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Characterization of Na^+ influx mediated by ATP^{4-}-activated P_2 purinoceptors in PC12 cells

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1 Micromolar levels of extracellular ATP increased cytosolic Na^+ concentration ($[Na^+]_i$) as well as cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) in PC12 cells.

2 Pretreatment of cells with tetrodotoxin, benzamil or thapsigargin did not alter the ATP-induced Na^+ influx.

3 Increased extracellular Mg^{2+} concentration decreased the ATP effect. Furthermore, when the extracellular ATP pool was treated to contain corresponding calculated concentrations of ATP^{4-}, the increase in $[Na^+]_i$ stayed linked to the ATP^{4-} concentration rather than to the total ATP concentrations in the stimulants.

4 Extracellular ATP does not create nonselective pores as shown by the fact that ethidium bromide does not enter the cells upon ATP stimulation.

5 Among the tested nucleotides, only adenosine 5'-O-(3-thiotriphosphate), 2-methylthio ATP and 2-chloro ATP also caused Na^+ influx.

6 Reactive Blue 2 specifically decreased the ATP effect in a concentration-dependent manner.

7 The results suggest that extracellular ATP triggers Na^+ influx through a P_2 purinoceptor which is activated by ATP^{4-} in PC12 cells.

Keywords: P_2 purinoceptor; ATP^{4-}; SBFI; intracellular sodium; Reactive Blue 2; PC12 cell

Introduction

Extracellular ATP has potent pharmacological actions in many neuronal cells such as sympathetic and parasympathetic neurones, sensory ganglia, hippocampal neurones, dorsal horn neurones, and PC12 cells (von Kügelgen & Starke, 1991; Dubyak & El-Moatassim, 1993; Illes & Nörenberg, 1993). The actions of ATP are mediated by P_2 purinoceptors (Burnstock, 1978), six subtypes of which have been classified: P_{2X}, P_{2Y}, P_{2U}, P_{2T}, P_{2Z}, and P_{2D} (Fredholm *et al.*, 1994).

In PC12 cells, extracellular ATP elevates the intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) through the activation of phospholipase C (PLC) and receptor operated Ca^{2+} channels (Fasolato *et al.*, 1990; Sela *et al.*, 1991; Majid *et al.*, 1992; Reber *et al.*, 1992; Kim & Rabin, 1994; Suh *et al.*, 1995). The ATP gated channel in PC12 cells is classified as a P_{2X} purinoceptor and has recently been cloned and sequenced in PC12 cells (Brake *et al.*, 1994). Inward currents are detected in oocyte and human embryonic kidney 293 cells transfected with P_{2X} purinoceptor cDNA cloned from PC12 cells (Evans *et al.*, 1995). Extracellular ATP also triggers inward currents in PC12 cells which are in part due to Na^+ influx (Nakazawa *et al.*, 1990a,b: Neuhaus *et al.*, 1991). However, the characteristics of the Na^+ influx have not yet been worked out. There may be several pathways for Na^+ influx: through a P_{2X} purinoceptor operated non-selective cation channel, a Na^+/Ca^{2+} exchanger, a voltage-sensitive Na^+ channel or an ATP-induced membrane pore. We investigated, whether the extracellular ATP-induced elevation of intracellular free Na^+ concentration ($[Na^+]_i$) occurs through a second pathway, concomitantly with the P_{2X} purinoceptor, in PC12 cells.

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Methods

Cell culture

PC12 cells were maintained at 37°C in RPMI 1640 supplemented with 10% (v/v) heat-inactivated bovine calf serum, 5% (v/v) heat-inactivated equine serum and 1% (v/v) penicillin-streptomycin in a humidified atmosphere of 95% air and 5% CO_2. The culture medium was changed every 2 days and cells were subcultured about once a week.

