, U.S.A.). 8-cyclopentyl-1,3-dimethylxanthine (8-CPT; A_1 antagonist) (Research Biochemicals Inc., MA, U.S.A.), CP66713 (A_2 antagonist) (Pfizer, CT, U.S.A.), EGTA, HEPES and fura-2AM (Dojin, Kumamoto, Japan). Other drugs were purchased from Hayashi Pure Chemical (Tokyo, Japan).

Statistics

Statistical differences in values for dopamine release and $[Ca]_i$ increase were determined by analysis of variance and Scheffe's test for multiple comparisons.

Results

Figure 1 shows the concentration-dependence of the adenosine-induced potentiation of ATP-evoked dopamine release from PC12 cells. Adenosine potentiated ATP (30 μM)-evoked dopamine release significantly in a concentration-dependent manner over a concentration-range from 10 to 100 μM. At the highest concentration, adenosine potentiated the ATP-evoked response by approximately 50%. Figure 2a shows the effect of adenosine (100 μM) on the concentration-dependent curves of ATP-evoked dopamine release from the cells. Adenosine (100 μM) shifted the ATP-response curve to the left but did not change the maximal response to ATP. Adenosine potentiated the response evoked by 30 μM ATP yet had no effect on the response evoked by 300 μM ATP (Figure 2b).

To examine the involvement of PTX-sensitive G-proteins on the adenosine-induced responses, the cells were preincubated with 2 ng ml^{-1} of PTX for 20 h at 37°C as described previously (Inoue & Kenimer, 1988). Figure 3 shows the

effects of PTX on the adenosine-induced potentiation of the ATP-evoked response. PTX abolished the effect of adenosine almost completely, suggesting that the adenosine-induced potentiation is mediated via a PTX-sensitive G-protein.

The effects of adenosine antagonists on the potentiation of the ATP-induced response was investigated. Figure 4 shows the effects of aminophylline, a non selective adenosine receptor antagonist, 8-cyclopentyltheophylline (8-CPT), a selective adenosine A_1 subtype antagonist, and CP66713, a selective adenosine A_2 subtype antagonist, on the adenosine-evoked potentiation. Aminophylline ($<100\,\mu M$) had no effect on dopamine release stimulated by $30\,\mu M$ ATP alone; however, it inhibited the potentiation seen with adenosine almost completely. The release was reduced to the control level

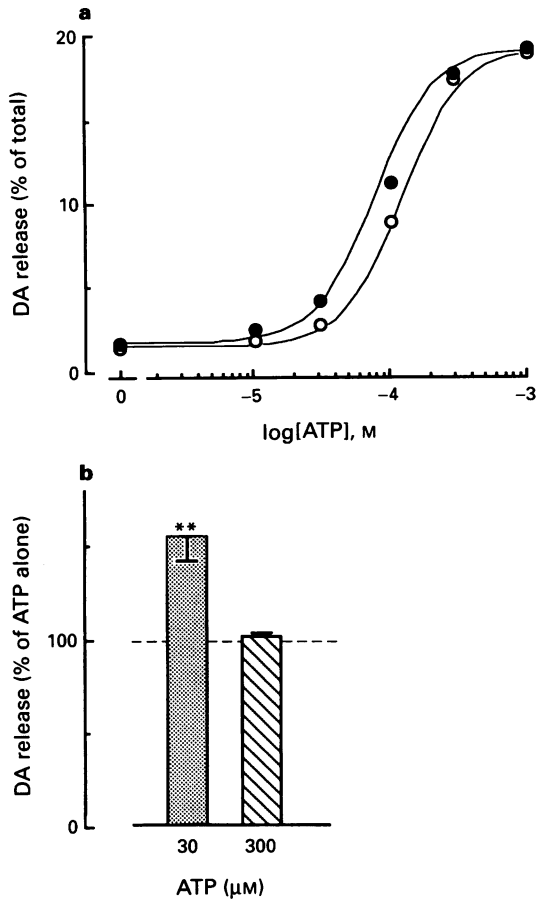

Figure 2 Effect of adenosine ($100\,\mu M$, ●) on the concentration-dependent curves of ATP-evoked dopamine release from the cells (a). Adenosine shifted the curves to the left without affecting the maximal response. Values represent the released dopamine (% of total content per min) of 3 separate experiments ($n=9$). (○) Control curve (b) Shows effect of adenosine ($100\,\mu M$) on $30\,\mu M$ ATP (stippled column) and $300\,\mu M$ ATP (hatched column). Values represent % of the response evoked by ATP alone. Each column indicates mean ± s.e.mean of 3–9 of dishes. Asterisks show significant difference from ATP alone: $^{**}P<0.01$.

