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Antagonism by nifedipine of contraction and Ca^{2+}-influx evoked by ATP in guinea-pig urinary bladder

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1 The effects of Ca^{2+}-antagonists, especially nifedipine, on contraction and increase of intracellular Ca^{2+} (Fura-2/AM method) evoked by ATP were evaluated in a thin outer layer segment of guinea-pig urinary bladder.

2 The ATP-evoked contraction was markedly inhibited by dihydropyridine-type Ca^{2+}-antagonists, such as nifedipine and nitrendipine, but not by D-600, ω-conotoxin and tetramethrin.

3 This antagonism by nifedipine of ATP-evoked contractions was competitive from the Schild plot analysis, the pA_2 value being 8.23. The reduction of ATP-evoked contraction by nifedipine (0.1 μM) was fully reversed by administration of Bay K 8644 (0.1 μM).

4 ATP (100 μM) caused an increase of fluorescence brightness after loading Fura-2/AM, which was coupled with a contraction of the bladder. Both the contraction and the elevation of intracellular Ca^{2+} evoked by the nucleotide were completely antagonized by nifedipine.

5 These results suggest that ATP may activate the dihydropyridine-sensitive, voltage-dependent Ca^{2+}-channels in a direct or indirect fashion and, thereby, elicit a contraction of the bladder.

Introduction

It has been shown that adenosine 5'-triphosphate (ATP) coexists with noradrenaline (Lagercrantz & Stjarne, 1974) or acetylcholine (ACh) (Dowdall *et al.*, 1974) in synaptic vesicles in peripheral autonomic nerves and may function as a neurotransmitter (Katsuragi & Furukawa, 1985; Burnstock, 1986; Lew & White, 1987; Katsuragi *et al.*, 1988). This nucleotide is capable of producing a contractile response of a variety of smooth muscles including the guinea-pig vas deferens (Sneddon & Burnstock, 1984; Sneddon & Westfall, 1984) and guinea-pig urinary bladder (Burnstock *et al.*, 1972; Kasakov & Burnstock, 1983; Westfall *et al.*, 1983; Katsuragi *et al.*, 1986) and blood vessels of the rabbit and dog (Von Kugelgen & Starke, 1985; Muramatsu, 1986). In addition, depolarization by ATP of the cell membranes from guinea-pig vas deferens (Wakui & Inomata, 1985), rat vas deferens (Nakazawa & Matsuki, 1987) and rat basilar artery (Byrne & Large, 1986) have been observed with a double sucrose gap method. Furthermore, miniature endplate potentials (m.e.p.ps) and excitatory junction potentials (e.j.ps) in guinea-pig vas deferens seem to be mediated not by noradrenaline but by ATP, because these m.e.p.ps and e.j.ps were largely blocked by α,β-methylene ATP, a desensitizer of P_{2x}-purinoceptors, and were not affected by prazosin (Sneddon & Burnstock, 1984; Stjärne & Åstrand, 1984).

To date there is no specific antagonist for postsynaptic P_{2x}-receptors (ATP-sensitive receptors). Further characterization of ATP as a neurotransmitter awaits the development of specific P_{2x}-purinoceptor antagonists. Early studies with rat vas deferens suggest that the nature of the contractile response to ATP, but not to noradrenaline, is similar to that of electrical stimulation in that it is antagonized by nifedipine, a Ca^{2+}-channel blocker (Stone, 1981). Blakeley and his colleagues (1981) showed that the electrically-evoked initial phase of the mechanical response in guinea-pig vas deferens was abolished by nifedipine, but the electrically-evoked late phase of the mechanical response was selectively inhibited by prazosin, an α_1-adrenoceptor antagonist, but not by nifedipine.

Therefore, in the present study we attempted to clarify the nature of the antagonism by Ca^{2+}-antagonists (especially nifedipine) of the ATP-evoked contraction and intracellular Ca^{2+} elevation in the guinea-pig urinary bladder.

