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The signalling pathway which causes contraction via P2-purinoceptors in rat urinary bladder smooth muscle

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1 The signalling pathway which causes contractions to adenosine 5′-O-2-thiodiphosphate (ADPβS) and α,β-methylene adenosine 5′-diphosphate (α,β-Me ADP) was investigated in rat urinary bladder smooth muscle by measuring isotonic tension.

2 The responses to 10 μM α,β-methylene adenosine 5′-triphosphate (α,β-Me ATP) in 0 and 3.6 mM Ca^{2+} were 5.9 ± 1.3 ($n=10$) and 122.2 ± 6.4 ($n=8$) % respectively of those obtained in 1.8 mM Ca^{2+}, whereas those to 100 μM ADPβS were 34.6 ± 3.3 ($n=8$) and 96.8 ± 7.2 ($n=8$) %, in 0 and 3.6 mM Ca^{2+}, respectively. In both experimental conditions, the responses to the two agonists expressed as % of the control responses were significantly different ($P<0.01$).

3 Indomethacin at high concentrations (>1 μM) decreased the responses to α,β-Me ATP (10 μM), ADPβS (100 μM) and α,β-Me ADP (100 μM). However, no significant difference was obtained between the responses to all the agonists at 30 μM indomethacin.

4 2-Nitro-4-carboxphenyl n,n-diphenylcarbamate (NCDC) at concentrations between 1 μM and 100 μM concentration-dependently decreased the responses to ADPβS (100 μM) and α,β-Me ADP (100 μM) and almost completely inhibited them at 100 μM. Although the responses to α,β-Me ATP (10 μM) were also inhibited by the drug, at 50 and 100 μM NCDC the responses to α,β-Me ATP were significantly larger than those to ADPβS and α,β-Me ADP ($P<0.01$).

5 NCDC 100 μM significantly inhibited the KCl-induced contraction to 65.9 ± 4.9% ($n=6$) of the control ($P<0.01$).

6 It is suggested that the contraction via ADPβS-sensitive receptors in the rat urinary bladder smooth muscle mainly depends on Ca^{2+} ions liberated from intracellular Ca^{2+} stores, though the contribution of Ca^{2+} ions from the extracellular space cannot be neglected. The release of Ca^{2+} ions from stores is mainly mediated by the production of inositol trisphosphate (IP_3) via the activation of phospholipase C.

Keywords: P2-purinoceptors; urinary bladder smooth muscle; α,β-methylene adenosine 5′-triphosphate; adenosine 5′-O-2-thiodiphosphate; NCDC; U73122; indomethacin

Introduction

In rat urinary bladder smooth muscle P1-purinoceptors mediate relaxation (Nicholls *et al*., 1992), whereas P2-purinoceptors mediate contraction (Bhat *et al*., 1989). At least two different P2-purinoceptors, P2X and adenosine 5′-O-2-thiodiphosphate (ADPβS)-sensitive purinoceptors, have been shown to exist in this tissue (Suzuki & Kokubun, 1994). The ADPβS-sensitive purinoceptor responds to either ADPβS or α,β-methylene adenosine 5′-diphosphate (α,β-Me ADP) but not to other adenine nucleotides, and is, not completely but significantly, antagonized by cibacron blue 3 GA or suramin. Recently we have also shown that approximately 20% of the neurogenic contraction of the rat urinary bladder smooth muscle is mediated via ADPβS-sensitive purinoceptors (Hashimoto & Kokubun, 1995). However, the signalling pathway which causes the contraction via ADPβS-sensitive purinoceptors has not yet been elucidated.