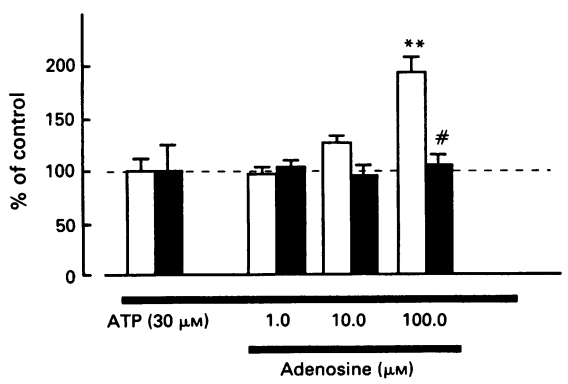

Figure 3 The involvement of pertussis toxin (PTX) on adenosine (1–100 μM)-induced potentiation in ATP (30 μM)-evoked dopamine release. The cells were treated with PTX as described in Methods. Values represent % of the response evoked by ATP alone in control (open columns) and the PTX (2 ng ml^{-1}, 20 h)-treated (solid columns) cells, respectively. Data are mean ± s.e.mean of 3 separate experiments ($n=9$). Symbols represent significant differences from the response to ATP alone ($^{**}P<0.01$) or corresponding response in intact cells ($\#P<0.05$), respectively.

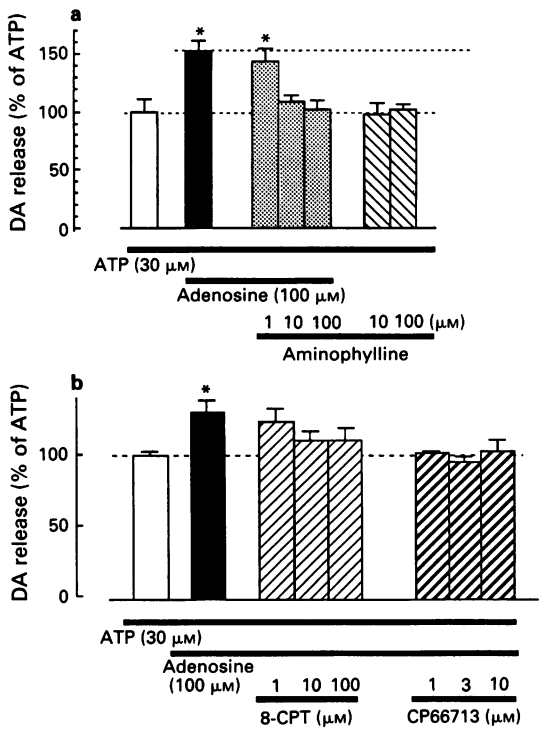

Figure 4 Inhibitory effect of adenosine antagonists on adenosine-induced potentiation of ATP-evoked dopamine (DA) release. Aminophylline (1–100 μM) (a), 8-cyclopentyltheophylline (8-CPT, 1–100 μM) and CP66713 (1–10 μM) (b) inhibited adenosine (100 μM)-induced potentiation of the ATP (30 μM)-evoked release. Values represent % of the response by ATP alone. Data are mean ± s.e. mean obtained from at least 3 separate experiments ($n=9-12$). Asterisks indicate significant difference from ATP alone: $^{*}P<0.05$.

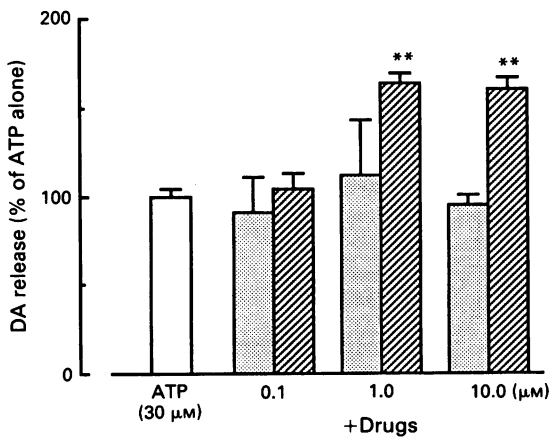

Figure 5 Effects of N^6-cyclohexyladenosine (CHA) (an adenosine A_1 agonist) or CGS22492 (an adenosine A_2 agonist) on ATP (30 μM)-evoked dopamine (DA) release from the cells. Stippled columns: CHA; hatched columns CGS 22492. Values represent % of the response by ATP alone. Data are mean ± s.e.mean obtained from 2 separate experiments ($n=6$). Asterisks indicate significant differences from ATP alone: $^{**}P<0.01$.

