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Inhibition by Zn^{2+} of uridine 5'-triphosphate-induced Ca^{2+}-influx but not Ca^{2+}-mobilization in rat phaeochromocytoma cells

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1 Uridine 5'-triphosphate (UTP)-evoked increase in intracellular Ca^{2+} concentration ($[Ca]_i$) and release of dopamine were investigated in rat phaeochromocytoma PC12 cells. UTP (1-100 μM) evoked an increase in $[Ca]_i$ in a concentration-dependent manner. This response was decreased to about 30% by extracellular Ca^{2+}-depletion, but not abolished. This $[Ca]_i$ rise was mimicked by 100 μM ATP but not by 100 μM 2-methyl-thio-ATP or α,β-methylene-ATP in the absence of external Ca^{2+}, suggesting that the response was mediated by P_{2U} purinoceptors, a subclass of P_2-purinoceptors.

2 The UTP-evoked $[Ca]_i$ rise consisted of two components; a transient and a sustained one. When external Ca^{2+} was removed, the sustained component was abolished while the transient component was decreased by about 70% but did not disappear. These results suggest that UTP induces Ca^{2+}-mobilization and, subsequently, Ca^{2+}-influx.

3 The UTP-evoked increase in $[Ca]_i$ was not affected by Cd^{2+} (100 and 300 μM) or nicardipine (30 μM), inhibitors of voltage-gated calcium channels, but was significantly inhibited by Zn^{2+} (10-300 μM) in the presence of external Ca^{2+}. Zn^{2+}, however, did not affect the Ca^{2+} response to UTP in the absence of external Ca^{2+}.

4 UTP (30 μM-1 mM) evoked the release of dopamine from the cells in a concentration-dependent manner. This dopamine release was abolished by Ca^{2+}-depletion or Zn^{2+} but not by Cd^{2+} or nicardipine.

5 Taken together, the data demonstrate that UTP stimulates P_{2U}-purinoceptors and induces a rise in $[Ca]_i$ both by Ca^{2+}-mobilization and Ca^{2+}-influx in PC12 cells. The dopamine release evoked by UTP requires external Ca^{2+} which may enter the cells through pathways sensitive to Zn^{2+} but insensitive to Cd^{2+} or nicardipine.

Keywords: Uridine 5'-triphosphate (UTP); intracellular Ca^{2+} concentration; dopamine release; Zn^{2+}; PC12 cells; Ca^{2+}-influx

Introduction

Several lines of evidence show that adenosine 5'-triphosphate (ATP) has a functional role as a neurotransmitter or modulator in the peripheral (Bean, 1992; Evans *et al.*, 1992) and the central nervous system (Inoue *et al.*, 1992; 1995; Edwards *et al.*, 1992). The receptors for ATP, purinoceptors, are classified into several subclasses according to a potency rank order for some agonists (Burnstock & Kennedy, 1985; Abbracchino & Burnstock, 1994; Fredholm *et al.*, 1992). Recently, the cDNAs encoding P_2-purinoceptors coupled with GTP-binding protein (G-protein) (Lustig *et al.*, 1993; Webb *et al.*, 1993) and those forming non-selective cation channels (Brake *et al.*, 1994; Valera *et al.*, 1994) have been cloned and characterized.

We have investigated ATP-evoked responses in PC12 cells, a cell line derived from a rat phaeochromocytoma (Greene & Tischler, 1976), and demonstrated that ATP activates non-selective cation channels ('P_{2X}-purinoceptors'), leading to Ca^{2+}-influx and the release of dopamine (Inoue *et al.*, 1989; Inoue & Nakazawa, 1992; Nakazawa & Inoue, 1992). Both the rise in intracellular Ca^{2+} concentration ($[Ca]_i$) and the release of dopamine evoked by ATP appear to be mediated by the influx of extracellular Ca^{2+} through P_{2X}-purinoceptor channels (Nakazawa & Inoue, 1992). It has been reported that uridine 5'-triphosphate (UTP), a pyrimidine nucleotide, stimulates another subclass of purinoceptors, P_{2U}-purinoceptors, leading to $[Ca]_i$ elevation in PC12 cells (Majid *et al.*, 1993; Raha *et al.*, 1993; Nikodijevic *et al.*, 1994; de Souza *et al.*, 1995). P_{2U}-purinoceptors have been identified in various types of cells including neuroblastoma x glioma NG108-15 cells (Lin *et al.*, 1993) and C6-2B rat glioma cells (Munshi *et al.*, 1993). P_{2U}-purinoceptors are coupled to IP_3 formation and Ca^{2+}-mobilization from IP_3-sensitive Ca^{2+}-stores, resulting in a rise in $[Ca]_i$ in PC12 cells (Murrin & Boarder, 1992; Majid *et al.*, 1993; Raha *et al.*, 1993; Barry & Cheek, 1994; de Souza *et al.*, 1995; Nikodijevic *et al.*, 1994). There have, however, been contradictory reports of the effect of UTP on catecholamine secretion from PC12 cells. Majid *et al.* (1993) found that UTP stimulates the secretion whereas Barry & Cheek (1994), de Souza *et al.* (1995) and Nikodijevic *et al.* (1994) did not observe such secretion.