Several types of P2X purinoceptors have been cloned. Although the potency of ATP and α,β-methylene adenosine 5′-triphosphate (α,β-Me ATP) as well as the channel kinetics varied, their physiological function is to act as intrinsic ion channels through which Na^+, K^+ and Ca^{2+} ions permeate (Fredholm *et al*., 1994). Therefore, in urinary bladder smooth muscle cells, the contraction via P2X purinoceptors requires extracellular Ca^{2+} ions (Bhat *et al*., 1989; Schneider *et al*., 1991). Various G-protein coupled P2Y purinoceptors, such as $P2Y_1$, $P2Y_2$ and $P2Y_4$ purinoceptors (Parr *et al*., 1994; Tokoyama *et al*., 1995; Communi *et al*., 1995), are known to increase the production of inositol triphosphate (IP_3) by the activation of phospholipase C. Since ADPβS-sensitive purinoceptors in the rat urinary bladder smooth muscle do not respond to α,β-Me ATP, it is plausible that the receptor has common features to P2Y receptors and that the contraction induced by ADPβS is less susceptible to the extracellular Ca^{2+} concentration ($[Ca^{2+}]_o$) than that inducecd by α,β-Me ATP.

In the present study, therefore, we first examined the susceptibility of ADPβS-induced contraction to $[Ca^{2+}]_o$ and then investigated the signalling pathway causing the contraction via ADPβS-sensitive purinoceptors in rat urinary bladder smooth muscle.

Methods

Male Wistar strain rats (weighing 200 to 250 g) were anaesthetized with pentobarbitone sodium (40 mg kg^{-1}), and the urinary bladder was rapidly removed. The bladder was transferred into control HEPES-Tyrode solution and then cut into small tissue strips of 7.5×2 mm. The strip was suspended in organ baths which contained 10 ml of control HEPES-Tyrode solution (composition in mM: NaCl 136.9, KCl 5.4, $CaCl_2$ 1.8, $MgCl_2$ 0.5, NaH_2PO_4 0.33, HEPES 5.0 and glucose 5.0; pH = 7.4). The solution was aerated with oxygen gas and maintained at 37°C. Responses to drugs were monitored by measuring isotonic tension under the resting load of 1.0 g. After each application of the drug, it was washed out by more than 100 ml of control HEPES-Tyrode solution and then we waited for at least 30 min until the next application of the drug. When the effects of extracellular Ca^{2+} ions on the agonist-induced contraction were examined, $CaCl_2$ was simply omitted from control HEPES-Tyrode solution (0 mM-Ca^{2+}

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HEPES-Tyrode solution) or increased to double the normal concentration (3.6 mM-Ca^{2+} HEPES-Tyrode solution).

When the concentration-response curves to blocking agents were performed, each dose was added separately and agonists were added after the incubation of a muscle strip for at least 60 min in the presence of blocking agents. To desensitize P2X receptors, tissues were pretreated with 10 μM α,β-Me ATP before the application of ADPβS or α,β-Me ADP. This desensitization procedure was shown to inhibit the response to the following application of α,β-Me ATP to nearly 20% of the control response (Suzuki & Kokubun, 1994).

The contractions induced by 100 μM ADPβS and 100 μM α,β-Me ADP after the desensitization of P2X receptors were 78.2 ± 3.2 and 60.4 ± 2.6 (mean $\pm$ s.e.mean) % of that induced by 10 μM α,β-Me ATP, respectively ($n = 20$).

Drugs

α,β-Me ADP, α,β-Me ATP, ADPβS and 2-nitro-4-carboxyphenyl n,n-diphenylcarbamate (NCDC) were purchased from Sigma Chemical Company. Indomethacin and U-73122 were purchased from Nacalai Tesque and Research Biochemicals International, respectively.

All agonists were prepared freshly before each experiment by dissolving them in 0 mM-Ca^{2+} HEPES-Tyrode solution. Indomethacin, NCDC and U-73122 were prepared by dissolving them in dimethyl sulphoxide (DMSO) (Kanto Chemical) and added to the bath.

In preliminary experiments, we examined the effects of DMSO on the agonist-induced contraction. DMSO concentrations lower than 1.0% in the bath did not significantly affect the contraction, though at concentrations higher than 1.0% it significantly increased the contraction. The responses to α,β-Me ATP (10 μM), ADPβS (100 μM) and α,β-Me ADP (100 μM), observed in the presence of 1.0% of DMSO, were 99.2 ± 2.5, 100.5 ± 2.7 and 104.6 ± 5.3% of those observed in the absence of DMSO, respectively ($n = 6$). Therefore, maximal concentrations of indomethacin and NCDC used in this study were determined in order that the concentration of DMSO did not exceed 1.0% in the bath. However, in the experiments with U-73122 we could not avoid the use of DMSO at concentrations higher than 1.0% (maximum 2.5%) due to the low solubility of the drug.

