Comparison of Contractile Mechanisms by Carbachol and ATP in Detrusor Strips of Rabbit Urinary Bladder

Ken-Ichi Kishii, Tetsuhiro Hisayama[#] and Issei Takayanagi[*]

Department of Chemical Pharmacology, Toho University School of Pharmaceutical Sciences, 2-2-1, Miyama, Funabashi, Chiba 274, Japan

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ABSTRACT — Contractile mechanisms by carbachol and ATP were compared in the detrusor strips of rabbit bladder. To exclude modulation of the agonists-induced responses by intramurally synthesized prostaglandins, all the experiments were done in the presence of the potent cyclooxygenase inhibitor flurbiprofen (1 μM). The concentration-response curves for carbachol and ATP were shifted to the right by 6–10-fold by verapamil (10 μM), which abolished the K-induced contraction of the atropinized detrusor completely. A similar curve for carbachol was obtained in the absence of extracellular Ca [$(Ca)_o$], but the contraction by ATP below 1 mM was more reduced by $(Ca)_o$-depletion than by verapamil. Under Ca-free conditions, repeated applications of ATP resulted in no response, but those of carbachol induced reproducible contractions. These results suggest that carbachol and ATP induces Ca-influx through L-type Ca channels and releases Ca from the Ca stores. However, while carbachol might increase the sensitivity of contractile machinery to Ca on the one hand, ATP would open additional, verapamil-insensitive Ca channels.

Much evidence indicates that smooth muscles have multiple sources of Ca ions which are utilized for contraction (1). In most smooth muscle cells, extracellular Ca enters the muscle cells through the Ca channels. Ca channels have been classified into L-, T- and N-types (2); and among them, the L-type channel with sensitivity to Ca antagonists is predominant in the smooth muscle cells, but the presence of the T-type like channels have been reported (2). In addition, release of intracellular Ca can be activated by several agonists via receptor-mediated signal transduction pathways (3), which have been also implicated in the recently found increased Ca sensitivity of the contractile machinery induced by some receptor agonists (4, 5).

Sources of Ca and change in Ca sensitivity of contractile elements have been widely investigated for vascular smooth muscle. Less information is available with regards to the detrusor muscle of the urinary bladder. Information in this field appears of particular interest because of the possible use of drugs in the management of bladder motility disorders such as detrusor hyperreflexia and bladder atonia.

It is well-known that cholinergic and non-cholinergic (possibly purinergic) neurons innervate most of the mammalian urinary bladder (6–8). Several studies have suggested that

[#]Present address: Department of Chemical Pharmacology, Faculty of Pharmaceutical Sciences, University of Tokushima, 1-78-1, Shomachi, Tokushima 770, Japan

[*]To whom all correspondence should be addressed.

multiple contractile mechanisms are utilized by cholinergic drugs and ATP. Ca entry via the L-type Ca channels has been proposed as a likely mechanism for bladder contraction induced by muscarinic activation in the detrusor muscle from rabbits, guinea pigs or rats (9–11), although the response to carbachol may also involve release of stored Ca in the rat and guinea pig (11–13). In rat detrusor, ACh and ATP show similar dependence on extracellular Ca [$(Ca)_o$] (12). Recent studies, however, suggested that multiple sourced of Ca for contraction may by utilized differentially, depending on the receptor agonist applied (14).

In the present study, the contractile mechanisms involved in the carbachol- and ATP-induced contraction were compared.

MATERIALS AND METHODS

Male albino rabbits weighing between 2 and 3 kg were reared on a standard diet and given tap water to drink.

The urinary bladder was rapidly removed and a longitudinal strip (about 2 × 20 mm) of the detrusor muscle was prepared. The strips were vertically suspended under a resting load of 10 mN in an organ bath which contained Krebs solution of the following composition: 118 mM NaCl, 4.75 mM KCl, 2.50 mM $CaCl_2$, 1.20 mM $MgSO_4$, 1.20 mM KH_2PO_4, 25.0 mM $NaHCO_3$ and 10.0 mM glucose. In some experiments, 1 μM flurbiprofen with or without 1 μM atropine was dissolved in the solution. The organ bath was maintained at 37°C and constantly gassed with 5% CO_2 in O_2. The responses to drugs were recorded isotonically. After the preparations had been allowed to equilibrate for at least 60 min, they were usually exposed twice to a submaximum concentration of carbachol (300 nM). Several concentration-response curves for carbachol were then determined; thereafter, the curve for purine nucleotides were also determined if necessary. The KCl-induced contraction was measured using solutions in which various concentrations of NaCl were replaced with KCl isosmotically. In some experiments, tissues were washed for some periods with Ca-free, 2 mM ethyleneglycol bis (β-aminoethylether)-*N*,*N*'-tetraacetic acid (EGTA)-containing solution, and carbachol and/or ATP was then applied.