In the present study, we first characterized $[Ca]_i$ rise evoked by UTP and its relation to dopamine release in PC12 cells. We found that UTP stimulated both Ca^{2+}-mobilization and Ca^{2+}-influx, and that the release of dopamine requires Ca^{2+}-influx. We next examined the effects of Zn^{2+} on the UTP-evoked responses because (1) Zn^{2+} is an endogenous trace element which is present in synaptic nerve endings in relatively high concentration in the brain (see Frederickson, 1989; Harrison & Gibbons, 1994 for reviews) and is released by excitatory stimulation (Assaf & Chung, 1984), and (2) Zn^{2+} has been shown to enhance the P_{2X}-purinoceptor-mediated responses in PC12 cells (Koizumi *et al.*, 1995). In contrast to the reported enhancement of P_{2X}-mediated responses, Zn^{2+} suppressed dopamine release mediated by P_{2U}-purinoceptors by inhibiting Ca^{2+}-influx, but not Ca^{2+}-mobilization, in these cells.

Methods

Cell culture

PC12 cells were the kind gift of Dr Terry Rogers, Johns Hopkins University School of Medicine, Baltimore, MD,

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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. A seed lot was frozen at passage 47 and stored in liquid nitrogen. All experiments described in this paper were performed with cells at passage numbers between 53 and 68. Cells were cultured in 75 cm^2 flasks 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 $\mu g\ ml^{-1}$ gentamicin 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 in a Ca^{2+}/Mg^{2+}-free solution containing 172 mM NaCl, 5.4 mM KCl, 1 mM NaH_2PO_4, and 5.6 mM glucose, pH 7.4. After 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 the 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.

$[Ca]_i$ 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 (Koizumi *et al.*, 1994). All the procedures including incubation, washing and drug application, were made with a balanced salt solution (BSS) of the following composition (in 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. For Ca^{2+}-free experiments, we used a medium where Ca^{2+} was removed and 1 mM glycoletherdiaminetetraacetic acid (EGTA) was added (Ca^{2+}-free BSS). The cells were washed with BSS and incubated with 10 μM fura-2 acetoxymethylester (fura-2 AM) in BSS at 37°C. After 30 min incubation, the cells were washed with 0.2 ml of BSS. The coverslips were mounted on an inverted epifluorescence microscope (TMD-300, Nikon, Tokyo, Japan) equipped with a 75 W xenon-lamp and band-pass filters of 340 nm wavelength (F340), for measurement of the Ca^{2+}-dependent signal, and 360 nm wavelength (F360), for measurement of the Ca^{2+}-independent signal. Measurements were carried out at room temperature. Image data, recorded by a high-sensitivity silicon intensifier target camera (C-2741-08, Hamamatsu Photonics, Co., Hamamatsu, Japan) were processed by Ca^{2+}-analyzing system (Furusawa Laboratory applicance Co., Kawagoe, Japan). The absolute $[Ca]_i$ was estimated from the ratio of emitted fluorescence (F340/F360) according to a calibration curve obtained by using Ca^{2+}-buffers (Molecular Probes Inc., C-3712 with 1 mM $MgCl_2$).

Dopamine release

Released dopamine was measured as previously described (Ohara-Imaizumi *et al.*, 1991). All the procedures including incubation, washing and drug application, were carried out using 1 ml/dish of BSS or Ca^{2+}-free BSS. Dishes were washed twice and incubated with BSS for 1 h at room temperature. After washing once with BSS, UTP or its related compounds were added to the dishes and incubated for 1 min. 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 h.p.l.c.-e.c.d. system (Bioanalytical systems, West Lafayette, IN, U.S.A.). The percentage of release was calculated from the values obtained for the dopamine content in the incubation solution (A) and the dopamine content remaining in the cells (B) with the following equation: % of total dopamine $= 100 \times A/(A + B)$.

Drugs

Drugs and chemicals were obtained from the following sources. Adenosine 5'-triphosphate disodium salt (ATP) was purchased from Yamasa Co. (Choshi, Japan), zinc acetate, uridine 5'-triphosphate disodium salt (UTP), nicardipine hydrochloride and cadmium chloride were from Sigma (St. Louis, MO, U.S.A.); α,β-methylene adenosine 5'-triphosphate (α,β-meATP) and 2-methyl-thio-adenosine 5'-triphosphate (2-meSATP) were from Research Biochemical International (Natick, MA, U.S.A.), and EGTA, HEPES and fura-2AM were from Dojin (Kumamoto, Japan). Other drugs were purchased from Wako Pure Chemical (Osaka, Japan). Nicardipine was dissolved in dimethyl sulphoxide at a concentration of 10 mM and then dissolved in BSS to appropriate concentrations. Other drugs were directly dissolved in BSS or Ca^{2+}-free BSS.