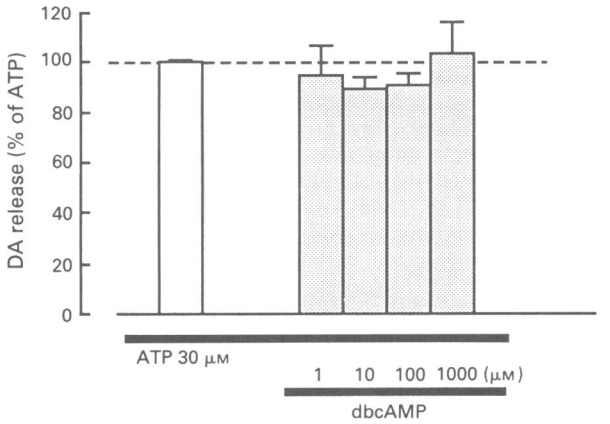

Figure 6 Effect of dibutyryl cyclic AMP (dbcAMP, 1–1000 μM) on ATP (30 μM)-evoked dopamine (DA) release from PC12 cells. Each column represents % of the response to ATP alone. Data are mean ± s.e.mean obtained from 2 separate experiments ($n = 6$).

(102.1 ± 7.3% of ATP alone, Figure 4a). Similarly, neither 8-cyclopentyltheophylline (8-CPT, 100 μM) nor CP66713 (10 μM) affected dopamine release stimulated by 30 μM ATP alone (data not shown), but they inhibited the adenosine-induced potentiation (Figure 4b). Next we examined the effects of CHA, a selective adenosine A_1 receptor agonist, and CGS22492, a selective adenosine A_2 receptor agonist, on ATP-evoked release (Figure 5). These agonists had no effects on spontaneous dopamine release. CHA failed to enhance the response, while CGS22492 potentiated over a concentration-range of 1 to 10 μM. These results suggest that adenosine-induced potentiation of ATP-stimulated dopamine release involves a subclass of adenosine A_2 receptors.

We investigated the effects of dibutyryl cyclic AMP, a cyclic AMP analogue, on ATP-evoked response because coupling between A_2 receptors and activation of adenylate cyclase has been suggested (Gilman, 1984). As shown in Figure 6, dibutyryl cyclic AMP did not affect ATP-evoked dopamine release from PC12 cells, even at concentrations up to 1 mM.