The sensitivity to each drug was expressed as its pD_2 value (the negative logarithm of molar concentration which produced 50% of its own maximum response), and the potency of verapamil as a noncompetitive antagonist against the K-induced contraction was expressed as the pD'_2 value (the negative logarithm of molar concentration which reduces the maximum response to 50%). These values were calculated from graphical analyses. All numerical data are expressed as means with S.E., and the number of experiments is shown in parentheses. Statistical analyses were performed by the *t*-test and Duncan's new multiple range test as appropriate. ATP value less than 0.05 was considered to indicate a significant difference.

Drugs used were carbachol chloride, vanadium-free adenosine 5'-triphosphate (ATP), α,β-methylene ATP, β,γ-methylene ATP, atropine sulfate, reactive blue 2, verapamil hydrochloride, tris (hydroxymethyl) aminomethane (Tris), flurbiprofen (Sigma Chemical Co., MO, USA) and EGTA (Wako Pure Chemical Industries, Ltd., Osaka, Japan). All drugs were in powder form, and they were dissolved in deionized, distilled water, except for EGTA and flurbiprofen. Stock solutions of EGTA (0.2 M) were dissolved by adding Tris to bring the pH to 7.4, and flurbiprofen (10 mM) was dissolved in 2% Na_2CO_3 in 50 mM Tris base followed by immediate titration with HCl to pH 8.0. They were directly diluted in appropriate physiological saline solutions. Other chemicals used were of analytical grade.

RESULTS

Effect of cyclooxygenase inhibitor on the carbachol- and ATP-induced contractions

A potent cyclooxygenase inhibitor flurbiprofen (1 μM) depressed the contraction by low concentrations of ATP (0.1–3 mM), without having any effect on the maximum re-

sponse. On the other hand, the maximum response to carbachol was increased by flurbiprofen, without any change in the sensitivity to the spasmogen (Fig. 1). These results suggest that the contractile responses to ATP and carbachol are modulated by intramurally synthesized prostaglandins (PGs) in a different manner. Although the precise mechanisms of these differential effects of the cyclooxygenase inhibitor on the two stimulants are not known, in the following experiments, to prevent complexity due to modulation of the carbachol- and ATP-induced Ca mobilizations by PGs, 1 μM flurbiprofen was included in the bathing solutions throughout.

Properties of purinoceptors in rabbit detrusor

Two congeners, α,β-methylene ATP and β,γ-methylene ATP, as well as ATP, produced concentration-dependent contractions of the detrusor strips. The maximum contractions by all these purine nucleotides were similar to that by K and smaller than that by carbachol. Among the nucleotides, the maximum responses to ATP and β,γ-methylene ATP were comparable to each other, but significantly larger than the α,β-methylene ATP-induced one (Fig. 2, Table 1). On the other hand, the rank order of sensitivities to these purines was α,β-methylene ATP > β,γ-methylene ATP > ATP (Fig. 2, Table 1). Adenosine (up to 10 mM) did not produce any detectable response. A putative P_{2y}-purinocepor antagonist, reactive blue 2, did not antagonize the ATP-induced contraction, but rather somewhat potentiated it (Fig. 3). A similar potentiation was observed in the carbachol-induced contraction to the same degree, suggesting that the potentiation was nonspecific in nature.

Table 1. The pD_2 values of ATP, its analogues and carbachol in flurbiprofen (1 μM)-treated rabbit bladder detrusor strips

Drug	PD_2
Carbachol	6.90 ± 0.03 (6)
α,β-methylene ATP	5.97 ± 0.19 (5)
β,γ-methylene ATP	4.51 ± 0.04 (6)
ATP	2.60 ± 0.05 (9)

Values are presented as means ± S.E. and the numbers of experiments are indicated in parentheses.