Statistics

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

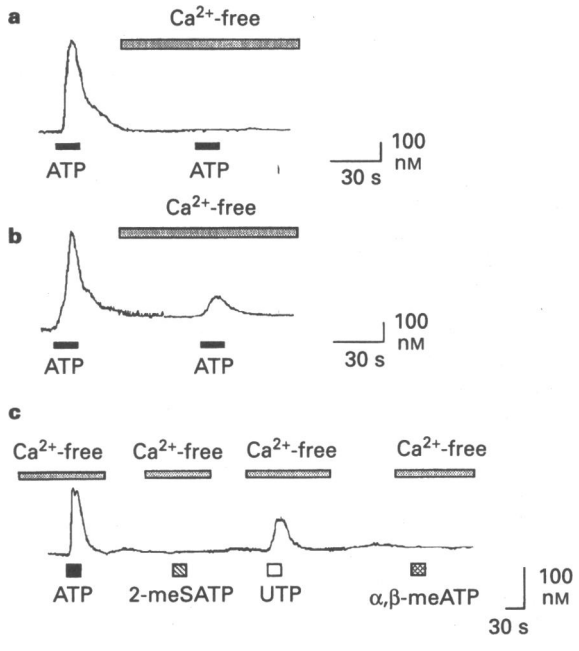

Figure 1 Typical Ca^{2+}-responses to ATP and its related compounds in PC12 cells. (a) A rise in $[Ca]_i$ evoked by 100 μM ATP in the presence and absence of external Ca^{2+} in a PC12 cell. ATP failed to produce the rise in $[Ca]_i$ when external Ca^{2+} was removed (horizontal stippled bar) in this cell. Horizontal solid bars show the application of 100 μM ATP. (b) A rise in $[Ca]_i$ evoked by 100 μM ATP in the presence and absence of external Ca^{2+} in another cell. Unlike the cell shown in (a), ATP produced the rise in $[Ca]_i$ even when the external Ca^{2+} was removed from the medium (horizontal stippled bar). Horizontal solid bars show the application of 100 μM ATP. (c) Changes in $[Ca]_i$ evoked by 100 μM ATP (solid bar), 2-meSATP (hatched bar), UTP (open bar) and α,β-meATP (cross-hatched bar) in the absence of external Ca^{2+} (horizontal stippled bars). ATP and UTP, but not 2-meSATP or α,β-meATP, produced a rise in $[Ca]_i$ in the same cell. Vertical and horizontal scale bars represent 100 nM and 30 s, respectively.

Results

Figure 1 shows typical Ca^{2+} responses to ATP and related compounds in the presence and absence of extracellular Ca^{2+} in PC12 cells. When ATP (100 μM) was applied to the cells for 15 s, it produced a rise in $[Ca]_i$ in the presence of external Ca^{2+} but failed to stimulate the $[Ca]_i$ rise in the absence of external Ca^{2+} in about 70% of cells tested which were responsive to ATP (62 out of 94 cells) (Figure 1a). The cells which responded to ATP in the absence of external Ca^{2+} (Figure 1b) also responded to 100 μM UTP but not to 2-meSATP (100 μM) or α,β-meATP (100 μM) ($n=32$) (Figure 1c). On the other hand, none of these compounds produced responses in those cells which did not respond to ATP in the absence of external Ca^{2+} ($n=62$).

Figure 2 shows the concentration-dependence of the UTP-evoked $[Ca]_i$ rise in the presence (Figure 2a(i)) and absence Figure 2a(ii)) of external Ca^{2+}. UTP stimulated the rise in $[Ca]_i$ in a concentration-dependent manner over a concentration range from 1 to 100 μM, either with or without external Ca^{2+}, though the response with Ca^{2+} was significantly larger than that without Ca^{2+} (Figure 2a(i)(ii) and 2b). The rise in $[Ca]_i$ evoked by UTP or ATP in the presence of external Ca^{2+} was decreased to about 30% or 15% by Ca^{2+}-depletion, respectively. In the absence of external Ca^{2+}, the $[Ca]_i$ rise evoked by 100 μM UTP was comparable to that evoked by 100 μM ATP (Figure 2b). Suramin (100 μM), a competitive antagonist at P_2-purinoceptors, abolished the $[Ca]_i$ rise evoked by 100 μM UTP (Figure 2a(iii)).

Figure 3a shows a typical time-course of the UTP-evoked $[Ca]_i$ rise. UTP (100 μM) was applied to the cells twice for a longer period (1 min) in the absence or presence of external Ca^{2+} (Figure 3a). The Ca^{2+} response to UTP in the absence of Ca^{2+} was scaled up to the same size as the response in the presence of Ca^{2+}, and then superimposed and their time-courses were compared (Figure 3b). In addition to the transient component that is observed in the absence of extracellular Ca^{2+}, UTP appears to evoke a sustained $[Ca]_i$ rise when extracellular Ca^{2+} is present. To evaluate this sustained component, the Ca^{2+} response to UTP at 1 min after UTP addition (indicated as # in Figure 3b) was calcuated as a percentage of the maximal response (Figure 3c). The sustained $[Ca]_i$ rise was significantly smaller in the absence of external Ca^{2+} (Figure 3c).

UTP evoked the release of dopamine from PC12 cells in a concentration-dependent manner over a concentration range of 30 to 300 μM in the presence of external Ca^{2+} (Figure 4). The dopamine release evoked by 100 or 300 μM UTP was abolished in the absence of extracellular Ca^{2+}, suggesting that extracellular Ca^{2+} is essential for the UTP-evoked dopamine release.

Figure 5 shows the effects of Zn^{2+}, Cd^{2+} and nicardipine on the release of dopamine evoked by 100 μM UTP. The voltage-gated Ca^{2+} channel blockers, Cd^{2+} (100–300 μM) and nicardipine (30 μM), had no effect on the UTP-evoked dopamine release. In contrast, Zn^{2+} significantly inhibited the release in a concentration-dependent manner over a concentration-range from 10 to 300 μM with an IC_{50} value of about 50 μM.