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Methods

Measurement of tension development

Male guinea-pigs weighing 250–350 g were stunned and bled. A muscle segment (about 1 mm in width and 1 mm in length) of the thin outer layer of the urinary bladder was dissected longitudinally as described previously (Usune *et al.*, 1986) and suspended in a bath (about 0.1 ml). The segment was superfused at 1.8 ml min^{-1} via a peristaltic pump (Gilson, HP-1) with a modified Krebs solution (pH 7.4) bubbled with 97% O_2 and 3% CO_2. The composition of the modified Krebs solution was as follows (mM): NaCl 121.4, KCl 5.9, $CaCl_2$ 2.5, $MgCl_2$ 1.2, $NaHCO_3$ 15.5 and glucose 11.5. The solution was kept at 32°C to inhibit spontaneous contraction. Contractions were recorded isometrically with a strain-gauge transducer (Toyo-Baldwin, T7-8-240). After each preparation had been loaded with 100 mg wt it was allowed to equilibrate for 60 min before the start of the experiment. When the contractions evoked by administration of ATP at 20 min intervals became constant (usually three administrations were needed), test Ca^{2+} antagonists, which were dissolved in either 0.1% ethanol or 0.1% dimethylsulphoxide, were introduced to the superfusate.

Measurement of fluorescence intensity

A thin outer layer muscle segment (about 2 mm width and 7 mm length) of the urinary bladder was dissected and suspended in a bath (about 0.7 ml), and loaded with Krebs solution containing 5 μM Fura-2/AM with 0.01% pluronic F-127 (a non-cytotoxic detergent, gift from Dojin, Japan) for 180 min at room temperature (23–25°C). After being loaded, the strip was superfused at 3.6 ml min^{-1} with the modified Krebs solution. To minimize movement, the segment was stretched and fixed at 200 μm intervals on a silicon board by fine insect pins, but one end of the tissue (about 1 mm length) remained free for the measurement of force development. Contractions of the tissue were recorded isometrically with a strain-gauge

transducer (HBM, WO.5T). The fluorescence signals and contractions were measured simultaneously in the same preparation.

As described previously (Usune *et al.*, 1989), fluorescence signals were measured by a single beam fluorometer (Farrand, Ratio fluorometer-2) which was equipped with a mercury arc lamp of 85 W. After exposure to an excitation wavelength of either 340 or 380 nm, fluorescence signals from the tissue were measured at an emission wavelength of 511 nm, by a photomultiplier tube (Hamamatsu photonics, R1527 SEL), and the output voltage was further amplified (16 Hz cut-off frequency). The excitation light was passed through a slit (about 1 mm width and 3 mm length) before reaching the tissue. It is well documented (Himpens & Somlyo, 1988) that during exposure to 340 nm light, stimulation of the tissue produces an increased fluorescence (511 nm) from the strip which is proportional to the concentration of intracellular free Ca^{2+} combined with Fura-2. In contrast, a decrease in fluorescence after excitation at 380 nm, reflects a reduction of intracellular free Fura-2 concentration following formation of a Fura-2-Ca^{2+} complex. Hence, after it had been confirmed that the fluorescence from a strip excited at a wavelength of 380 nm was decreased by ATP (300 μM), the present fluorescence measurement was carried out with a single beam excitation light of 340 nm (not by the fluorescence ratio method, Usune *et al.*, 1989). Addition of ATP or nifedipine in the absence of Fura-2 caused no autofluorescence with excitation and emission wavelengths at 340 and 511 nm, respectively (data not shown).

Drugs

The drugs used here were: ATP benzeneacetonitrile hydrochloride (Boeringer Mannheim), nifedipine, nitrendipine (Sigma), D-600 (benzene acetonitrile hydrochloride; gift from Knoll), ω-conotoxin (Peptide Inst.), tetramethrin (gift from Sumitomo Chem.), Bay K 8644 (methyl 1,4-dihydro-2,6-dimethyl-3-nitro-4-(2-trifluoromethylphenyl)-pyridine-5-carboxylate; gift from Bayer), tetrodotoxin (Sankyo) and Fura-2/AM (Dojin).