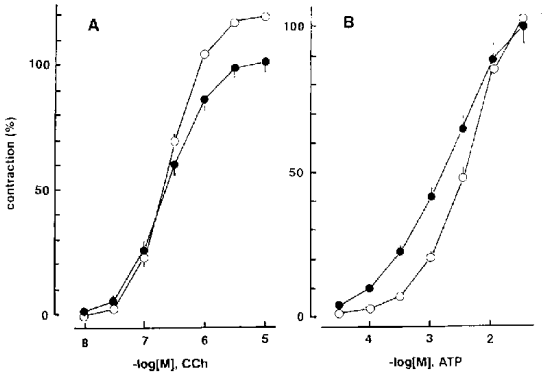

Fig. 1. Effects of flurbiprofen on carbachol (A)- and ATP (B)-induced contractions in the rabbit bladder detrusor strips. ●, control; ○, after a 60-min treatment of 1 μM flurbiprofen. Abscissa, concentrations of carbachol (A) and ATP (B); ordinate, % maximum contraction by each drug in the absence of flurbiprofen. Each value is expressed as a mean ± S.E. (bar) of 6 experiments.

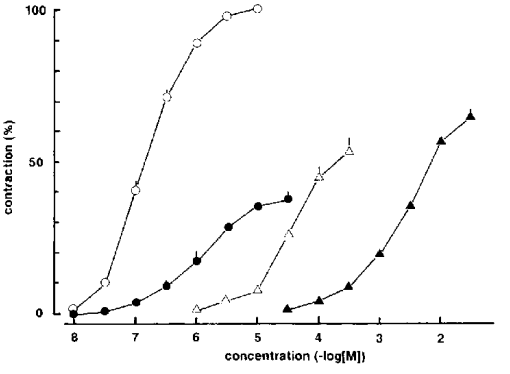

Fig. 2. Concentration-response curves for ATP, its analogues and carbachol in the flurbiprofen (1 μM)-treated rabbit bladder detrusor strips. ○, carbachol; ▲, ATP; △, β,γ-methylene ATP; ●, α,β-methylene ATP. Abscissa, concentrations of drugs; ordinate, % maximum contraction by carbachol. Each value is expressed as a mean ± S.E. (bar) of 6–7 experiments.

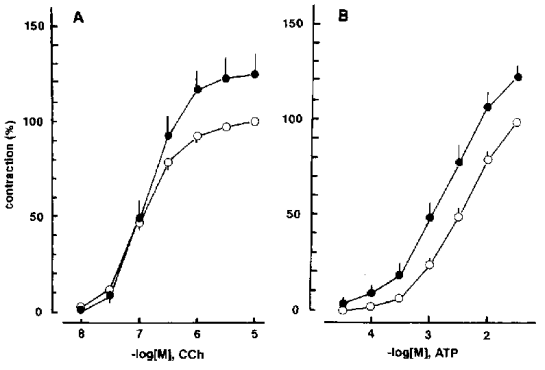

Fig. 3. Effects of reactive blue 2 (100 μM) on the carbachol (A)- and ATP (B)-induced contractions of flurbiprofen (1 μM)-treated rabbit bladder detrusor stips. ○, control; ●, in the presence of reactive blue 2 (100 μM). Each value is expressed as a mean ± S.E. of 6 experiments.

Verapamil sensitivities in K-, carbachol- and ATP-induced contractions

A Ca antagonist verapamil depressed the concentration-response curve for K in a non-competitive manner ($\geq$ 0.1 μM, data not shown), and the pD'_2 value for verapamil was 56.1 ± 0.15 (n = 5). However, 1 μM atropine reduced the response to higher concentrations of K ($\geq$ 45.87 mM), and it inhibited the maximum contraction produced by 85.87 mM K to 71.3 ± 3.4% (n = 5) of the control value (Fig. 4A, $P < 0.001$). These results suggest that K-depolarization, whilst contracting the muscle directly, indirectly stimulated it via a release of acetylcholine from parasympathetic nerve endings in this tissue. When 1 μM atropine was present, the K-induced contration was more readily inhibited by verapamil and completely abolished by 10 μM concentration of the drug (Fig. 4B). The pD'_2 value for verapamil was 6.85 ± 0.12 (n = 6, significantly different from that obtained without atropine), and the potency of verapamil in inhibiting the K-induced contraction was increased 17-fold (antilog of the difference between the pD'_2 values for verapamil determined in the absence and presence of atropine).