Finally, the effects of Zn^{2+} on the Ca^{2+} responses to UTP (100 μM) in the absence (EGTA was omitted to avoid chelating Zn^{2+}) and presence of external Ca^{2+} were examined (Figure 6). UTP (100 μM) was applied to the cells twice for 15 s separated by a 2 min interval and the first and second responses to UTP were defined as S1 and S2, respectively. The first treatment was always with UTP alone and the second treatment was given under various conditions. 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. When UTP was applied to the cells twice under Ca^{2+}-free conditions, the Ca^{2+} response to the second UTP application was drastically attenuated (data not shown). To avoid this attenuation, Ca^{2+} (1.8 mM) was added to the cells for 30 s between the first and the second UTP-application. The Ca^{2+} responses to the second

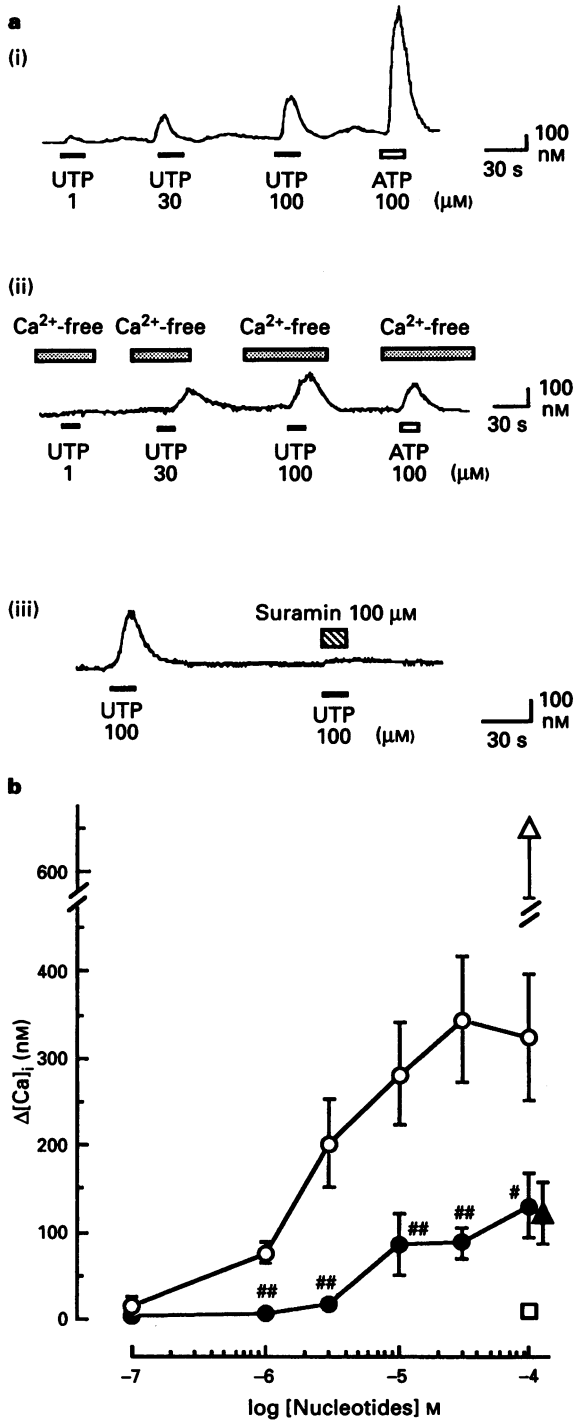

Figure 2 Concentration-dependence of UTP-evoked peak rise in $[Ca]_i$ in PC12 cells. (a) Representative traces of the rise in $[Ca]_i$ evoked by various concentrations of UTP and 100 μM ATP in the presence (i) and absence (ii) (horizontal stippled bars) of extracellular Ca^{2+}. Horizontal solid and open bars show the application of UTP and ATP, respectively. (iii) The effect of suramin (100 μM) on the UTP (100 μM)-evoked $[Ca]_i$ rise in the presence of external Ca^{2+}. Horizontal hatched bar shows the application of 100 μM suramin. Vertical and horizontal scale bars show 100 nM and 30 s, respectively. (b) Concentration-response relationships of the UTP-evoked $[Ca]_i$ rise in the presence (control: ○) and absence (●) of external Ca^{2+}; (△) and (▲) show the rise in $[Ca]_i$ evoked by 100 μM ATP in the presence and absence of extracellular Ca^{2+}, respectively; (□) shows the Ca^{2+}-response to 100 μM UTP with 100 μM suramin in the presence of external Ca^{2+}. Values show the maximal $[Ca]_i$ rise above basal. Data are mean ± s.e.mean of 30–32 cells tested. Significant differences from control: #$P<0.05$; ##$P<0.01$).