Finally, we observed the effects of adenosine on ATP-

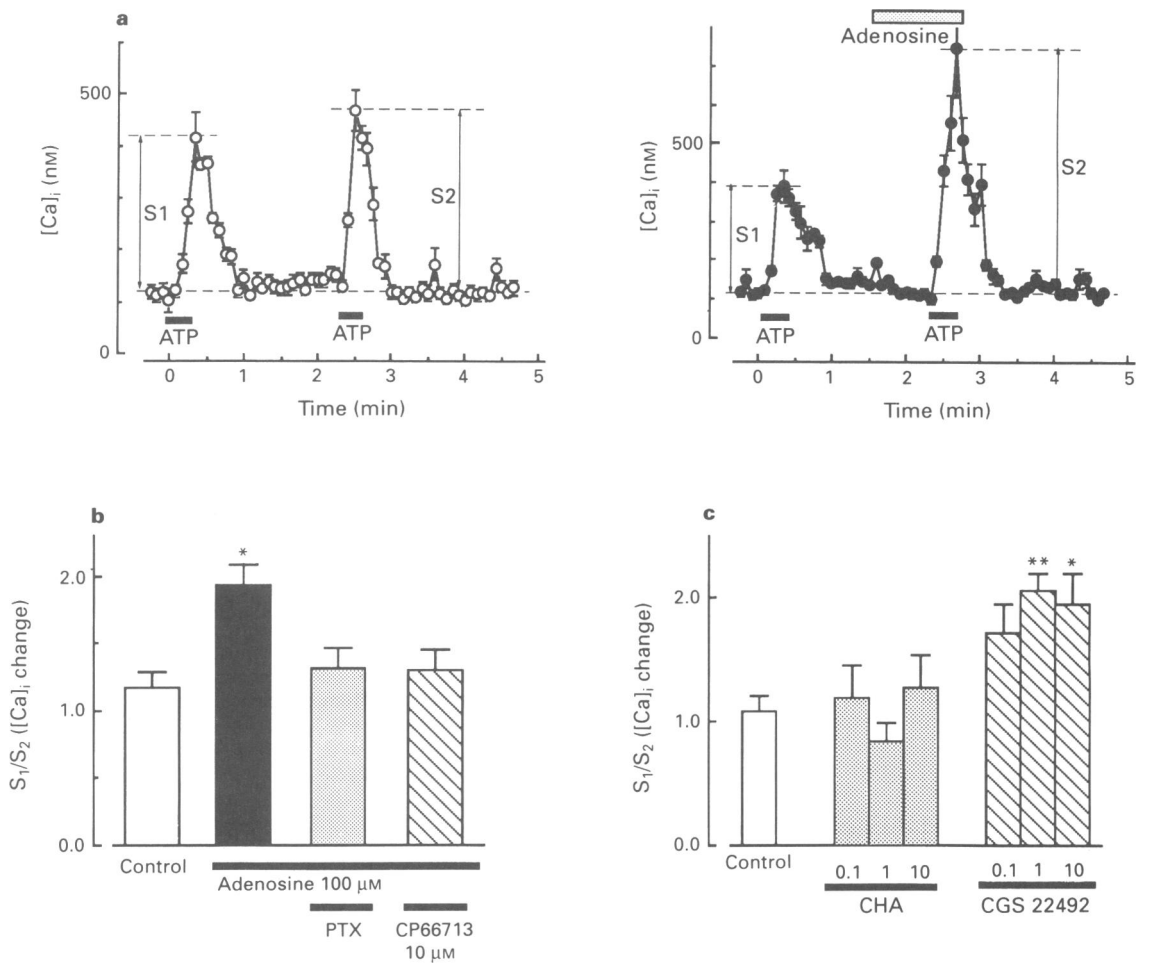

Figure 7 Potentiation by adenosine and its analogue of the ATP-evoked increase in $[Ca]_i$ in PC12 cells. (a) Ca^{2+} response to repeated applications of 30 μM ATP (15 s) to the same cells. In the left panel, both of the ATP applications were made in the absence of adenosine. In the right panel, adenosine was applied for 1 min before and during the second ATP application. Each symbol represents the mean ± s.e.mean from 14 (control) and 8 (100 μM adenosine) cells tested. (b) Comparison of the increase in $[Ca]_i$ upon the second application of 30 μM ATP. The Ca^{2+} response upon the first (S_1) and that upon the second (S_2) were measured as shown in (a), and the ratio of S_2/S_1 was determined in individual cells. Adenosine (100 μM) and CP66713 (10 μM) was applied 1 min before and during the second ATP stimulation. Each column represent mean ± s.e.mean from 5 (adenosine), 7 pertussis toxin (PTX) and 8 (CP66713) cells tested. (c) Effects of adenosine agonists on ATP-evoked $[Ca]_i$ rise. Values indicate the ratio (S_2/S_1) of the first and the second Ca^{2+} response to ATP (30 μM) as described in (b). The agents were applied 1 min before and during the second ATP stimulation. Data are mean ± s.e.mean from 13–19 (N^6-cyclohexyladenosine, CHA) and 11–14 (CGS22492) cells tested. Asterisks indicate significant differences from control: $*P < 0.05$; $**P < 0.01$.

evoked increase in $[Ca]_i$. Figure 7a shows the time-course of ATP-evoked $[Ca]_i$ rise in the cells. ATP was applied twice for 15 s with a 2 min interval between applications. ATP (30 μM, 15 s) stimulated transient increase in $[Ca]_i$ in the cells and the S_2/S_1 ratio was 1.2 ± 0.11 ($n = 5$). In PTX-treated PC12 cells, the ratio of repeated ATP application was unaffected (1.2 ± 0.11, $n = 7$). The first response to ATP was measured in the absence of adenosine (Figure 7a, left panel) and the second response to ATP was measured in the presence of adenosine (Figure 7a, right panel), and the ratio of S_2/S_1 was determined in individual cells. Adenosine (10 and 100 μM) potentiated the magnitude of $[Ca]_i$ rise (S_2/S_1 ratio: 10 μM; 1.6 ± 0.18, $n = 6$; 100 μM 1.9 ± 0.16, $n = 8$). These values were significantly larger compared with ATP alone ($P < 0.05$). Figure 7b indicates the effects of PTX and CP66713 (10 μM) on adenosine (100 μM)-induced potentiation of the ATP-evoked $[Ca]_i$ rise. PTX and CP66713 significantly attenuated the potential $[Ca]_i$ rise by adenosine. Furthermore, CGS22492 (1 and 10 μM) potentiated the ATP-evoked response significantly whereas CHA did not (Figure 7c).