Statistical analysis

Results are expressed as mean $\pm$ s.e.mean. One-way analysis of variance (one-way anova) was used to test for statistical significance in Figures 1 to 4a, while in Figure 4 paired t test was used for the data obtained with U-73122. A probability value of 0.05 or less was considered significant.

Results

Effects of various extracellular Ca^{2+} concentrations on the contractions induced by α,β-Me ATP and ADPβS

We first investigated the effect of subtraction of extracellular Ca^{2+} ions on the contraction induced by either α,β-Me ATP or ADPβS. As shown in Figure 1a, in 1.8 mM Ca^{2+} (control HEPES-Tyrode solution) 10 μM α,β-Me ATP strongly contracted the muscle strip by 3.2 mm. The following application of 100 μM ADPβS, 13 min after the application of α,β-Me ATP, also contracted it by 1.5 mm. After incubation of the muscle strip in 0 mM-Ca^{2+} HEPES-Tyrode solution for 6 min, 10 μM α,β-Me ATP contracted the tissue by less than 0.1 mm, though 100 μM ADPβS contracted it by 0.9 mm 2 min after the application of α,β-Me ATP. When the incubation time in 0 mM-Ca^{2+} HEPES-Tyrode solution was further prolonged to 15 min, 100 μM α,β-Me ATP failed to contract the muscle strip. However, under these conditions, the following application of ADPβS (100 μM) 2 min after the application of α,β-Me ATP still contracted the muscle by 0.7 mm. As shown in the figure, the duration of the contraction induced by ADPβS was decreased as the incubation time in 0 mM-Ca^{2+} HEPES-Tyrode solution was increased.

Although an obvious contraction of the muscle strip by ADPβS after 15 min of incubation in 0 mM-Ca^{2+} HEPES-Tyrode solution was observed in two out of 8 experiments, we always observed a contraction of the muscle by the drug when the incubation time in 0 mM-Ca^{2+} solution was within 6 min. Therefore, we compared the effects of subtracting Ca^{2+} ions on the contraction induced by α,β-Me ATP and ADPβS quantitatively by incubating muscle strips in 0 mM-Ca^{2+} solution for 6 min.

Figure 1b shows the responses of muscle strips to α,β-Me ATP (10 μM) and ADPβS (100 μM) in 0 mM-Ca^{2+} and 3.6 mM-Ca^{2+} HEPES-Tyrode solutions. Data are expressed as percentage of the responses to each agonist obtained in the control HEPES-Tyrode solution. In 0 mM-Ca^{2+} HEPES-Tyr-