Statistical analysis

Values are expressed as mean ± s.e. and n indicates the number of experiments. The statistical significance was assessed by paired t test for values from the fluorescence experiment and by Dunnett's t test for multiple comparisons between several values obtained with different concentrations of an antagonist and a control value. Differences were considered to be significant when $P < 0.05$.

Results

Effects of Ca^{2+}-antagonists on the ATP-evoked contraction

ATP administered at 20 min intervals, produced phasic contractions of guinea-pig urinary bladder in a concentration-dependent manner, in the range of 1.0 to 1000 μM (Figure 1). The ATP-evoked contraction (100 μM) was virtually unaffected by exposure to tetrodotoxin (0.3 μM) for 40 min (data not shown). However, this ATP-evoked contraction was completely abolished by exposure to Ca^{2+}-free medium containing 10 μM EGTA for 20 min (data not shown). In the presence of Mn^{2+} at concentrations of 100 and 300 μM, the ATP-evoked contractions were reduced to 83.2 ± 8.3% ($n = 8$) and 56.9 ± 5.2% ($n = 6$, $P < 0.05$), respectively. The effects of various organic Ca^{2+}-channel blockers in concentrations ranging from 0.1 to 1 μM on ATP (100 μM)-evoked contraction (100%) are expressed as alterations of % contraction. The inhibition by nifedipine (0.3 μM) was most potent. Nitrendipine exhibited a moderate blockade of this ATP-evoked contraction. However, the Ca^{2+}-antagonists ω-conotoxin, D-600 and tetramethrin did not inhibit the ATP-evoked contraction even at a concentration of 1 μM (Figure 2).

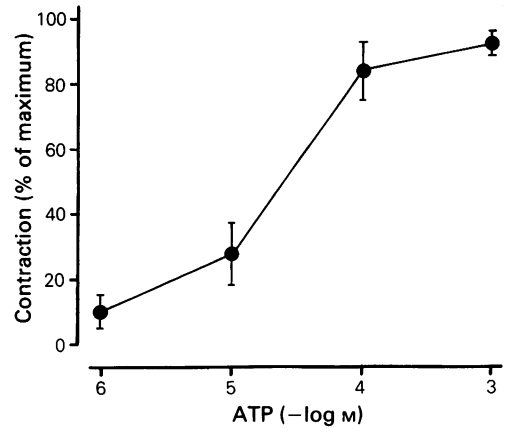

Figure 1 The concentration-response curve for the contraction induced by ATP in guinea-pig urinary bladder. A series of ATP concentrations was administered at 20 min intervals to the perfusate. Contractions were expressed as % of the maximum response in each preparation. Each point indicates mean and vertical lines show s.e. ($n = 4$). The maximum ATP (1000 μM)-induced contraction was 2.07 ± 0.04 mN.

Antagonism by nifedipine of the ATP-evoked contraction

Concentration-response curves for ATP shifted to the right in the presence of nifedipine (0.03 to 0.3 μM) (data not shown). In order to analyse the mode of inhibition by nifedipine from the data, Schild plots were made by plotting the log