Measurements of $[Na^+]_i$

The level of intracellular Na^+ was determined by use of sodium-binding benzofuran isophthalate tetra-acetoxymethyl ester (SBFI/AM), the fluorescent sodium indicator (Minta & Tsien, 1989). Cells were harvested and incubated in serum-free RPMI 1640 medium with 15 μM SBFI/AM, 0.2% pluronic acid and 250 μM sulfinpyrazone at 37°C for 90 min under continuous stirring. Then the cells were washed with serum-free RPMI 1640 medium containing 250 μM sulphinpyrazone. The cells were kept at room temperature for 30 min to allow completion of the hydrolysis of SBFI/AM in serum-free RPMI 1640 solution with 250 μM sulphinpyrazone. Before measurement, a small portion of cells (0.5×10^6 cells) were divided into aliquots for assay, centrifuged and, after the supernatant was removed, resuspended in Locke buffer solution (composition mM: NaCl 154, KCl 5.6, $MgCl_2$ 1.2, $CaCl_2$ 2.2, HEPES 5, and glucose 10, at pH 7.3), Ca^{2+}-free Locke solution (composition mM: NaCl 158.4, KCl 5.6, $MgCl_2$ 1.2, HEPES, 5, glucose 10, and 200 μM EGTA at pH 7.3), Na^+-free Locke solution (choline chloride 154, KCl 5.6, $MgCl_2$ 1.2, $CaCl_2$ 2.2, HEPES 5, and glucose 10 at pH 7.3) or Mg^{2+}-free Locke solution (composition mM NaCl 156.4, KCl 5.6, $CaCl_2$ 2.2, HEPES 5 and glucose 10 at pH 7.3). For these experiments, the increase of cytosolic Na^+ was measured as an increase in the fluorescence ratio

determined at the dual excitation wavelengths of 340 nm and 380 nm and the emission wavelength of 550 nm at 37°C. Since the calibrations of the obtained fluorescence ratios for Na^+ concentrations are not absolute (Chueh *et al.*, 1994), we usually expressed our results as fluorescence ratios. In these experiments, the baseline Na^+ concentrations were calculated to be near 5 mM and to increase up to approximately 7 mM upon 300 μM ATP treatment in the absence of extracellular Mg^{2+}.

Measurement of $[Ca^{2+}]_i$

The level of intracellular Ca^{2+} was determined with fura-2 penta-acetoxymethyl ester (fura-2/AM) as previously described (Suh & Kim, 1994). Briefly, a cell suspension was harvested and incubated in fresh serum-free RPMI 1640 medium with 3 μM fura-2/AM at 37°C for 50 min under continuous stirring. Then the cells were washed with serum-free RPMI 1640 medium. Sulphinpyrazone (250 μM) was added to all solutions to prevent dye leakage. For measurement, a small portion of the cells (0.5×10^6 cells) was removed, pelleted, and resuspended in Locke's buffer solution. Changes in the fluorescence ratio were measured at dual excitation wavelengths of 340 nm and 380 nm and the emission wavelength of 500 nm. The calibration of the fluorescence ratio in terms of $[Ca^{2+}]_i$ was performed according to Grynkiewicz *et al.* (1985).

Measurement of membrane permeabilization

The membrane permeabilization was determined using ethidium bromide (EtBr) as previously reported by Chueh *et al.* (1994). Briefly, cells were harvested in serum-free RPMI 1640 medium. Before the measurement, 0.5×10^6 cells were aliquoted, centrifuged, and the pellets resuspended in Locke buffer solution containing 25 μM EtBr. In order to measure the membrane permeability after the addition of the indicated reagents, changes in fluorescence intensity were recorded at an excitation wavelength of 310 nm and an emission wavelength of 580 nm.

Materials

RPMI 1640 and penicillin-streptomycin were purchased from GIBCO (Grand Island, NY, U.S.A.). Bovine calf serum and equine serum were obtained from HyClone Laboratories (Logan, UT, U.S.A.). Fura-2/AM SBFI/AM, 1,2-*bis*(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetoxymethyl ester (BAPTA/AM) and pluronic acid were obtained from Molecular Probes (Eugene, OR, U.S.A.). ATP, ADP, AMP, adenosine, GTP, CTP, UTP, ITP, XTP, adenosine 5′-O-(3-thiotriphosphate) (ATP-γ-S), adenosine 5′-O-(2-thiodiphosphate) (ADP-β-S), adenosine 5′-(β,γ-methylene) triphosphate (AMPPCP), 5′-adenylylimidodiphosphate (AMPPNP), 3′-O-(4-benzoyl)benzoyl ATP (BzATP), tetrodotoxin, thapsigargin, bradykinin, sulfinpyrazone, monensin, EGTA, and EtBr were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Adenosine 5′-(α,β-methylene) triphosphate (AMPCPP), 2-chloro ATP (2-Cl ATP) and 2-methylthio ATP (2-MeS ATP) were obtained from Research Biochemicals Inc. (Natick, MA, U.S.A.).