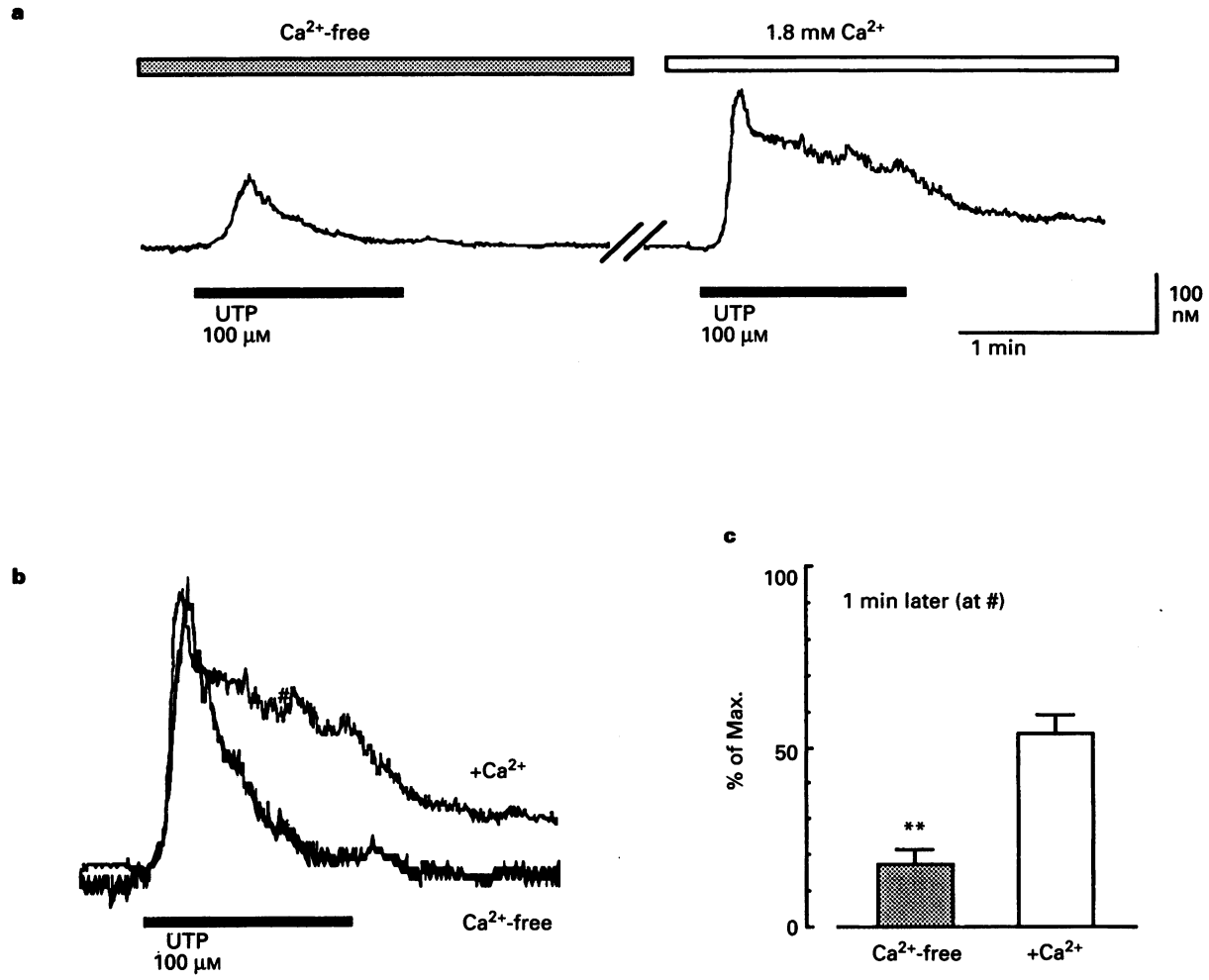

Figure 3 The time-course of UTP-evoked increase in $[Ca]_i$ in PC12 cells. (a) Typical Ca^{2+}-responses to 100 μM UTP in the absence and presence of external Ca^{2+}. UTP was applied to the cells twice for 1 min (horizontal solid bars) separated by a 3 min interval. The first and the second stimulation were performed in the absence (horizontal stippled bar) and presence (horizontal open bar) of external Ca^{2+}. Vertical and horizontal scale bars represent 100 nM and 1 min, respectively. (b, c) The traces shown in (a) are superimposed and their time-courses are compared in (b). #s show the $[Ca]_i$ at 1 min after UTP-application in the presence ($+Ca^{2+}$) and absence (Ca^{2+}-free) of external Ca^{2+}, which are summarized in (c). Values show % of the maximal responses at # in the presence (open column) and absence (stippled column) of external Ca^{2+}. Data are mean ± s.e.mean of 10 ($+Ca^{2+}$) and 7 (Ca^{2+}-free) cells tested. Asterisks show a significant difference from the response in the presence of external Ca^{2+}: $^{**}P<0.01$.

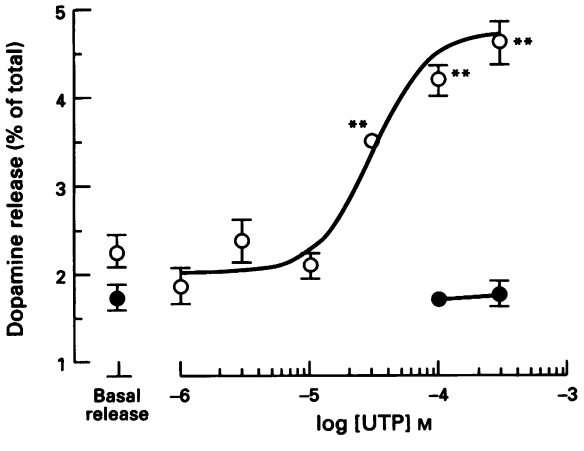

Figure 4 Concentration-dependence of UTP-evoked dopamine release from PC12 cells (○). These are results from a typical experiment with each data point being the mean ± s.e.mean of triplicate measurements. The curve was fitted to the Hill equation with a Hill coefficient of 2 and ED_{50} of about 30 μM. Two such experiments were performed; (●) responses in the absence of external Ca^{2+}. Significant difference from basal release: $^{**}P<0.01$.

