Selective and competitive antagonism by suramin of ATP-stimulated catecholamine-secretion from PC12 phaeochromocytoma cells

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- 1 Suramin, a putative P₂-antagonist, (10 to 300 μ M) inhibited the adenosine 5'-triphosphate (ATP)-stimulated secretion of [³H]-noradrenaline or endogenous dopamine from phaeochromocytoma PC12 cells in a concentration-dependent manner. Suramin (300 μ M) did not affect the dopamine-secretion stimulated by high K⁺ or nicotine.
- 2 Suramin shifted the concentration-response curve for ATP to the right. The antagonism was competitive with a pA₂ value of 4.52.
- 3 ATP also stimulated an increase in intracellular Ca²⁺ concentration as determined by fura-2 methods. Suramin antagonized this effect over the same concentration range that antagonized the ATP-stimulated catecholamine secretion.
- 4 These results suggest that suramin can be used as a selective and competitive antagonist of ATP in experiments concerning mechanisms of catecholamine-secretion.

Introduction

Extracellular adenosine and adenine nucleotides have been regarded as modulators in various kinds of cells (Burnstock, 1978; Gordon, 1986; Bean & Friel, 1990). Receptors mediating the effects of adenosine and adenine nucleotides are classified broadly into two types (P₁ and P₂) based mainly upon the order of potency of various agonists (Burnstock & Kennedy, 1985). It was reported recently that suramin blocks the effects of adenosine 5'-triphosphate (ATP) in smooth muscles and it was suggested that suramin acts as a P₂-purinoceptor antagonist (Dunn & Blakeley, 1988; Den Hertog *et al.*, 1989). We have previously reported that suramin inhibits an inward current activated by ATP in PC12 phaeochromocytoma cells (Nakazawa *et al.*, 1990b). However, it was not known whether suramin acts purely as a P₂-purinoceptor antagonist or exerts some other effects on neuronal responses, including catecholamine-secretion.

In the present study, we have measured catecholamine-secretion from PC12 cells and Ca²⁺-concentrations in these cells to investigate the selectivity and the mechanism of action of suramin.

Methods

Cell culture

PC12 cells were prepared as previously described by Inoue & Kenimer (1988). Briefly, PC12 cells were cultured in Dulbecco's modified Eagle's medium containing 7% foetal bovine serum, 7% heat-inactivated horse serum, 2 mM L-glutamine, and 50 μ g ml⁻¹ gentamicin sulphate at 37°C with an atmosphere of 10% CO₂, and then plated into collagen-coated polystyrene dishes (35 mm in diameter) at 1 × 10⁶ cells per dish. After an additional 2 to 3 day-culture, the cells were used for experimentation.

Dopamine- and noradrenaline-secretion

Secretion of dopamine from PC12 was measured by a modification of a procedure described by Inoue & Kenimer (1988). The dishes were washed and incubated with 1 ml of balanced salt solution (BS) containing (in mM): NaCl 150, KCl 5, CaCl₂ 1.8, MgCl₂ 1.2, NaH₂PO₄ 1.2, glucose 10, EDTA 0.1, and HEPES 25 (pH adjusted to 7.4 with NaOH) for 1 h at room temperature. After washing once with BS (1 ml), the cells were stimulated by ATP, nicotine, or high K (155 mM Na in BS was substituted with K) in the absence or presence of suramin. The incubation solution (0.8 ml) was removed 1 min after addition, and mixed with 0.2 ml of 1 N perchloric acid. The cells remaining in the dishes were incubated with 1 ml of 0.2 N perchloric acid for 5 min, removed from the dish, and disrupted by sonication. After centrifugation (1 min, 1000 g), supernatants of both the incubation solution and the cell suspension were collected for measurement of dopamine contents.

Dopamine contents were determined with a high-performance liquid chromatography-electrochemical detector (h.p.l.c.-e.c.d.) system (Bioanalytical Systems). The electrode potential of the detector was set at +0.7 V. A part of the supernatant (10 μ l) was applied to the h.p.l.c. system which consisted of a pre-column (4.6 mm × 30 mm, ODS 5 μ m, Bioanalytical Systems) and an analytical column (4.6 mm × 150 mm, ODS 5 μ m, Bioanalytical Systems). The mobile phase consisted of a monochloroacetate buffer (140 mM, pH 3.1) containing 10% methanol, 0.003% sodium octanesulphonate and 0.15 mM EDTA (flow rate, 1.0 ml min⁻¹). The chromatography was performed at 30°C. The data were analyzed with a chromatographic data processor (Shimadzu C-R3A).

The ratio of secretion was calculated from the dopamine content in the incubation solution (A) and that remaining in the cells (B) by the following equation: % of total dopamine min⁻¹ = 100 × A/(A + B).