Analysis of data

All quantitative data are expressed as mean ± s.e. mean. The results were analyzed for differences using Student's unpaired *t* test. We calculated EC_{50} and IC_{50} by the graphical method of described by Turner (1965). Differences were considered to be significant only for $P < 0.05$.

Results

Extracellular ATP-induced $[Na^+]_i$ and $[Ca^{2+}]_i$ elevation in PC12 cells

Extracellular ATP is known to activate phospholipase C (PLC) and elicit ion fluxes across the plasma membrane in PC12 cells. Figure 1 shows that extracellular ATP triggered the elevation of both $[Ca^{2+}]_i$ and $[Na^+]_i$. ATP elevated $[Ca^{2+}]_i$ only slightly in the absence of extracellular Ca^{2+} (dotted trace of Figure 1a), and did not increase $[Na^+]_i$ when extracellular Na^+ was substituted with choline (dotted trace in Figure 1b). This suggests that the ATP-induced $[Na^+]_i$ elevation is caused only by influx from the extracellular space. Activation of bradykinin receptors, which are also linked to PLC, did not change $[Na^+]_i$ although it triggered a $[Ca^{2+}]_i$ rise by intracellular mobilization and influx across the membrane. However, monensin, the Na^+ specific ionophore, did elevate $[Na^+]_i$ in these cells. ATP raised $[Na^+]_i$ in a concentration-dependent manner (Figure 2) with an EC_{50} of 40.75 ± 4.11 μM (95% confidence limit was 13.08).

Lack of involvement of voltage-sensitive Na^+ channel, Na^+/Ca^{2+} exchanger, and Ca^{2+} release-activated channel in the ATP-induced Na^+ influx

We tested the possibility that extracellular ATP-induced Na^+ influx may occur through a voltage-sensitive Na^+ channel by using the voltage-sensitive Na^+ channel blocker, tetrodotoxin. Tetrodotoxin had no effect on the ATP-induced Na^+ influx (Figure 3a).

There still remained the other possibility that extracellular ATP could modulate the activity of the Na^+/Ca^{2+} exchanger. We tested this possibility with benzamil, the blocker of the Na^+/Ca^{2+} exchanger. Pretreatment with 5 μM benzamil did not influence the ATP effect (Figure 3b). To study the in-

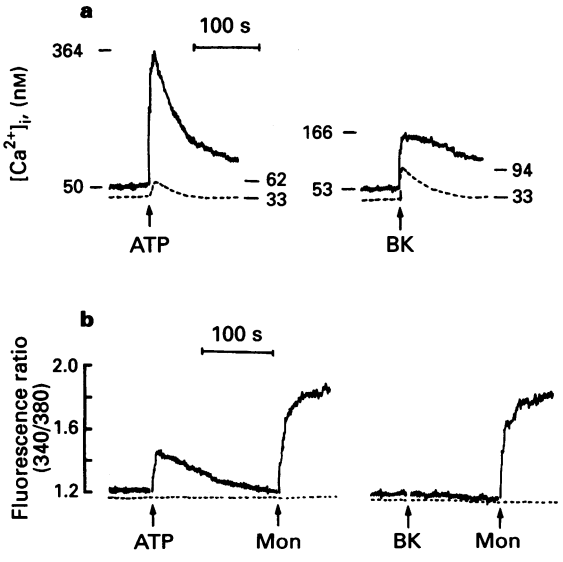

Figure 1 Increases in $[Na^+]_i$ and $[Ca^{2+}]_i$ by extracellular ATP in PC12 cells. (a) PC12 cells were loaded with the fluorescent Ca^{2+} indicator, fura-2/AM, and then treated with 300 μM ATP (left) or 5 μM bradykinin (BK; right). Dotted traces show $[Ca^{2+}]_i$ elevations under Ca^{2+}-free conditions using Ca^{2+}-free Locke solution. (b) PC12 cells loaded with the fluorescent Na^+ indicator, SBFI/AM, were challenged with 300 μM ATP (left) or 5 μM bradykinin (BK; right). Monensin (Mon), 40 μM, the Na^+ specific ionophore, was used in order to reveal the elevation of $[Na^+]_i$ as a positive control. Dotted traces show responses under Na^+-free conditions using Na^+-free Locke solution. Data shown in (a) and (b) are representative of four to eight separate experiments. The results from every experiment were reproducible.