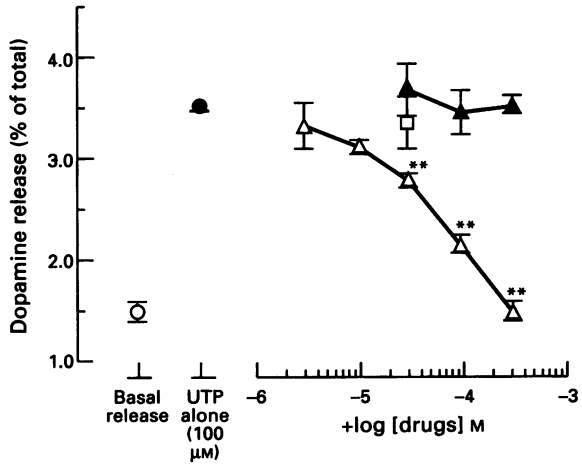

Figure 5 The effects of Zn^{2+}, Cd^{2+} and nicardipine on the UTP-evoked dopamine release form PC12 cells: (○) and (●) show spontaneous dopamine release and the release evoked by 100 μM UTP alone, respectively; the effects of Zn^{2+}, Cd^{2+} and nicardipine on the UTP (100 μM)-evoked responses are indicated: Zn^{2+} (△), Cd^{2+} (▲) and nicardipine (□). These are results from a typical experiment and each data point is the mean ± s.e.mean of triplicate measurements. Three such experiments were performed. Asterisks show significant difference from the response evoked by UTP alone: $^{**}P<0.01$.