In some experiments, noradrenaline-secretion was measured instead of dopamine-secretion according to Inoue & Kenimer (1988). Briefly, the cells were incubated with [³H]-noradrenaline (43.7 Ci mmol⁻¹, 0.125 μ Ci per dish) for 1 h at 37°C and

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then incubated with BS for 1 h at room temperature. After washing with BS, the cells were used in experiments. The ratio of noradrenaline-secretion was calculated from radioactivity in the incubation solution and that remaining in the cells as in the case of the dopamine-secretion described above.

Intracellular Ca^{2+} concentrations

Intracellular Ca^{2+} concentrations were measured with fura-2, a fluorescent Ca^{2+} indicator (Grynkiewicz *et al.*, 1985). The cultured cells were removed from dishes by pipetting and loaded with fura-2 for 30 min by incubation with the membrane permeable dye, fura-2/AM (acetoxymethyl ester form, 10 μM) dissolved in BS. The cells were then rinsed with BS and were placed in the spectrometer (3×10^6 cells ml^{-1}) for intracellular Ca^{2+} measurements (Japan Spectroscopic, CAF-100). Fluorescence was excited at 340 nm and 380 nm, and intensity of the fluorescence (F340 and F380) at 500 nm was measured from the cells at 37°C. Ca^{2+} concentrations were calculated from the ratio of the fluorescence intensity ($R = \text{F340}/\text{F380}$) and a K_d value of 224 nm according to Grynkiewicz *et al.* (1985). R_{max} and R_{min} were obtained after treatment with ionomycin (10 μM) and EGTA (10 mM), respectively. ATP or ATP plus suramin was applied to the cell suspension as a solution containing 100 fold the final concentrations. The final concentrations were readily attained because the cell suspension was continuously stirred during the Ca^{2+} measurement.

Drugs

Drug used were ATP, nicotine (Sigma) and suramin monosodium salt (Bayer). [^3H]-noradrenaline was from New England Nuclear, and fura-2/AM (dissolved in DMSO) was from Dojin.

Data analysis

Data analysis was made according to Tallarida & Jacob (1979). In the curve-fitting for concentration-response data, curves were fitted to the data using an equation, $E/(E_{\text{max}} - E) = ([\text{ATP}]/\text{EC}_{50})^n$, where E is an effect induced by each concentration of ATP. In Schild analysis, the concentration of ATP (A) which induced half-maximal response without suramin and the concentration (A') which induced the same response with suramin of each concentration (B) were obtained from the concentration-response curves, and $\log(A'/A - 1)$ was plotted against $-\log B$.

Statistical analyses were made by Student's t test. Comparison was made within same batches (i.e., the data in a series of experiments obtained in a day). Significant difference ($P < 0.05$) was indicated by an asterisk in figures.

Results

Effect of suramin on secretion of noradrenaline and dopamine

We previously characterized ATP-stimulated catecholamine-secretion by loading PC12 cells with [^3H]-noradrenaline (Inoue *et al.*, 1989; 1990). The receptors mediating the noradrenaline secretion can be classified as the P_2 -type (Burnstock & Kennedy, 1985), judging from the order of agonist potency (Inoue *et al.*, 1990). Suramin (10 to 300 μM) inhibited the [^3H]-noradrenaline-secretion in a concentration-dependent manner (Figure 1a), suggesting that suramin antagonized the P_2 -purinoceptor-mediated response in these cells. Figure 1b shows the effects of suramin on ATP-stimulated dopamine-secretion from PC12 cells as determined with h.p.l.c.-e.c.d. (see Methods). Suramin blocked the ATP-stimulated dopamine-secretion with a concentration-

Figure 1 Effects of suramin on noradrenaline (NA) (a) and dopamine (DA)-secretion (b, c) from PC12 cells. (a, b) Concentration-dependent inhibition by suramin (10 to 300 μM) of extracellular ATP (100 μM)-stimulated noradrenaline (a) or dopamine-secretion (b). (c) Lack of effects of suramin (300 μM) on dopamine-secretion induced by high-K (155 mM) or nicotine (10 μM). Each column represents the mean from three dishes of the same batch. Bars are s.e.mean. Data shown were representative of three (a, b) or two (c) experiments. The blank was obtained without stimulation.

dependency similar to that seen for blockade of the ATP-stimulated noradrenaline secretion (Figure 1a). We characterized the effects of suramin further by measuring its effect on stimulated dopamine-secretion.

Suramin (300 μM) did not affect the dopamine-secretion stimulated by high-K (155 mM) or nicotine (10 μM) (Figure 1c). These results suggest that the blockade is selective for ATP-stimulated secretion. Figure 2 shows the concentration-response curves for the ATP-stimulated dopamine-secretion. Suramin (30 and 100 μM) shifted the curve to the right in a concentration-dependent manner (Figure 2). The maximal response was not altered in the presence of 30 μM suramin (Figure 2), suggesting that the antagonism is competitive.