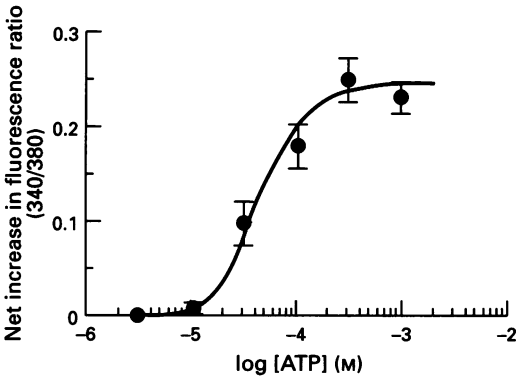

Figure 2 Concentration-dependent elevation of $[Na^+]_i$ by extracellular ATP. Various concentrations of ATP were applied to SBFI-loaded cells and changes in the fluorescence ratio at the peak height were monitored. Data are expressed as mean±s.e. mean for four separate experiments.

volvement of the Na^+/Ca^{2+} exchanger more clearly, we pre-incubated cells with 10 μM BAPTA/AM for 30 min to chelate intracellular Ca^{2+}. However, the ATP-induced $[Na^+]_i$ increase was not changed by the BAPTA/AM treatment, whereas the ATP-induced $[Ca^{2+}]_i$ elevation was completely blocked under these conditions (data not shown).

We also tested whether Na^+ influx might occur with Ca^{2+} influx evoked by the messenger generated from depleted Ca^{2+} stores. Thapsigargin, which is a potent inhibitor of microsomal Ca^{2+}-ATPase and depletes Ca^{2+} stores, did not change the ATP-induced Na^+ influx (Figure 3C).

ATP^{4-}-induced Na^+ influx

Several reports suggested that ATP^{4-} creates nonselective membrane pores that allow passage of ions and small molecules below 831 Dalton (Steinberg *et al.*, 1987). We examined the effect of ATP^{4-} on Na^+ flux. The calculation of $[ATP^{4-}]$ was carried out according to the following equation of Cockcroft & Gomperts (1979):

$$[ATP^{4-}]_{free} = [ATP]_{total}/(10^{3.94} \times [Ca^{2+}] + 10^{4.28} \times [Mg^{2+}])$$

Figure 4 clearly shows that increase in $[Na^+]_i$ was affected by the changes in extracellular Mg^{2+} concentration. The increments of $[Na^+]_i$ caused by treatments with the corresponding calculated concentrations of ATP^{4-} were almost the same even when the total concentrations of extracellular ATP were different. In the first two columns of Figure 4 containing the responses to 5.22 μM ATP^{4-}, both $[Na^+]_i$ increases are similar, although the total concentrations of ATP were 219 μM in one case and 100 μM in the other. The experiments with 0.52 μM ATP^{4-} showed similar increases in $[Na^+]_i$ in an ATP^{4-}-dependent manner, as demonstrated in the last two columns.

Effect of ATP^{4-} on membrane pore formation in PC12 cells

In order to test whether ATP^{4-} makes membrane pores, we examined the effect of ATP on EtBr uptake indicating membrane permeabilization. We employed various concentrations of ATP in the presence or absence of extracellular Mg^{2+}. Figure 5 shows that extracellular ATP did not lead to any increase in fluorescence intensity at either 300 μM, which is the maximal concentration for Na^+ mobilization as shown in Figure 2, nor at 10 mM ATP (equivalent to 240 μM of ATP^{4-}, data not shown). In addition, treatment with the above concentrations of ATP in the absence of extracellular Mg^{2+} also did not trigger entry of EtBr (data not shown). On the other hand, treatment with 10 μM and 50 μM digitonin, which generally permeabilizes the cell membrane, markedly increased the fluorescence intensity.