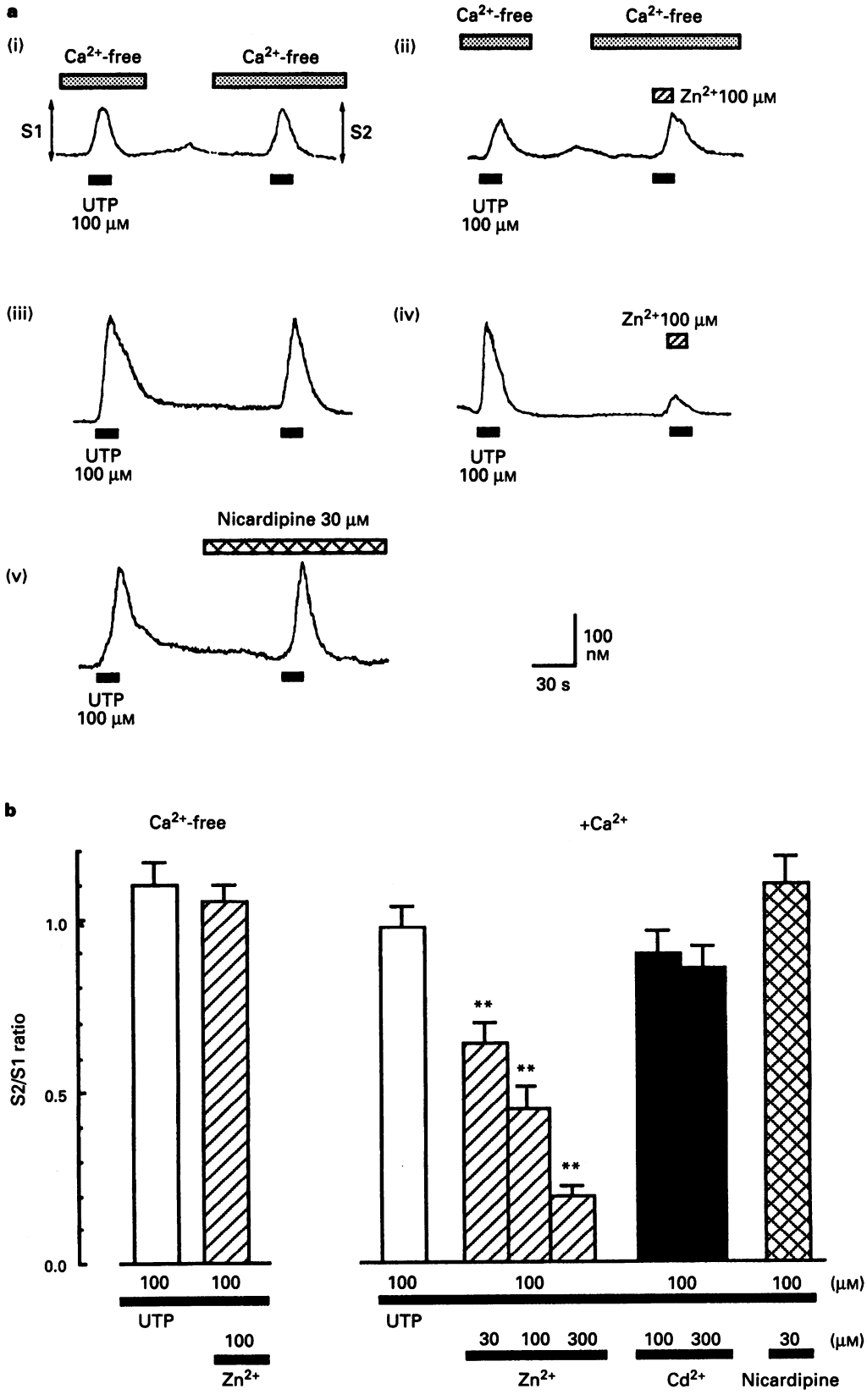

Figure 6 The effects of Zn^{2+} on the UTP (100 μM)-evoked rise in $[Ca]_i$ in the presence and absence of extracellular Ca^{2+} in PC12 cells. (a) Typical Ca^{2+} responses to UTP in the absence (i and ii) and presence (iii, iv and v) of external Ca^{2+}. UTP was applied to the cells twice for 15 s separated by a 2 min interval. Typical Ca^{2+} responses to UTP alone (i) and UTP with Zn^{2+} (ii) in the absence of external Ca^{2+} and those to UTP alone (iii), UTP with 100 μM Zn^{2+} (iv) and UTP with 30 μM nicardipine (v) in the presence of external Ca^{2+} are shown. The agents were applied simultaneously with UTP except that nicardipine was applied to the cells 30 s before and during UTP application. Horizontal solid bars show the UTP (100 μM)-application. Horizontal stippled, hatched and cross-hatched bars show the removal of extracellular Ca^{2+}, the application of Zn^{2+} (100 μM) and nicardipine (30 μM), respectively. Vertical and horizontal scale bars show 100 nM and 30 s, respectively. (b) Summary of the ratio of the second response to the first response (S2/S1), as obtained in (a). Data are mean ± s.e.mean of 15–23 cells tested. Significant differences from the ratio obtained with UTP alone $^{**}P<0.01$.

UTP application in the absence (Figure 6a(i)) and presence (Figure 6a(iii)) of external Ca^{2+} were similar to those to the first UTP application, and the S2/S1 ratios were 1.1 ± 0.07 ($n=15$) and 1.0 ± 0.06 ($n=23$), respectively. Zn^{2+} (100 μM) had no effect on the Ca^{2+} response to UTP in the absence of external Ca^{2+} (Figure 6a(ii)), but inhibited the response in the presence of external Ca^{2+} (Figure 6a(iv)). The S2/S1 ratios obtained under various conditions are summarized in Figure 6b. Zn^{2+} (30–300 μM) significantly inhibited the UTP-evoked responses only in the presence of external Ca^{2+}. Cd^{2+} (100 and 300 μM) or nicardipine did not affect the UTP-evoked $[Ca]_i$ rise in the presence of external Ca^{2+} (Figure 6a, b), in agreement with the dopamine release data shown in Figure 5.

Changes in emitted fluorescence intensity excited by a 360 nm ultraviolet ray (F360), a Ca^{2+}-independent signal, were investigated to examine whether Zn^{2+} influenced Ca^{2+} signals by interacting directly with fura-2 molecules (see Discussion). When UTP was applied to the cells with 1.8 mM Ca^{2+}, the F360 remained unchanged in the absence or presence of Zn^{2+} (100 and 300 μM) (data not shown).

Discussion

ATP stimulated a rise in $[Ca]_i$ in the absence of extracellular Ca^{2+} in about 30% of PC12 cells, and the response was mimicked by UTP but not by α,β-meATP or 2-meSATP (Figure 1). These results suggest that functional P_{2U}-purinoceptors are present in PC12 cells and that activation by ATP or UTP of these receptors induces Ca^{2+}-release from intracellular Ca^{2+}-storage sites. This Ca^{2+}-release may result from IP_3 formation linked to P_{2U}-purinoceptors as has been suggested for PC12 cells by other investigators (Majid *et al.*, 1993; Raha *et al.*, 1993; Barry & Cheek, 1994; Nikodijevic *et al.*, 1994; de Souza *et al.*, 1995). When UTP was applied to the cells for 1 min, it caused not only a transient $[Ca]_i$ rise but also a sustained rise in the presence of external Ca^{2+}, whereas it produced only the transient Ca^{2+} response in the absence of external Ca^{2+} (Figure 3). Thus, it is suggested that, in addition to Ca^{2+}-mobilization which causes the transient Ca^{2+} response, UTP also stimulates Ca^{2+}-influx. It is, however, still unclear whether the transient or the sustained response observed with external Ca^{2+} exclusively arises from Ca^{2+}-mobilization or Ca^{2+}-influx, respectively.

UTP stimulated the release of dopamine from the cells, though the amount of dopamine evoked by 100 μM UTP was less than 50% of that evoked by 100 μM ATP. In contrast to our results, several groups have reported that UTP fails to stimulate the catecholamine secretion from PC12 cells (Barry & Cheek, 1994; Nikodijevic *et al.*, 1994; de Souza *et al.*, 1995). The Ca^{2+} response to UTP was found in only about 30% of PC12 cells in the present study. Thus, one possible explanation for this discrepancy is that only a small proportion of PC12 cells have functional P_{2U}-purinoceptors. The amount of the UTP-evoked dopamine or noradrenaline release is generally measured as a sum of the response of about 10^6 to 10^7 cells. If functional P_{2U}-purinoceptors were present only in a small population of cells, the secretory response, unlike our present study, may not be detected. The second possible explanation for the discrepancy is that the rise in $[Ca]_i$ evoked by UTP may not reach a level sufficient to cause the secretion in PC12 cells used by other investigators because of the small number of functional P_{2U}-purinoceptors in individual cells. The third possible explanation for the discrepancy is that intracellular signalling mechanisms linked to P_{2U}-purinoceptors may be different among PC12 cells. In any case, PC12 cells may be heterogeneous with respect to sensitivity to UTP.