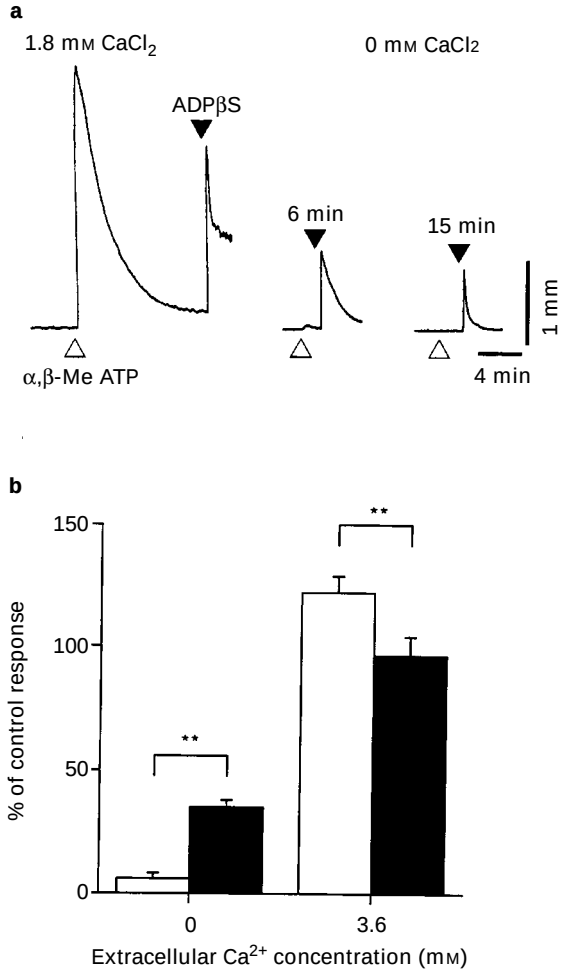

Figure 1 Contractile responses of rat urinary bladder smooth muscle to α,β-Me ATP and ADPβS in various extracellular Ca^{2+} concentrations. (a) Original records of the contractile responses to α,β-Me ATP and ADPβS. The panel on the left-hand side shows the contraction induced by α,β-Me ATP (10 μM) and ADPβS (100 μM) in the presence of 1.8 mM $CaCl_2$. Drugs were applied at the points indicated by triangles. The middle panel shows a similar experiment performed after incubating muscle strip for 6 min in the absence of $CaCl_2$, while the panel on the right-hand side shows the experiment performed after 15 min incubation in the absence of $CaCl_2$. In this panel the concentration of α,β-Me ATP was increased to 100 μM. These experiments were done in the same muscle strip. (b) The responses to 10 μM α,β-Me ATP (open columns) and 100 μM ADPβS (solid columns) in 0 mM and 3.6 mM extracellular $CaCl_2$. The responses are expressed as a percentage of the response obtained in the presence of 1.8 mM $CaCl_2$. Each column indicates mean and s.e.mean ($n = 8-10$). $^{**}P < 0.01$.

ode solution, the responses to α,β-Me ATP and ADPβS were decreased to 5.9±1.3 ($n=10$) and 34.6±3.3% ($n=8$), respectively. These were significantly different ($P<0.01$). When the responses to drugs were observed in 3.6 mM-Ca^{2+} HEPES-Tyrode solution, those to α,β-Me ATP and ADPβS were 122.2±6.4 and 96.8±7.2%, respectively ($n=8$). These were also significantly different ($P<0.01$).

Effects of indomethacin on the responses to α,β-Me ATP, ADPβS and α,β-Me ADP

We examined the concentration-dependent effects of indomethacin on the responses to α,β-Me ATP (10 μM), ADPβS (100 μM) and α,β-Me ADP (100 μM) (Figure 2).

At 0.01 μM indomethacin the responses to any agonists expressed as percentage of control response did not significantly differ from those obtained in the presence of DMSO. At indomethacin concentrations higher than 0.01 μM the responses to α,β-Me ATP were significantly smaller than those obtained in the presence of DMSO (0.1 μM: $P<0.05$, >0.1 μM: $P<0.01$), whereas those to ADPβS were significantly smaller at concentrations of indomethacin higher than 1 μM ($P<0.01$). The responses to α,β-Me ADP were significantly smaller than those obtained in the presence of DMSO at 1 μM ($P<0.05$) and 30 μM ($P<0.01$) indomethacin. At 0.01 and 10 μM indomethacin, the responses to α,β-Me ADP were significantly larger than those to α,β-Me ATP ($P<0.05$), while at all other concentrations of indomethacin those to the three agonists were not significantly different.

Contraction by α,β-Me ATP, ADPβS and α,β-Me ADP in the presence of NCDC

We examined whether NCDC inhibited the contraction induced by α,β-Me ATP, ADPβS and α,β-Me ADP (Figure 3). As shown in the upper panel in the figure, 100 μM NCDC inhibited the contraction induced by 10 μM α,β-Me ATP to 73.3% of the control, whereas that induced by 100 μM ADPβS was inhibited to 1.5% of the control. NCDC also inhibited the contraction induced by 100 μM α,β-Me ADP to 5% of the control, although in the same muscle strip the contraction to 10 μM α,β-Me ATP was reduced to 61% by the drug (the lower panel in Figure 3).