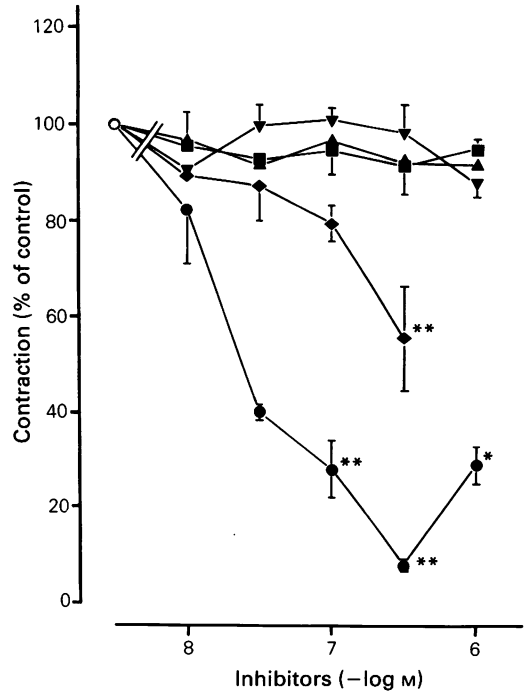

Figure 2 Effects of organic Ca^{2+}-antagonists on ATP-evoked contraction. Contractions are expressed as a percentage of the control contraction induced by ATP (100 μM) without antagonists. (○) Control; (■) ω-conotoxin; (▲) D-600; (▼) tetramethrin; (◆) nitrendipine; (●) nifedipine. After the contractions evoked by repeated administrations of ATP at 20 min intervals had attained a constant amplitude, various concentrations of the respective Ca^{2+}-antagonist were introduced to the perfusate and their effects on the ATP-evoked contractions were examined in the same preparation. Each point indicates the mean and vertical lines show s.e. ($n = 4$ to 10). * $P < 0.05$; ** $P < 0.01$ from the control (Dunnett's t test).

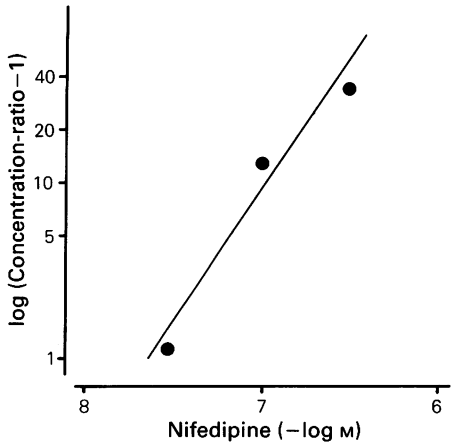

Figure 3 Schild plot of antagonism by nifedipine of ATP-evoked contraction. Concentration-ratio of ATP denotes the ratio of the 50% responses elicited by the agonist in the presence and absence of various concentrations of nifedipine. Each point indicates the mean value from ten experiments.

(concentration-ratio − 1) of ATP against −log molar concentration of nifedipine (Figure 3). The apparent pA_2 value of the Ca^{2+} antagonist for ATP obtained from this plot was 8.23 and the slope 0.977 (correlation coefficient, $r = 100$), suggesting a competitive antagonism.

In the presence of nifedipine (0.3 μM), the ATP (100 μM)-evoked contraction was almost abolished. However, after subsequent introduction of Bay K 8644 (0.3 μM), the contraction of the bladder evoked by ATP reappeared. A recording from a single experiment is shown in Figure 4.

Effect of nifedipine on the ATP-evoked contraction and the fluorescence signals

The thin outer layer preparation of guinea-pig urinary bladder previously exposed to Krebs solution containing 5 μM Fura-2/AM for 180 min was used for this experiment. ATP (100 μM) produced a contraction coupled with an increased fluorescence (340 nm/511 nm), indicating an increased intracellular Ca^{2+}-concentration. Typical recordings of the contractions and the fluorescence signals (340 or 380 nm/511 nm) evoked by ATP administered at 20 min intervals in the presence or absence of nifedipine are shown in Figure 5. When the fluorescence intensities at 340 nm (excitation) and 511 nm (emission) caused by the first application of ATP to the tissue after loading with Fura-2/AM were expressed as 100%, the intensities produced by the second application of the nucleotide after 20 min with and without nifedipine (0.1 μM) amounted to 28.2 ± 3.4% ($n = 4$) and 80.0 ± 7.7% ($n = 4$), respectively. The difference of the values between the second applications was statistically significant ($P < 0.05$ by paired t test).