The UTP-evoked dopamine release was inhibited by Ca^{2+}-depletion and Zn^{2+} but not by nicardipine or Cd^{2+}. These results suggest that the dopamine release evoked by UTP requires an influx of extracellular Ca^{2+} through an ionic pathway that is different from voltage-gated calcium channels. Furthermore, Zn^{2+} inhibited the UTP-evoked $[Ca]_i$ rise only in the presence of external Ca^{2+}, with no effect observed in the absence of external Ca^{2+}. Thus, Zn^{2+} may suppress the dopamine release by inhibiting the Ca^{2+}-permeable ionic pathway; not by affecting the UTP molecule itself or P_{2U}-purinoceptors. P_{2U}-purinoceptors do not form ion channels, but they are coupled with G-proteins (Abbracchino & Burnstock, 1994; Fredholm *et al.*, 1994). The mechanism of Ca^{2+}-influx that is activated by this type of receptor is totally unknown. It has been reported that the depletion of intracellular Ca^{2+}-stores can induce Ca^{2+}-influx without any receptor activation in non-excitable cells (Puteny, 1986). Recently, this store-dependent Ca^{2+}-influx was also observed in excitable cells such as smooth muscle cells (Pacaud *et al.*, 1993). Randriamampita & Tsien (1993) have reported that the depletion of Ca^{2+}-stores stimulates the release of an intracellular molecule, named Ca^{2+}-influx factor (CIF), which induces Ca^{2+}-influx from extracellular spaces. Thus, the UTP-induced Ca^{2+}-influx in PC12 cells may be mediated by the depletion of Ca^{2+}-stores or a CIF-like substance. Another possibility is that some cellular signals resulting from activation of P_{2U}-purinoceptors promote the Ca^{2+}-influx independent of Ca^{2+}-stores, as suggested for bradykinin- or carbachol-evoked Ca^{2+}-influx in PC12 cells (Clementi *et al.*, 1992). In any case, it is emphasized that UTP induces Ca^{2+}-influx via a Zn^{2+}-sensitive, Cd^{2+}- and nicardipine-insensitive, pathway. Zn^{2+} may be a key substance to characterize Ca^{2+}-influx mediated by activation of P_{2U}-purinoceptors or other G-protein coupled receptors.

We used a fluorescent dye, fura-2, as an indicator of $[Ca]_i$ changes. Fura-2 binds Zn^{2+} with a dissociation constant around 2 nM which is about 100 times lower than that for Ca^{2+} (Grynkiewicz *et al.*, 1985). Zn^{2+} may enter cells and affect the fluorescence intensity of the Ca^{2+}-fura-2 complex. If Zn^{2+} enters upon UTP-stimulation, the emitted fluorescence signals excited at 360 nm (F360) should be increased because the Zn^{2+}-fura-2 complex, unlike the Ca^{2+}-fura-2 complex, increases the emitted fluorescence intensity excited at this wave length as well as that at 340 nm. Thus, we investigated the effects of Zn^{2+} on F360 intensity, a Ca^{2+}-independent signal. Zn^{2+} did not affect F360 signals, suggesting that, even if Zn^{2+} enters the cells, the amount may be negligible.

Zn^{2+} has been reported to potentiate the ATP-evoked responses mediated by Ca^{2+}-permeable P_{2X}-purinoceptor channels in superior cervical (Cloues *et al.*, 1993; Li *et al.*, 1993) and coeliac ganglia (Li *et al.*, 1993) and PC12 cells (Koizumi *et al.*, 1995). On the contrary, our present observation suggested that Zn^{2+} inhibits the Ca^{2+}-influx mediated by G-protein-coupled P_{2U}-purinoceptors. Zn^{2+} may have opposite effects on the ATP-evoked Ca^{2+}-influx mediated by these two types of P_2-purinoceptors. The concentration of Zn^{2+} at synapses in the hippocampus has been estimated to be as high as 100–300 μM (see Frederickson, 1989; Harrison & Gibbons, 1994 for reviews) and these two types of regulation occur at concentrations $\leqslant 100$ μM (Figures 5 and 6 and Koizumi *et al.*, 1995). Thus, this reciprocal regulation by Zn^{2+} of P_2-purinoceptor-mediated responses may be an event of great physiological consequence when it modulates actions evoked by endogenous ATP. Similar reciprocal regulation has been reported for glutamate receptor channels. Zn^{2+}-inhibited NMDA receptor channels, but potentiated non-NMDA receptor channels expressed in *Xenopus* oocytes (Rassendren *et al.*, 1990). Zn^{2+} inhibits various ligand-gated channels, including the channels gated by GABA, NMDA, 5-hydroxytryptamine, and voltage-gated Ca^{2+}, Na^+ and K^+ channels in other neuronal cells (see Frederickson, 1989 for review). It remains to be clarified whether or not Zn^{2+} inhibits a Ca^{2+}-influx mediated by stimulation of any other G-protein-coupled receptors.

In summary, we characterized the $[Ca]_i$ rise and dopamine release mediated by P_{2U}-purinoceptors in PC12 cells. The UTP-evoked rise in $[Ca]_i$ consists of two components, namely, a transient and a sustained component. The transient $[Ca]_i$ rise may arise from intracellular Ca^{2+}-stores and the sustained rise

may be caused by Ca^{2+}-influx from extracellular spaces via a Zn^{2+}-sensitive ionic pathway which is distinct from voltage-gated calcium channels or P_{2X}-purinoceptor channels.

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