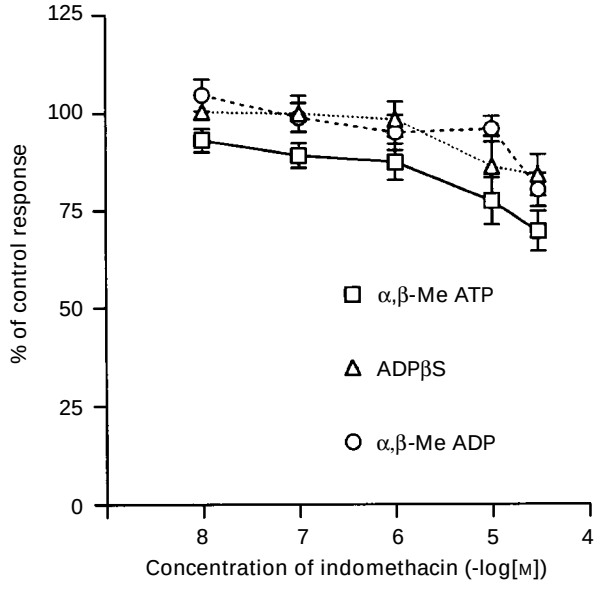

Figure 2 Effects of various concentrations of indomethacin on the responses to 10 μM α,β-Me ATP, 100 μM ADPβS and 100 μM α,β-Me ADP. The responses are expressed as a percentage of the control response to each agonist obtained in the absence of indomethacin. Symbols and vertical lines indicate mean and s.e.mean ($n=6-15$). Abscissa scale indicates the concentration of indomethacin (−log[M]).

In 8 experiments, the concentration-dependent inhibitory effect of NCDC on the agonist-induced contraction was examined (Figure 4a). At 50 and 100 μM NCDC the responses to α,β-Me ATP expressed as a percentage of control responses were significantly larger than those to either ADPβS or α,β-Me ADP ($P<0.01$), whereas no significant differences were obtained between responses to ADPβS and those to α,β-Me ADP at 50 and 100 μM NCDC.

We also examined the effect of U-73122 on the responses to α,β-Me ATP and α,β-Me ADP (Figure 4b). The drug showed obvious inhibition of the responses to both agonists at concentrations higher than 10 μM. At 25 μM U-73122 the responses to the two agonists were significantly different ($P<0.01$) when expressed as percentage of control responses.

Effects of NCDC on KCl-induced contraction

We examined whether NCDC inhibited KCl-induced contractions. The contraction of the muscle strip was induced by increasing the extracellular K^+ concentration to 60 mM. In the absence of NCDC, the contraction was 4.7±0.6 mm, whereas in the presence of NCDC (100 μM) it was 3.1±0.4 mm ($n=6$). When the contraction in the presence of the drug was expressed as the percentage of that in its absence, it was 65.9±4.9%. The contractile response was significantly smaller in the presence of NCDC ($P<0.01$).

When the same experiment was done in the presence of 25 μM U-73122, the drug inhibited the KCl-induced contraction to 59.4% of the control value ($n=3$).

Discussion

This study showed that the contraction induced by ADPβS was less susceptible to the change in $[Ca^{2+}]_o$ concentration than that induced by α,β-Me ATP. In the absence of extracellular Ca^{2+} the response to α,β-Me ATP was significantly smaller than that to ADPβS, although in the presence of 3.6 mM Ca^{2+} it was significantly larger than that to ADPβS. Since α,β-Me ATP induces contraction of urinary bladder smooth muscle via P2X receptors (Suzuki & Kokubun, 1994), which mediate both the depolarization of the membrane causing the influx of Ca^{2+} ions through voltage-dependent Ca^{2+} channels and the influx of Ca^{2+} ions directly through the receptor (Schneider *et al*., 1991), the response to α,β-Me ATP would be expected to be small (<6% of the control) in 0 mM Ca^{2+} and large (>120% of the control) in the presence of 3.6 mM Ca^{2+}, as has been observed in this study.