In contrast, verapamil did not abolish either the carbachol- or ATP-induced contraction.

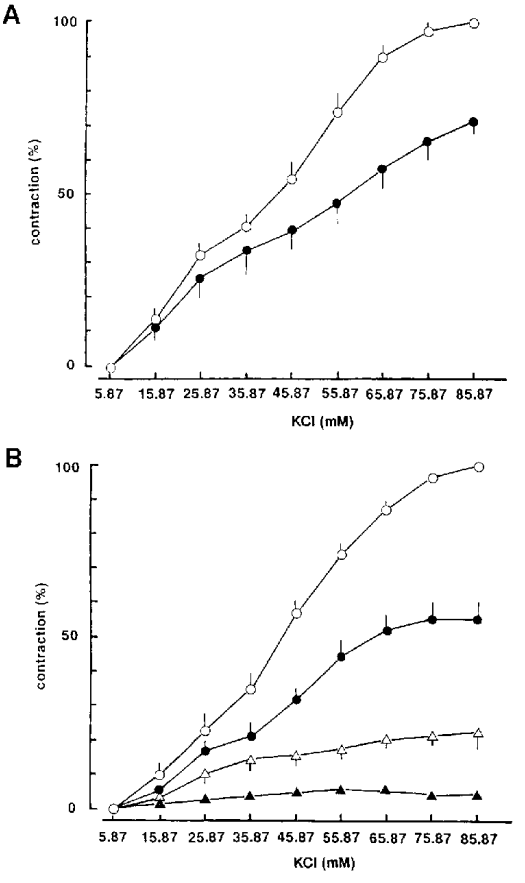

Fig. 4. Effects of atropine (A) and verapamil in the presence of atropine (1 μM; B) on the K-induced contractions of flurbiprofen (1 μM)-treated rabbit bladder detrusor strips. (A) ○, control; ●, in the presence of atropine (1 μM). (B) ○, control; ●, △, ▲, in the presence of 0.1, 1, 10 μM verapamil, respectively. Abscissa, concentrations of K; ordinate, % maximum contraction by K in the absence (A) and presence (B) of atropine (1 μM). Each value is expressed as a mean ± S.E. of 6 experiments.

The concentration-response curve for carbachol was shifted to higher concentrations by only 6.4 ± 0.8-fold (n = 6) at the control EC_{50} level, with a slight but significant reduction of the maximum response to 86.1 ± 2.5% (n = 6) of the controls by 10 μM verapamil (Fig. 5A, B), which was a sufficient concentration to abolish the K-induced contraction (Fig. 4B). Similarly, the concentration-response

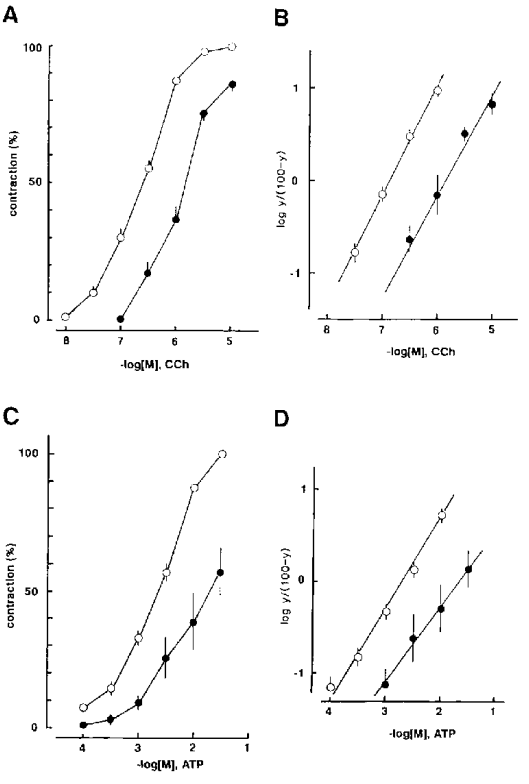

Fig. 5. Effects of verapamil (10 μM) on the carbachol (A, B)- and ATP (C, D)-induced contractions of flurbiprofen (1 μM)-treated rabbit bladder detrusor strips. ○, control; ●, in the presence of verapamil (10 μM). B and D are logit-log representations of the data shown in A and C, respectively. Abscissa, concentrations of carbachol (A, B) and ATP (C, D); ordinate, % maximum contractions by carbachol (A) and ATP (C); log y/(100−y), where y is % maximum contractions by carbachol (B) and ATP (D). Each value is expressed as a mean ± S.E. of 6 expreriments.

