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# EFFECTS OF β-ADRENERGIC STIMULATION ON CALCIUM MOVEMENTS IN RABBIT AORTIC SMOOTH MUSCLE: RELATIONSHIP WITH CYCLIC AMP

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SUMMARY

1. The effects of isoprenaline ($10^{-6}$ M) on relaxation, unidirectional as well as net $Ca^{2+}$ fluxes, and cyclic AMP levels were investigated in rabbit aorta under the condition of high-$K^+$ depolarization in the presence of phentolamine ($10^{-5}$ M).

2. Isoprenaline ($10^{-6}$ M) caused significant inhibition of $Ca^{2+}$ influx stimulated by 145 mM-$K^+$ (0 $Na^+$) solution. The time courses of $Ca^{2+}$ influx inhibition and relaxation by isoprenaline were parallel. Isoprenaline also caused a significant inhibition of high-$K^+$-induced gain in net $Ca^{2+}$ content.

3. Ro 20-1724 (1 mM), a phosphodiesterase inhibitor, also caused relaxation and $Ca^{2+}$ influx inhibition in high-$K^+$-depolarized rabbit aorta. Pre-treatment with Ro 20-1724 potentiated isoprenaline-induced $Ca^{2+}$ influx inhibition and relaxation.

4. Isoprenaline and Ro 20-1724 each alone increased cyclic AMP levels. Furthermore pre-treatment with Ro 20-1724 caused potentiation of isoprenaline-induced increases in cyclic AMP levels.

5. At submaximal concentration, D600 ($10^{-7}$ M) caused partial inhibition of high-$K^+$-stimulated $Ca^{2+}$ influx and produced relaxation. However, unlike Ro 20-1724, it did not potentiate isoprenaline-induced $Ca^{2+}$ influx inhibition and relaxation. D600 does not increase cyclic AMP levels in smooth muscle.

6. Dibutyryl cyclic AMP (1 mM), a lipid-soluble analogue of cyclic AMP, caused relaxation and inhibited high-$K^+$-stimulated $Ca^{2+}$ influx.

7. Isoprenaline failed to cause stimulation of $Ca^{2+}$ efflux in high-$K^+$-depolarized rabbit aorta.

8. It is concluded that the inhibition of $Ca^{2+}$ influx may be one of the mechanisms by which β-receptor stimulation can reduce intracellular free $Ca^{2+}$ to promote relaxation of smooth muscle. The data support the involvement of cyclic AMP in this action of the β-agonist.

9. Since the experiments were conducted in 145 mM-$K^+$ (0 $Na^+$) depolarizing conditions, the role of hyperpolarization or of a $Na^+$–$Ca^{2+}$ exchange mechanism in isoprenaline-induced $Ca^{2+}$ influx inhibition and/or relaxation can be excluded.

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## INTRODUCTION

According to current views, relaxation of smooth muscle occurs when there is a significant reduction in cytoplasmic $Ca^{2+}$ activity or when there is an inhibition of actin–myosin interaction due to phosphorylation of myosin light chain kinase (Adelstein & Hathaway, 1979). Either one or both mechanisms may operate during $\beta$-adrenoceptor stimulation to produce smooth muscle relaxation. The present report deals with a possible mechanism by which isoprenaline can reduce intracellular free $Ca^{2+}$ concentration to promote relaxation. A decrease in cytoplasmic $Ca^{2+}$ concentration can be achieved by one or any combination of the following mechanisms (van Breemen, 1976): (*a*) increase in $Ca^{2+}$ efflux; (*b*) a decrease in $Ca^{2+}$ influx, and (*c*) intracellular sequestration of $Ca^{2+}$. Marshall & Kroeger (1973) found that isoprenaline caused a decrease in net tissue $Ca^{2+}$ content in rat uterus depolarized by high-$K^+$. In a subsequent report by the same group, isoprenaline was found to cause an increase in $Ca^{2+}$ efflux (Kroeger, Marshall & Bianchi, 1975). Enhanced efflux of $^{45}Ca$ by isoprenaline was also demonstrated in guinea-pig taenia coli (Tomiyama, Takayanagi & Takagi, 1973; Bülbring & den Hertog, 1980). On the other hand, Mueller & van Breemen (1979) obtained evidence indicating that intracellular $Ca^{2+}$ sequestration was the mechanism involved in $\beta$-receptor-induced relaxation of guinea pig taenia coli. Casteels & Raeymaekers (1979) also found in the guinea-pig taenia coli that an increase uptake of $Ca^{2+}$ into an intracellular store contributes to the relaxing effect of isoprenaline. In addition, they functionally characterized this intracellular store of $Ca^{2+}$ as the one which is released by excitatory muscarinic agonists and is sensitive to monovalent ions such as $Na^+$. The ability of smooth muscle sarcoplasmic reticulum (s.r.) to accumulate $Ca^{2+}$ has been convincingly demonstrated by Somlyo (1980). Thus, more than one cellular mechanism of $Ca^{2+}$ removal may operate during isoprenaline induced relaxation in smooth muscle. In the present report we have investigated the effects of $\beta$-receptor activation on $Ca^{2+}$ influx and extrusion in the rabbit aortic smooth muscle. The data presented support the involvement of inhibition of stimulated $Ca^{2+}$ influx as a mechanism by which isoprenaline relaxes high-$K^+$ contracture. No stimulation of $Ca^{2+}$ efflux by isoprenaline was observed under this condition. The involvement of cyclic AMP in the inhibitory action of isoprenaline on stimulated $Ca^{2+}$ influx and relaxation was also investigated.