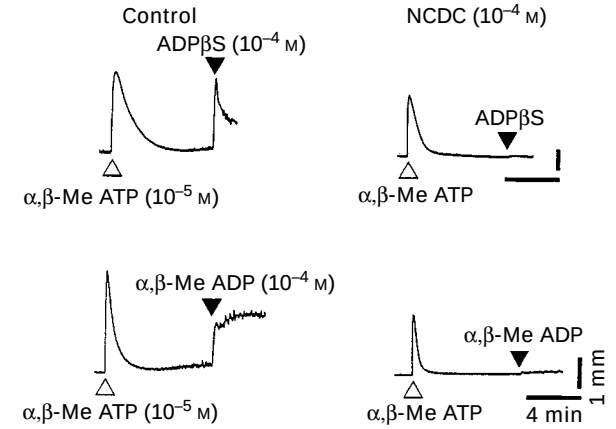

Figure 3 The effects of NCDC (100 μM) on the responses to 10 μM α,β-Me ATP, 100 μM ADPβS and 100 μM ADP. Original contractile responses to agonists in the absence (left-hand side) and the presence (right-hand side) of NCDC are shown. Agonists were applied at the points indicated by triangles. Data in the upper and lower panels were obtained from different muscle stips.

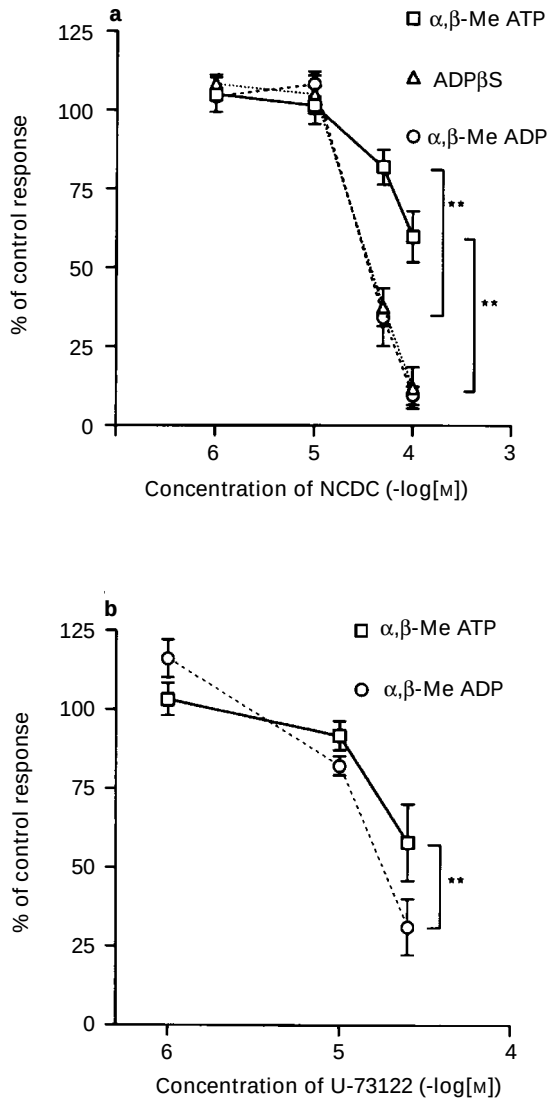

Figure 4 (a) Effects of NCDC at various concentrations on the responses to 10 μM α,β-Me ATP, 100 μM ADPβS and 100 μM α,β-Me ADP. The responses are expressed as percentage of the control response to each agonist obtained in the absence of NCDC ($n=8$). (b) Effects of U-73122 at various concentrations on the responses to 10 μM α,β-Me ATP and 100 μM α,β-Me ADP. The responses are expressed as percentage of the control response to each agonist obtained in the absence of U-73122 ($n=6$). In both (a) and (b), symbols and vertical lines indicate mean and s.e.mean. Abscissa scale indicates the concentration of blockers ($-\log$[M]). $^{**}P<0.01$.

The response to ADPβS in 0 mM Ca^{2+} was approximately 35% of the control. However, in 3.6 mM Ca^{2+} the response of the drug was nearly the same as the control and significantly smaller than that to α,β-Me ATP. These findings suggest that in rat urinary bladder smooth muscle, ADPβS induces a contraction mainly by the release of Ca^{2+} from intracellular Ca^{2+} store sites. The decrease in the peak and the duration of the contraction induced by ADPβS in the absence of extracellular Ca^{2+} could be explained by assuming that the amount of Ca^{2+} ions in intracellular store sites might be decreased by incubation of the muscle strip in 0 mM-Ca^{2+} solution. However, the possibility that ADPβS induces the influx of extracellular Ca^{2+} through unknown pathways, in addition to the release of Ca^{2+} from intracellular store sites, cannot be excluded.