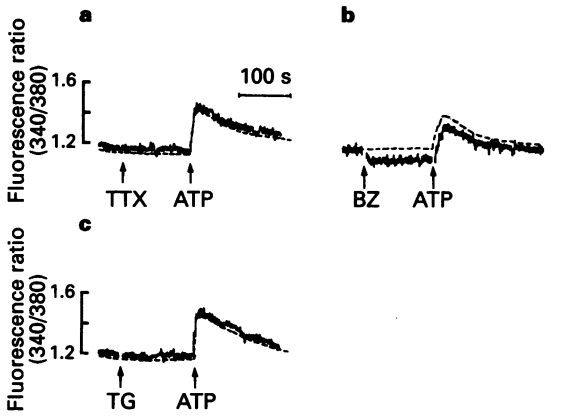

Figure 3 Effects of tetrodotoxin, benzamil, and thapsigargin on the ATP-induced changes in $[Na^+]_i$. SBFI-loaded PC12 cells were preincubated for 3 min with each agent before stimulation with 300 μM ATP; (a) 3 μM tetrodotoxin (TTX); (b) 5 μM benzamil (BZ); (c) 1 μM thapsigargin (TG). Dotted traces in (a), (b) and (c) show the stimulations obtained by 300 μM ATP without the pretreatment with TTX, BZ, and TG, respectively. Data are representative of more than five separate experiments. The results were reproducible.

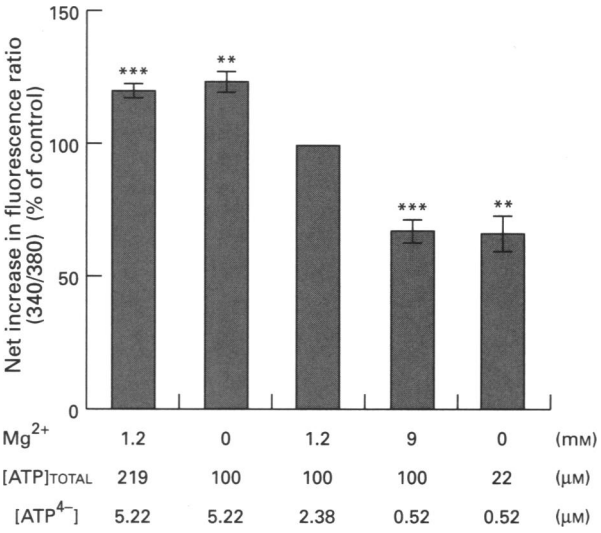

Figure 4 Extracellular Mg^{2+}-dependence of the ATP-induced $[Na^+]_i$ increase. SBFI-loaded PC12 cells were resuspended in Mg^{2+}-free Locke solution to which various concentrations of Mg^{2+} were added subsequently. The amount of calculated tetrabasic form of ATP and the total ATP concentration used are indicated. The responses were converted to percentage of the control, the treatment with 100 μM ATP in the presence of 1.2 mM Mg^{2+}. Data are expressed as mean±s.e. mean for six separate experiments. Statistical significance was found at the indicated level between the control and each response. $^{**}P<0.005$; $^{***}P<0.001$.

Inhibition of ATP-induced Na^+ influx by Reactive Blue 2

Reactive Blue 2 specifically inhibited the ATP-induced elevation of $[Na^+]_i$ with an IC_{50} of 39±4.35 μM (95% confidence limit was 18.72) and Hill slope of 1.29 (Figure 6). Reactive Blue 2 also inhibited the ATP-induced elevation of $[Ca^{2+}]_i$, whereas it did not inhibit the bradykinin-induced $[Ca^{2+}]_i$ increase (data not shown).

Effects of nucleotides and ATP analogues on Na^+ influx

We tested the effects of other nucleotides and ATP analogues on $[Na^+]_i$ elevation. When we applied 100 μM of each analogue

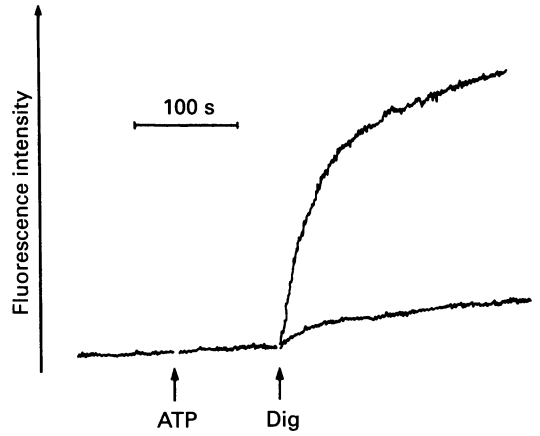

Figure 5 Effect of ATP on the uptake of EtBr. PC12 cells were resuspended in Locke buffer solution containing 25 μM EtBr and then treated with 300 μM ATP: 10 μM (lower trace) and 50 μM (upper trace) digitonin (Dig) were used in order to reveal the entrance of EtBr as positive controls. The entry of EtBr was measured as the fluorescence of the DNA-EtBr complex. Data shown are representative of four separate experiments.