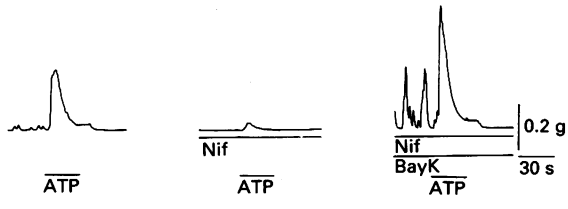

Figure 4 Effects of Bay K 8644 on the antagonism by nifedipine of the ATP-evoked contraction. Nifedipine (Nif, 0.3 μM) alone and in combination with Bay K 8644 (Bay K, 0.3 μM) was added to the perfusate 20 min before administration of ATP (100 μM) in the same preparation. This single experiment was chosen from four experiments showing similar results.

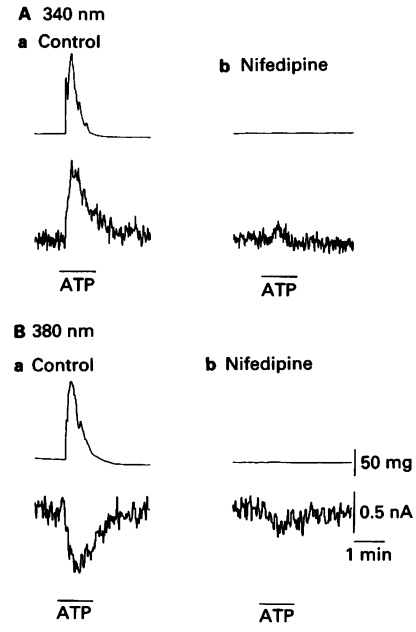

Figure 5 Typical traces of contractions and intracellular Ca^{2+} elevation evoked by ATP (100 μM) in the presence or absence of nifedipine (0.1 μM). (A) 340 nm (excitation); 511 nm (emission). (B) 380 nm (excitation); 511 nm (emission). (A) and (B) were taken from the same preparation. Upper traces; contractions. Lower traces; fluorescence signals. Repeated administrations of ATP were made at 20 min intervals to the perfusate. Nifedipine was introduced to the perfusate 20 min before ATP.

Discussion

When electrical stimulation is applied to the nerves of guinea-pig or rat urinary bladder, an atropine-resistant twitch contraction occurs (Dahlen & Hedqvist, 1980). ATP administered exogenously to this tissue also evokes a transient contraction. α,β-Methylene ATP, a desensitizer of P_{2x}-purinoceptors, is able to inhibit both the ATP-evoked contraction and the atropine-resistant component of the nerve-evoked contraction (Kasakov & Burnstock, 1983; Katsuragi *et al.*, 1986). From such facts, Burnstock and his coworkers (1978) have postulated that the non-cholinergic innervation of this tissue may be 'purinergic'. Recent double sucrose gap studies provided further evidence that electrically evoked e.j.ps in guinea-pig, rabbit or pig urinary bladder were abolished by desensitization of P_{2x}-purinoceptors with α,β-methylene ATP, but not by atropine, (Fujii, 1988). In the present work, we verified that contraction of the guinea-pig bladder could be evoked by ATP in a concentration-dependent fashion. This ATP-evoked contraction was inhibited by L-type Ca^{2+}-channel blockers such as nitrendipine and nifedipine, the latter being a more potent and competitive antagonist for the ATP evoked-contraction. On the other hand, ω-conotoxin, (L- and N-type Ca^{2+}-channel blocker, McCleskey *et al.*, 1987), tetramethrin, (T-type Ca^{2+}-channel blocker, Hagiwara *et al.*, 1988), and D-600 (L-type blocker) did not display such antagonism.

According to Triggle *et al.* (1979), the potencies of nifedipine for inhibition of muscarinic agonist- and KCl-evoked phasic contractions in guinea-pig ileum were 50 and 4 times, respectively, more than those of D-600. Thus, our finding that nifedipine was about 10 times more potent than D-600, suggests that the comparative potencies of inhibition vary with the different stimulants used to evoke a given response.