We then investigated the signal transduction pathway which caused the release of intracellular Ca^{2+} ions via ADPβS-sensitive purinoceptors. Since it has been found in rabbit ileal longitudinal smooth muscle that α,β-Me ADP caused contraction via the production of prostaglandins (Frew & Baer, 1979), we first performed experiments with indomethacin to inhibit the production of prostaglandins. At high concentrations of indomethacin, the responses to α,β-Me ATP, ADPβS and α,β-Me ADP were significantly suppressed. However, even at 30 μM indomethacin the responses to all three agonists expressed as percentage of control responses were not significantly different, indicating that the arachidonic acid cascade did not play a significant role in the liberation of Ca^{2+} from intracellular Ca^{2+} store sites in the contraction mediated via ADPβS-sensitive receptors.

In various tissues, including smooth muscle, the increase in the production of IP_3 by the activation of phospholipase C via P2Y-purinoceptors has been demonstrated (Fredholm *et al.*, 1994; Sawai *et al.*, 1996). NCDC has been shown to inhibit the activity of phosphoinositide-specific phospholipase C (Walenga *et al.*, 1980) and also to inhibit both the phosphoinositide hydrolysis and the contraction of the rat aorta induced by 5-hydroxytryptamine (5-HT) (Nakaki *et al.*, 1985). Therefore, in this study we used NCDC to inhibit the activity of phospholipase C. At concentrations of NCDC higher than 10 μM the responses to ADPβS and α,β-Me ADP expressed as a percentage of control responses were significantly smaller than that to α,β-Me ATP. The range of concentrations of NCDC over which we observed the significant reduction of responses by the drug was similar to that used in the rat aorta to inhibit the contraction to 5-HT. This indicates that the contractions induced by ADPβS and α,β-Me ADP might be mainly mediated via the production of IP_3.

Although the response to α,β-Me ATP in the presence of 100 μM NCDC was significantly larger, when expressed as a percentage of the control responses, than those to ADPβS and α,β-Me ADP, it was also inhibited to less than 60% of the control response. In order to investigate the mechanisms underlying the inhibition of the response to α,β-Me ATP by NCDC, we examined whether NCDC inhibited KCl-induced contractions. The result clearly indicated that NCDC at 100 μM significantly inhibited KCl-induced contraction of the rat urinary bladder smooth muscle, suggesting that the drug inhibited the response to α,β-Me ATP by blocking voltage-dependent Ca^{2+} channels in this tissue. However, this is in contrast to the previous findings in the rat aorta where 75 μM NCDC was shown not to affect the KCl-induced contraction significantly (Nakaki *et al.*, 1995).

U-73122 has recently been used as a specific inhibitor of phospholipase C (Smallridge *et al.*, 1992; Yule & Williams, 1992). In the present study, with 25 μM U-73122 the response to α,β-Me ADP was significantly smaller than that to α,β-Me ATP when expressed as percentage of the control responses. This concentration of U-73122 has been shown to inhibit effectively the activity of phospholipase C in the presence of carbachol in rat pancreatic acinar cells (Yule & Williams, 1992). This further supports our idea that the contraction induced by α,β-Me ADP might be mainly mediated by the production of IP_3.

U-73122 has been shown to inhibit completely voltage-dependent Ca^{2+} channels in differentiated NG108-15 cells (Jin *et al.*, 1994). This could account for the finding in the latter study that 25 μM U-73122 inhibited the response to α,β-Me ATP to less than 60% of the control. In fact, at the same concentration U-73122 also inhibited the KCl-induced contraction to 59.4% of the control in this study.

The present study suggests that the contraction mediated via ADPβS-sensitive receptors in the rat urinary bladder smooth muscle mainly depends on Ca^{2+} ions liberated from intracellular Ca^{2+} stores, though the possible contribution of Ca^{2+} ions from extracellular space cannot be excluded. The release of Ca^{2+} ions from stores is mainly mediated by the production of IP_3 via the activation of phospholipase C.

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