to the cells, ADP, ADP-β-S, AMP, adenosine, CTP, GTP, ITP, XTP, UTP, AMPPCP, AMPCPP, AMPPNP, and BzATP had no effect on Na^+ mobilization. However, 2-MeS ATP, 2-Cl ATP, and ATP-γ-S caused an increase in $[Na^+]_i$ by $75.8 \pm 5.6\%$ ($P<0.05$), $66.4 \pm 7.1\%$ ($P<0.01$), and $53.5 \pm 11.9\%$ ($P<0.01$) of the ATP effect, respectively.

Discussion

Extracellular ATP causes the elevation of $[Ca^{2+}]_i$ by several pathways in PC12 cells. The ATP-induced $[Ca^{2+}]_i$ rise occurs via the voltage-sensitive Ca^{2+} channel (Fasolato *et al.*, 1990), the ATP^{4-}-gated receptor operated channel (Kim & Rabin, 1994), and the Ca^{2+} release-activated Ca^{2+} channel triggered by the depletion of IP_3-sensitive internal Ca^{2+} stores (Suh *et al.*, 1995; Fasolato *et al.*, 1994; Kim & Rabin, 1994). Ca^{2+} influx through Ca^{2+} release-activated channels is evoked by an unidentified messenger which is generated by Ca^{2+} store depletion (Randriamampita & Tsien, 1993; Parekh *et al.*, 1993).

It has been reported that there are voltage-sensitive Na^+ channels and Na^+/Ca^{2+} exchangers in PC12 cells (Stallcup, 1979) and that extracellular ATP causes membrane depolarization (Magoski & Walz, 1992; Kim & Rabin, 1994). We excluded the possibility that ATP-induced Na^+ influx may occur through a voltage-sensitive Na^+ channel opened by membrane depolarization, because tetrodotoxin did not effect the ATP-induced Na^+ influx. Furthermore, bradykinin, which also depolarizes PC12 cells (Fasolato *et al.*, 1990), did not increase intracellular sodium. We also excluded the possibility of a direct activation of the Na^+/Ca^{2+} exchanger by ATP as the Na^+/Ca^{2+} exchanger inhibitor, benzamil, did not influence the ATP effect. Also it was possible that the $[Na^+]_i$ increase could occur indirectly via activation of the Na^+/Ca^{2+} exchanger following the increase in $[Ca^{2+}]_i$. Chelation of cytosolic free Ca^{2+} by BAPTA/AM did not affect the elevation of $[Na^+]_i$ by ATP (data not shown), suggesting that a Na^+/Ca^{2+} exchanger is not involved in the ATP response.

Hoth & Penner (1993) reported that the Ca^{2+} release-activated calcium current mediated by an unidentified messenger is highly selective for calcium. However, Tepel *et al.*, (1994) reported that the filling state of intracellular thapsigargin-sensitive Ca^{2+} pools regulates trans plasma membrane Na^+ and Ca^{2+} influx. We additionally tested whether Na^+ influx occurs through the above channel. The treatment of cells with thapsigargin did not evoke Na^+ influx and did not affect the ATP-induced $[Na^+]_i$ increase, thus excluding the involvement of this channel.