curve for ATP also seemed to be shifted to the right by verapamil, although, unfortunately, the maximum response to ATP could not be obtained, because higher ATP concentrations than 30 mM were not applied to avoid hypertonicity (Fig. 5C). The logit-log analyses of the curves in the absence (control) and presence of 10 μM verapamil, however, showed that there was statistically significant parallelism between the two curves ($P < 0.05$, Fig. 5D). The shift of the concentration-response curve for ATP by 10 μM verapamil was 9.8 ± 2.1 fold ($n = 5$) at the control EC_{50} level and was not significantly different from the value for carbachol ($P > 0.05$).

Effects of extracellular Ca deprivation on K-, carbachol- and ATP-induced contractions

The K-induced contraction (in the presence of 1 μM atropine) totally disappeared 5 min after incubation of the atropinized tissue in Ca-free, 2 mM EGTA-containing solution (Fig. 6A). However, as the results with verapamil, a substantial amplitude of contractions by carbachol and ATP remained even in the absence of extracellular Ca (Fig. 6B, C). The concentration-response curve for carbachol in the absence of $[Ca]_o$ fairly agreed with the curve in the presence of 10 μM verapamil (Fig. 6B). The amplitude of contraction by 30 mM ATP was the same whenever it was applied in the presence of 10 μM verapamil or in the absence of $(Ca)_o$. However, when lower ATP concentrations ($\leq$ 10 mM) were used, the contraction was more readily inhibited by $(Ca)_o$ deprivation than by treatment with 10 μM verapamil (Fig. 6C): the amplitudes of responses to ATP were significantly greater in the verapamil-treated muscles than in the $[Ca]_o$-deprived ones at each concentration of ATP ($P < 0.05$). Increasing the verapamil concentration up to 100 μM did not result in further depression of the ATP-induced contraction ($n = 6$, data not shown).

We then tried to learn the properties of carbachol- and ATP-induced contractions of the $(Ca)_o$-deprived tissues in more detail. After changing the normal Krebs solution to Ca-free, 2 mM EGTA-containing solution, carbachol (10 μM) or ATP (30 mM) was applied at timed intervals, washing the muscle with Ca-free solution when the contraction by each application of the drug reached a plateau (Fig. 7A). When carbachol was used as a stimulant, the first contraction was slightly reduced [90.6 ± 2.1% ($n = 10$)], compared to the controls observed in normal medium, but the following sequential applications resulted in small but reproducible contractions that were constant in size and sustained until at least 60