Discussion

We have demonstrated here that adenosine (100 μM) potentiated ATP (30 μM)-evoked dopamine release in PC12 cells. Adenosine (100 μM) shifted the concentration-dependent curve of ATP-evoked dopamine release to the left without affecting the maximal ATP response, suggesting that adenosine does not increase the number of functional P_2-purinoceptors, but facilitates the efficiency of the response. Adenosine acts on at least two types of receptors (Stiles, 1992), namely, A_1 receptors that inhibit adenylate cyclase and A_2 receptors that stimulate adenylate cyclase. Nazarea et al. (1991) has shown that adenosine A_1 receptors attenuate ATP-evoked phosphoinositide turnover and arachidonate release in rat thyroid FRTL-5 cells, and that this inhibition involves a PTX-sensitive G-protein. In PC12 cells, pretreatment of the cells with PTX inhibited the adenosine-induced potentiation almost completely (Figure 3). This observation suggests that the modification by adenosine of ATP-evoked release is mediated by a PTX-sensitive pathway. Thus, our present data are in agreement with those of Nazarea et al. (1991) as to sensitivity to PTX. However, although 8-CPT, an A_1 antagonist, inhibited the potentiation, CP66713, an A_2 antagonist, abolished the potentiation and CGS22492, an A_2 agonist, potentiated ATP-evoked responses in the present study (Figures 4, 5, 7). These results suggest that the adenosine A_2 subclass receptors are involved in the potentiation. The observation that 5'-(N-cyclopropyl)-carboxamidoadenosine, another selective A_2 receptor agonist, also potentiated ATP-evoked dopamine release supports this idea (S. Koizumi & K. Inoue, unpublished data). The fact that 8-CPT also depressed adenosine-evoked potentiation may be explained by its poor selectivity: 8-CPT may also affect another subclass of A_2 receptors. Alternatively, this potentiation may be caused by an unknown adenosine receptor, sensitive to these antagonists and CGS22492.

It is suggested that stimulation of A_2 receptors activates adenylate cyclase, and causes cytoplasmic cyclic AMP accumulation (Gilman, 1984). An elevated cyclic AMP by forskolin can enhance carbachol-evoked dopamine release from PC12 cells (Meldolesi et al., 1988). We found that dibutyryl cyclic AMP, a membrane permeable analogue of cyclic AMP, had no effect on ATP-evoked dopamine release (Figure 6). The observation suggests that the potentiation of ATP-evoked dopamine release may not be mediated by the accumulation of cytoplasmic cyclic AMP. The lack of contribution of cyclic AMP seems to be reasonable since the subclass of adenosine A_2 receptors that mediates cyclic AMP accumulation are PTX-insensitive (Gilman, 1984) whereas the potentiation by adenosine in the present study was abolished by PTX-treatment (Figures 3 and 7b).

Adenosine (100 μM) potentiated the ATP-evoked increase in $[Ca]_i$ in PC12 cells (Figure 7a,b). As with the potentiation of the evoked release, PTX abolished the potentiation of $[Ca]_i$ increase and CGS22492 potentiated the Ca^{2+} response to ATP (Figure 7b,c). It is likely that the facilitation of ATP-evoked dopamine release by adenosine is due to an enhancement of the $[Ca]_i$ increase. The enhancement of $[Ca]_i$ increase, may at least partly, be mediated by an increase in Ca^{2+}-influx from extracellular media. This view is supported by our recent results using whole-cell voltage-clamp (Inoue et al., 1993) where adenosine (100 μM) potentiated the ATP (30 μM)-evoked inward current by a PTX-sensitive G-protein-mediated mechanism. As the ATP-activated channels are Ca^{2+}-permeable (Inoue & Nakazawa, 1992), this current enhancement may result in augmentation of Ca^{2+}-influx.

In conclusion, adenosine potentiates ATP-evoked dopamine release by facilitating the $[Ca]_i$ rise in PC12 cells. Evidence is most consistent with a role for a subclass of adenosine A_2 receptors in the observed potentiation. However, we cannot exclude the possibility that the potentiation may be mediated via a new type of adenosine receptor which is sensitive to PTX, and cannot readily be classified into the A_1 or A_2 subclasses. Detailed cellular mechanisms and the receptor subtypes are currently under investigation.

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