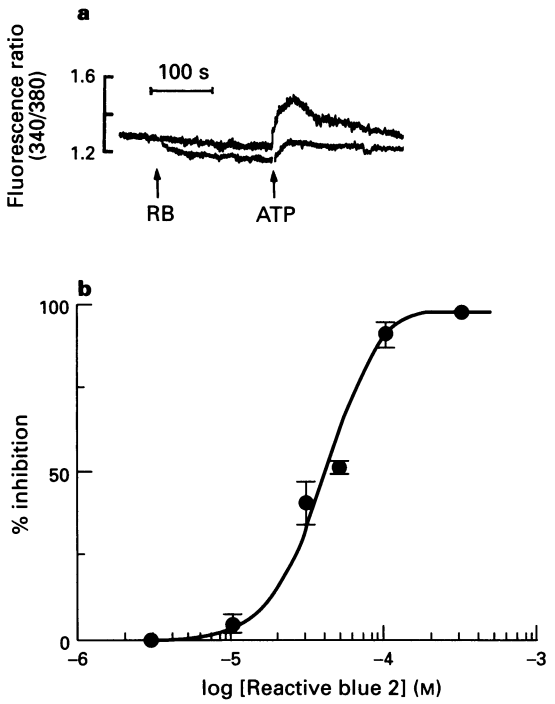

Figure 6 Effect of Reactive Blue 2 on the ATP-evoked $[Na^+]_i$ increase. SBFI-loaded PC12 cells were preincubated for 3 min with various concentrations of Reactive Blue 2 (RB) before stimulation with 300 μM ATP. (a) The response when pretreated with 50 μM Reactive Blue 2 (lower trace) and of the untreated control (upper trace) are shown. Data represent six independent experiments. (b) Concentration-dependent inhibition of $[Na^+]_i$ rise by Reactive Blue 2 is shown. The responses were converted to percentage of the control, the 300 μM ATP-evoked response without Reactive Blue 2 pretreatment. Data are expressed as mean $\pm$ s.e. mean for four separate experiments.

We demonstrated that Na^+ influx occurs through the activation of a P_2 purinoceptor triggered by ATP^{4-}. In physiological buffers and in biological fluids, ATP exists as a mixture of several species including ATP^{4-}, $MgATP^{2-}$ and $CaATP^{2-}$ (Dahlquist & Diamant, 1974). Although the predominant form of ATP in physiological buffers is $MgATP^{2-}$, some studies reported that ATP^{4-} is the receptor ligand that mediates the effect of the P_2 purinoceptor in many kinds of cells including rat mast cells (Dahlquist & Diamant, 1974; Cockcroft & Gomperts, 1979), mouse macrophages (Steinberg *et al.*, 1987), human fibroblasts (Fine *et al.*, 1989), smooth muscle of the guinea-pig vas deferens (Fedan *et al.*, 1990), sheep and rat pituitary cells (Davidson *et al.*, 1990), vascular endothelial cells (Lustig *et al.*, 1992), bovine aortic endothelial cells (Motte *et al.*, 1993), and PC12 cells (Kim & Rabin, 1994). If ATP^{4-} acts as the specific ligand for the P_2 purinoceptor, the effects induced by extracellular ATP should be modulated in an extracellular Ca^{2+} or Mg^{2+} concentration-dependent manner. Our results strongly suggest that Na^+ influx is mediated by an ATP^{4-} receptor in PC12 cells.

Nakazawa *et al.* (1990a) and Neuhaus *et al.* (1991) have shown that a high concentration of extracellular Ca^{2+} reduces the Na^+ current caused by ATP in PC12 cells. The above effect correlates with our observation that a high concentration of external Mg^{2+} reduces the ATP-induced Na^+ influx.

ATP^{4-} permeabilizes cells not only to cations but also to low molecular weight solutes such as EtBr in mast cells, macrophages, and thymocytes (Steinberg *et al.*, 1987; El-Moatassim *et al.*, 1990; Wiley *et al.*, 1993). ATP, however, did not create any increase in cellular fluorescence intensity, even at 10 mM which is 30 times higher than the maximal effective concentration in PC12 cells. In addition, removal of extra-

cellular Mg^{2+} also did not evoke fluorescence changes at the above concentrations of ATP. The results thus indicate that extracellular ATP does not create nonspecific membrane pores.

We conclude that the elevation of $[Na^+]_i$ is mediated by the opening of receptor operated non-selective cation channels activated by ATP^{4-}. This conclusion is supported by the antagonism between the ATP and Reactive Blue 2 (for review see Fedan & Lamport, 1990). Reactive Blue 2 has been studied as an antagonist of ATP-induced inward current and dopamine secretion in PC12 cells (Inoue *et al.*, 1991). It has also been reported that Reactive Blue 2 inhibits [^{32}P]-BzATP photoaffinity labelling in PC12 cells (Majid *et al.*, 1992). The data suggest that Na^+ influx and Ca^{2+} influx occur through the same cation channels. This suggestion is strongly supported by the reported observation that Reactive Blue 2 inhibits the ATP-induced membrane current through an ionotropic P_{2X} purinoceptor cloned from PC12 cells, which has an AMPPCP-insensitive and nondesensitizing manner (Brake *et al.*, 1994; Evans *et al.*, 1995; Kennedy & Leff, 1995). It seems likely that the P_{2X} purinoceptor mainly contributes to the Na^+ influx.

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