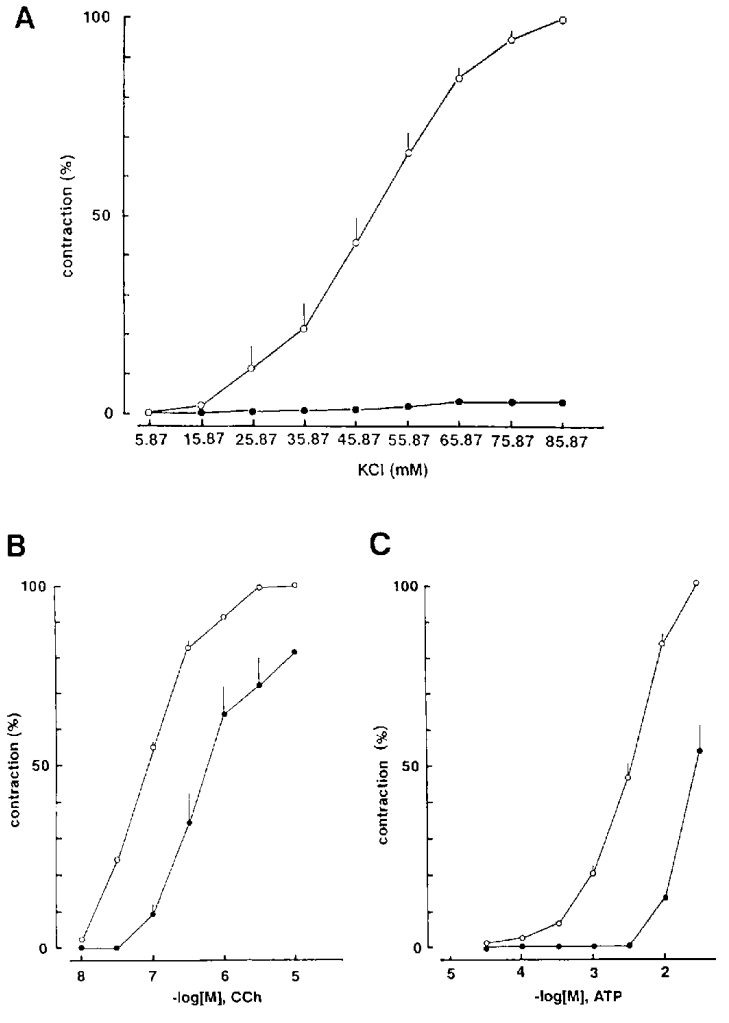

Fig. 6. Effects of depletion of extracellar Ca on the isosmotic K (A)-, carbachol (B)- and ATP (C)-induced contractions of flurbiprofen (1 μM)-treated rabbit bladder detrusor strips. ○, control; ●, responses 5 min after changing the medium to Ca-free, 2 mM EGTA-containing solution. Abscissa, concentrations of K (A), carbachol (B) and ATP (C); ordinate, % maximum contraction by each stimulant. Each value is expressed as a mean ± S.E. of 6 experiments.

min after $(Ca)_o$ deprivation. When the preparation remained unstimulated for 60 min in the Ca-free medium, the amplitude of the carbachol-induced contraction was the same as that of the contraction obtained after sequential applications described above.

When ATP was used as a spasmogen, the contraction by ATP was transient and the amplitude of the response was reduced to 55.2 ± 5.2% (n = 6) of the controls obtained in normal media, 5 min after $(Ca)_o$ deprivation. The contractile response to the following sequential application of ATP was abolished (Fig. 7B). After the preparation remained unstimulated for 30 min in the Ca-free medium, the contraction was not induced by ATP (Fig. 7B).

The contraction by 10 μM carbachol 10 min

after $(Ca)_o$ deprivation was 67% of the controls (Fig. 8B, hatched columns). When 10 or 30 mM ATP was applied 5 min after $(Ca)_o$ deprivation, a contraction was produced in a concentration-dependent manner (Fig. 8A, hatched column), and the following response to 10 μM carbachol, applied 10 min after $(Ca)_o$ deprivation, was reduced correspondingly (Fig. 8B, dotted columns). To the contrary, after carbachol was first applied, the following response to 30 mM ATP completely disappeared (n = 5, data not shown).

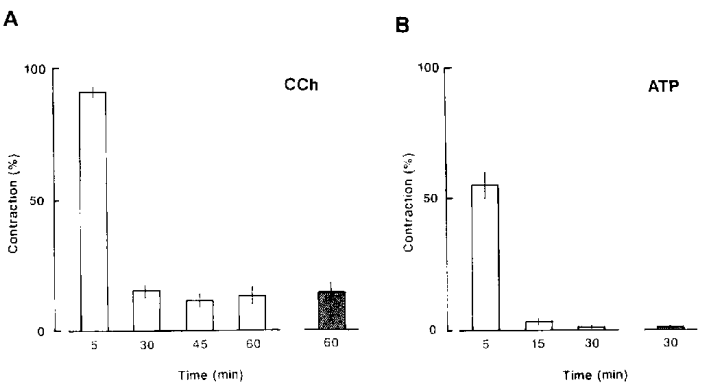

Fig. 7. Time courses of the amplitudes of contractions induced by repeated and one-shot applications of carbachol (A) and ATP (B) in flurbiprofen (1 μM)-treated rabbit bladder detrusor strips deprived of extracellular Ca. (A) open columns, determined as follows: 5 min after changing the normal Krebs solution to Ca-free, 2 mM EGTA-containing solution (Ca-free solution), 10 μM carbachol was applied and the muscle was washed with Ca-free solution. Then, carbachol was applied 30 min after extracellular Ca $[(Ca)_o]$ depletion without Ca loading of the preparation. The same procedures were repeated 45 and 60 min after $(Ca)_o$ depletion. Filled column, the amplitude of the contraction by carbachol, which was determined 60 min after $(Ca)_o$ depletion without preceding stimulations by carbachol. Abscissa, the time in minutes after $(Ca)_o$ depletion, when carbachol was applied; ordinate, % contraction by carbachol in the normal medium. (B) ATP (30 mM)-induced contractions determined by the same procedures as for A. Abscissa, the time in minutes after $(Ca)_o$ depletion, when each agonist was applied; ordinate, % contraction induced by carbachol (10 μM, A) and ATP (30 mM, B) in the normal medium. Each value is expressed as a mean ± S.E. (bar) of 6 experiments.