Figure 2 Concentration-response curves for ATP-stimulated dopamine (DA)-secretion in the absence (●) and presence of 30 (▲) or 100 μM (■) suramin. Each symbol represents the mean from three dishes. Vertical lines are s.e.mean. Smooth curves were fitted to the data using the values: $n = 2.5$, and $\text{EC}_{50} = 50$ (control), 102 (30 μM suramin) or 234 μM (100 μM suramin). Inset: Schild plot for the antagonism by suramin.

Figure 3 Increase in intracellular free Ca^{2+} concentrations ($[\text{Ca}]_i$) induced by ATP and its suppression by suramin. (a) Time course of changes in Ca^{2+} concentrations obtained from cell suspension (3×10^6 cells ml^{-1}). ATP (100 μM) with or without suramin was applied at the time indicated by an arrow, and continuously present in cell suspension. (b) Concentration-dependent antagonism by suramin of ATP-induced increase in Ca^{2+} concentrations. Each column represents the mean of peak Ca^{2+} concentrations after administration of 100 μM ATP with or without suramin. Data were obtained with cells from three dishes of the same batch. Bars are s.e.mean. The data shown were representative of three experiments. The basal Ca^{2+} concentration was measured just before application of drugs.

Competitive antagonism was also indicated by the slope obtained from a Schild plot (Figure 2, inset): the calculated slope (1.08) was close to the value (1.00) predicted from an ideal competitive antagonism (Tallarida & Jacob, 1979). The pA_2 value for suramin determined from the Schild plot was 4.52.

Effect of suramin on the ATP-stimulated increase of intracellular Ca^{2+}

Figure 3a shows a typical recording of intracellular Ca^{2+} concentrations in PC12 cells obtained with fura-2. The intracellular Ca^{2+} concentration was increased from a basal value of about 60 nM to about 700 nM by extracellular ATP (100 μM). Simultaneous administration of suramin suppressed the increment of Ca^{2+} concentration increase without affecting the time-course of the increase (Figure 3a). Suramin antagonized the intracellular Ca response to ATP in a concentration-dependent manner (Figure 3a, b).

Discussion

We have studied the effects of suramin on the ATP-stimulated catecholamine-secreted from PC12 cells. Suramin antagonized the effects of ATP on secretion, in a manner similar to its reported antagonism of ATP-induced smooth muscle contraction (Dunn & Blakeley, 1988) or relaxation (Den Hertog *et al.*, 1989). Thus, suramin may generally antagonize responses mediated through P_2 -purinoceptors.

Suramin antagonized the ATP-stimulated catecholamine-secretion but not that stimulated by nicotine (Figure 1). Lack of blocking effects on the nicotine-induced secretion is in accordance with our previous result showing that suramin blocks an inward current activated by ATP but not that activated by nicotine (Nakazawa *et al.*, 1990b). Suramin thus discriminates P_2 -purinoceptors from nicotinic receptors as well as from muscarinic receptors (Dunn & Blakeley, 1988; Nakazawa *et al.*, 1990b) and α_1 -adrenoceptors (Den Hertog *et al.*, 1989).

The slope of the concentration-response curve for ATP increases in the presence of suramin in the smooth muscle cells (Dunn & Blakeley, 1988; Den Hertog *et al.*, 1989) whereas such an increase was not observed in the present study (Figure 2). The discrepancy suggests that the response to ATP is almost completely mediated through suramin-sensitive receptors in PC12 cells but not in smooth muscle cells.

Suramin suppressed the stimulation of intracellular Ca^{2+} concentration by ATP at the same concentrations that inhibited the ATP-stimulated catecholamine-secretion (Figure 3). These results suggest that the inhibition of catecholamine-secretion by suramin is due to a decrease in free Ca^{2+} concentrations. Suramin also blocked the ATP-activated inward current in PC12 cells at these concentrations (Nakazawa *et al.*, 1990b). The ATP-activated channels are Ca-permeable (Nakazawa *et al.*, 1990a) and the Ca-influx through the channels directly causes the catecholamine-secretion (Inoue *et al.*, 1990). Therefore, it is probable that suramin inhibits the ATP-stimulated catecholamine-secretion by blocking the activation of the channels coupled to the receptors (Bean & Friel, 1990) rather than some subsequent process. This may be indirect evidence that suramin does not exert significant effects other than competition with ATP.

In conclusion, suramin selectively and competitively antagonized the ATP-stimulated catecholamine-secretion from PC12 cells. The antagonistic effect of suramin may arise from its competition with ATP at receptors coupled to the ion channels. Suramin, at the concentrations used in the present study, can be regarded as a pure P_2 -purinoceptor antagonist in experiments concerning catecholamine-secretion.

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