In an earlier study with rat vas deferens, French & Scott (1981) first presented the possibility that nifedipine blocked selectively the electrically-evoked non-adrenergic contraction from the prostatic end of the rat vas deferens without affecting the adrenergic contraction. From a series of studies on this nucleotide, it has been proposed that this non-adrenergic con-

traction is mediated by ATP. In the guinea-pig vas deferens, the initial and late phases of the mechanical response to nerve stimulation were selectively inhibited in the presence of nifedipine and prazosin, respectively (Blakeley *et al.*, 1981). The study by Stone (1981) showed that contractions of guinea-pig vas deferens evoked by ATP and nerve stimulation were inhibited with a similar time-course after addition of nifedipine (5 μM). It has also been found that nifedipine blocks the purinergic rather than the adrenergic nerve-mediated vasopressor responses in the pithed rat (Bulloch & McGrath, 1988). In the guinea-pig urinary bladder, we found that the ATP-evoked contraction was competitively antagonized by nifedipine with a pA_2 value of 8.23 from the Schild plot analysis. In addition, the inhibition of the ATP-evoked contraction by nifedipine was reversed by administration of Bay K 8644, a Ca^{2+}-agonist. As well as causing this contraction, ATP simultaneously produced an increased fluorescence intensity of the tissue due to a rise in the intracellular Fura-2-Ca^{2+} complex. This evoked fluorescence brightness was also antagonized by nifedipine (0.3 μM).

It has been shown that exogenously applied ATP depolarizes the vas deferens of the rat (Nakazawa & Matsuki, 1987) or guinea-pig (Wakui & Inomata, 1985) and single cells (Benham *et al.*, 1987), as well as intact preparations (Miyahara & Suzuki, 1987), from the rabbit ear artery. Patch-clamp studies have demonstrated ATP-sensitive K^+-channels on guinea-pig or rabbit cardiac cells (Noma, 1983). Similarly, in guinea-pig myenteric neurones. ATP seems to induce a membrane hyperpolarization in S-neurones and depolarization in AH-neurones which are due to activation and inactivation of a Ca^{2+}-sensitive K^+-conductance, respectively (Katayama & Morita, 1989). Accordingly, the membrane depolarization elicited by ATP might result from a decrease of K^+-outward current. On the other hand, ATP seems to open several types of channel directly. Benham & Tsien (1987) have shown that a receptor gated non-selective cation channel of smooth muscle cells from rabbit ear artery can be activated by ATP and can mediate inward Ca^{2+} current. In single vascular smooth muscle cells isolated from guinea-pig mesenteric artery, intracellularly applied ATP activated the slow Ca^{2+}-channels, but not the fast Ca^{2+}-channels, in a concentration-related manner (Ohya & Sperelakis, 1989). A fluorescence study with Indo-I indicated that ATP stimulates transsarcolemmal influx of Ca^{2+} in rat cardiac myocytes (Danziger *et al.*, 1988).

From these findings, the following possible mechanism may account for the nifedipine-sensitive fluorescence brightness developed by ATP. ATP could first depolarize the cell membrane of guinea-pig urinary bladder via the opening of a non-selective cation channel or by inactivating Ca^{2+}-sensitive potassium conductance. Subsequently, the voltage-gated Ca^{2+} channels must be opened by this membrane depolarization and these Ca^{2+}-channels are primarily nifedipine-sensitive. There is also the possibility that an increase in Ca^{2+}-influx by ATP is brought about directly by the alteration of an active site on the molecule of the voltage-gated Ca^{2+}-channels (Ohya & Sperelakis, 1989), or through activation of a G-protein as shown with adenosine, a breakdown metabolite of ATP (Kurachi *et al.*, 1986). Further patch-clamp studies are needed to define the precise mode of action of ATP involved in the facilitation of the Ca^{2+}-influx.

However, the lack of sensitivity of the response to D-600, which is thought to have a similar effect on L-type Ca^{2+} channels to nifedipine, leaves open the possibility that nifedipine may be a competitive antagonist for the P_{2x}-purinoceptors. This question remains to be solved.

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