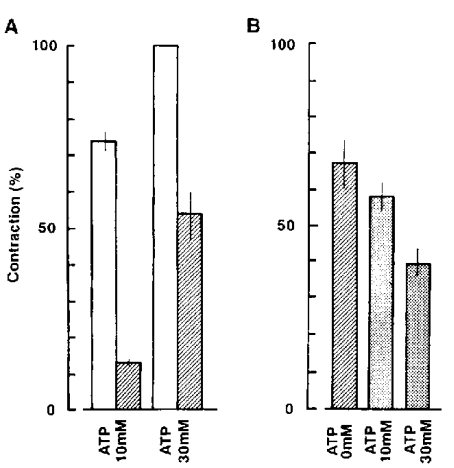

Fig. 8. Concentration-dependence of ATP-induced contractions in the absence and presence of extracellular Ca $[(Ca)_o]$ (A) and the amplitude of contractions by the following applications of carbachol (B) in flurbiprofen (1 μM)-treated rabbit bladder detrusor strips. (A) ATP (10 and 30 mM, indicated below abscissa)-induced contractions in the presence (open columns) and absence (hatched columns) of $(Ca)_o$. Under Ca-free conditions, ATP was applied 5 min after $(Ca)_o$ depletion. (B) Carbachol (10 μM)-induced contractions 10 min after $(Ca)_o$ depletion with (dotted columns) and without (hatched column) application of ATP (10 and 30 mM, indicated by abscissa) at 5 min $(Ca)_o$ deprivation. Ordinate, % contraction induced by ATP (30 mM, A) and carbachol (10 μM, B) in the normal medium. The value is expressed as mean ± S.E. (bar) of 6 experiments.

DISCUSSION

Endogenous PGs have been reported to modulate the intensity of contraction by stimulants in many smooth muscle preparations (15–17). Furthermore, ATP is a well-known to stimulate synthesis of PGs (18, 19). Therefore, before investigating the mechanisms of the contractile action of carbachol and ATP, we observed the effect of the potent cyclooxygenase inhibitor flurbiprofen (20) in the rabbit detrusor strips. The drug inhibited the contraction by lower concentrations of ATP and enhanced the maximum response to carbachol. It might be that the spontaneously released PGs suppress the muscle contractility, whereas the PGs released by ATP enhance the mechanical activity, but we did not carry out a further study in this respect. We investigated the Ca-mobilization and contractile mechanism utilized by carbachol and ATP in the presence of flurbiprofen, to exclude these indirect or secondary action by endogenous PGs.

In this tissue, high concentrations of ATP (milimolar range) was shown to be necessary to elicit the contractile response, as observed in other smooth muscles, such as guinea pig detrusor and vas deferens (19, 21, 22). Recent studies revealed that purinoceptors could be divided into at least two types, P_1 and P_2 receptors, which interact with adenosine and ATP, respectively (19). The latter receptor types have been further subclassified into two subtypes, P_{2x} and P_{2y}, which have different rank order of potencies for ATP and its analogues: α,β-methylene ATP > β,γ-methylene ATP > ATP for the P_{2x} subtypes and ATP > β,γ-methylene ATP > α,β-methylene ATP for the P_{2y} subtypes (19). The P_{2x} subtypes are also known to be desensitized by high concentrations of α,β-methylene ATP (6, 19, 22).

The ATP-induced contraction in this muscle was not due to interaction with P_1 receptors of adenosine which was produced by degradation of the applied ATP, since 10 mM adenosine did not evoke a contraction. Our results suggest that there predominantly prevail the P_{2x}-subtypes in the detrusor, since the rank order of potencies of the ATP analogues was α,β-methylene ATP > β,γ-methylene ATP > ATP that agreed with the order observed in the P_{2x}-subtypes, and since reactive blue 2, a selective P_{2y} receptor antagonist (23), did not inhibit but rather enhanced the ATP-induced contraction. The same degree of potentiation by reactive blue 2 was observed in the carbachol-induced contraction, and consequently, it was suggested that the potentiating action of reactive blue 2 was nonspecific in nature. The maximum contraction by α,β-methylene ATP was somewhat less than those by ATP and β,γ-methylene ATP in this muscle, suggesting the self-desensitization of P_{2x}-receptors by application of high concentrations of α,β-methylene ATP.

Verapamil as well as other Ca antagonists, dihydropyridines and diltiazem, are well-known to inhibit Ca-entry into the cell through the L-type, voltage-dependent Ca channels (24). Using this drug, we then determined to what extent the Ca influx contributes to the carbachol- and ATP-induced contractions. At first, since K-depolarization has been reported to release transmitters from the nerve elements in many isolated smooth muscle preparations, we observed the effect of atropine on the K-induced contraction. Treatment of the rabbit detrusor with atropine reduced the contraction mainly by high concentrations of K and increased the potency of verapamil to inhibit the K-induced contraction. This suggests that high concentrations of K released ACh and the ACh-induced contraction was rather resistant to verapamil, as observed with carbachol (see below). The potency of verapamil in the atropinized detrusor is comparable to those reported in other smooth muscles (25, 26). Verapamil (10 μM), whilst blocking the K-induced contraction completely, shifted the concentration-response curves for carbachol and ATP by only 6–10 fold, suggesting that contractile mechanisms other than the Ca-influx via the L-type Ca channels contribute to the responses to these agonists. Since the concentration-response curve for carbachol in the presence of 10 μM

verapamil well-superimposed with that in the absence of $(Ca)_o$, the Ca entering the cells is pissibly influxed almost exclusively through the L-type Ca channels. On the other hand, the contraction by low concentrations (≤ 1 mM) of ATP was significantly larger in the verapamil-treated preparations than in $(Ca)_o$-deprived ones. ATP would open additional Ca channels other than the verapamil-sensitive, L-type channels. This may be plausible, since P_{2x} receptors have been reported to open non-selective cation channels with a high-conductance (8, 27–31). Although such a possibility has been proposed in vascular smooth muscle (28), further study concerning these points is required.

The contraction observed in the absence of $(Ca)_o$ is generally accepted to be caused by the Ca released from the intracellular Ca stores. The contraction by carbachol, applied after the challenge with ATP, was reduced as the concentration of ATP was increased, and the contraction by ATP was abolished by the preceding application of carbachol. These results suggest that carbachol has a greater capability to release stored Ca than ATP and that these agonists share common Ca storage sites to a considerable degree. With either stimulant, the first application produced a large contraction. However, after the muscle was washed with Ca-free solution, the second application of the same drug resulted in no response in the case of ATP and caused only a small contraction in the case of carbachol. The reduction in the amplitude of or abolition of contraction seems to be due to no reuptake by the stores of the Ca released from the stores. However, carbachol repeatedly induced contractions of a constant size. Some part of the Ca released by carbachol may be recycled during contraction/relaxation processes. Or, more plausibly, carbachol but not ATP induces a contraction in the absence of $(Ca)_o$ by sensitization of the contractile machinery (4, 5). Simultaneous measurement of mechanical activity and intracellular Ca $[(Ca)_i]$ level using aequorin and fura-2 has revealed the discrepancy between the two parameters (32, 33). Some authors found that some receptor agonists shifted the $[Ca]_i$-tension curve to the left (e.g., refs. 33–35). A similar case may apply to the carbachol-induced contraction, but not the ATP-induced one in rabbit detrusor muscle. If so, although the carbachol-induced contractions were small, the contribution of the sensitization of contractile elements by carbachol to contraction may be underestimated, since Ca-free solution would lower the cytoplasmic Ca concentration to below the resting level (e.g., ref. 33). Determination of intracellular Ca ion concentration with fura-2 is needed to delineate the mechanisms of the repeatedly inducible contractions by carbachol under $(Ca)_o$-free conditons.

In conclusion, both carbachol and ATP induce Ca-influx through the L-type Ca channels and releases Ca from the Ca stores. However, only carbachol might increase the sensitivity of the contractile machinery to Ca on the one hand, and only ATP would open the additional, verapamil-insensitive Ca channels on the other. These may be of interest when considering treatment of motor acitivity of the urinary bladder by drugs such as Ca antagonists and intracellular Ca-modulating drugs.

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