## METHODS

The preparation of rabbit aortic rings and measurement of isometric tension have been described previously (Meisheri, Palmer & van Breemen, 1980). The rings were maintained in a warm (37 °C) physiological salt solution (PSS) at pH 7·2 and bubbled with 100% $O_2$. The composition of PSS (in mM) is as follows: NaCl, 140·0; $CaCl_2$, 1·5; $MgCl_2$, 1·0; KCl, 4·6; glucose, 10; HEPES, 5·0. High-$K^+$ depolarizing solution contained either 80 mM-$K^+$ (with 65 mM-$Na^+$) or 145 mM-$K^+$ (with 0 $Na^+$).

### *$Ca^{2+}$ influx*

Measurement of unidirectional $Ca^{2+}$ influx was carried out by exposing the aortic rings to $^{45}Ca$-containing solution for short periods of time (1–6 min). The amount of $^{45}Ca$ entering the tissue during such short periods can be assumed to be primarily due to $Ca^{2+}$ influx (Meisheri, Hwang & van Breemen, 1981). This experimental approach allowed us selectively to determine the influence of isoprenaline on high-$K^+$-stimulated $Ca^{2+}$ influx. The aortic rings were incubated in non-radioactive

PSS for up to 2 hr. The rings were then exposed to control or experimental solutions containing $^{45}Ca$ and tissues were taken out at different time periods as indicated in the Results section. The separation of $^{45}Ca$ contained in the extracellular and intracellular compartments was obtained by bathing the tissue for 45 min in ice-cold $Ca^{2+}$-free PSS containing 2 mM-EGTA (Meisheri *et al.* 1980). The $^{45}Ca$ uptake was calculated from the counts left in the tissue after a 45 min wash in ice-cold $Ca^{2+}$-free PSS containing 2 mM-EGTA.

*Net $Ca^{2+}$ uptake*

One experiment was also carried out to investigate the effect of isoprenaline on high-$K^+$-induced net $Ca^{2+}$ uptake. The method of measurement of net $Ca^{2+}$ uptake has been described previously (Meisheri *et al.* 1980). Briefly, the muscle rings were exposed to $^{45}Ca$-labelled PSS for 90 min which was followed by exposure to an experimental solution also labelled with $^{45}Ca$ at the same specific activity. At the end of the incubation period, the tissues were bathed in ice-cold $Ca^{2+}$-free PSS with 2 mM-EGTA for 45 min. The tissues were then blotted and weighed and their $^{45}Ca$ was extracted with a hypotonic EDTA solution overnight. The extract and tissues were counted in a liquid scintillation counter.

*$Ca^{2+}$ efflux*

Aortic rings were labelled with $^{45}Ca$ for 2 hr in normal PSS at 37 °C. They were then quickly rinsed in a large volume of non-labelled PSS and passed through a series of test tubes each containing 145 mM-K-PSS with $10^{-5}$ M-phentolamine. The tissues were left for 3 min in each tube and at 33 min $10^{-6}$ M-isoprenaline was added to the experimental rings.

*Cyclic AMP determination*

The extraction of cyclic AMP from tissues by using 5% trichloroacetic acid was carried out as described previously (Meisheri & McNeill, 1979). The determination of cyclic AMP in the extract was carried out using a commercial competitive protein binding assay kit from Amersham/Searle.

*Statistics*

Student's $t$ test for unpaired data was used; a probability of $P < 0.05$ was considered to indicate significantly different populations.

*Drugs and chemicals*

Isoprenaline hydrochloride (Sigma); Ro 20-1724 (Hoffman-La Roche); D600 (A. K. Knoll, West Germany); Dibutyryl cyclic AMP (Sigma). $^{45}Ca$ (specific activity 12·9 m-c/mg) was obtained from New England Nuclear.

## RESULTS

### *Inhibition of high-$K^+$-stimulated $Ca^{2+}$ influx by isoprenaline*

In the first series of experiments, the effect of isoprenaline ($10^{-6}$ M) on $Ca^{2+}$ influx stimulated by either 80 mM-$K^+$ (65 mM-$Na^+$) or 145 mM-$K^+$ (0 $Na^+$) was investigated. The high-$K^+$-stimulated $Ca^{2+}$ influx was measured by exposing the tissues to $^{45}Ca$ for short periods of time (2, 4 and 6 min) (Fig. 1). Isoprenaline ($10^{-6}$ M) caused a significant decrease in stimulated $Ca^{2+}$ influx at 4 and 6 min in both high-$K^+$ solutions. Both 80 mM-$K^+$ and 145 mM-$K^+$ produced similar increases in $Ca^{2+}$ influx over control which is consistent with our previous observation (Meisheri *et al.* 1981). Isoprenaline at $10^{-6}$ M did not affect the control (passive) $Ca^{2+}$ influx. It was found that concentrations of isoprenaline above $10^{-6}$ M produced contraction of rabbit aorta and caused stimulation of $Ca^{2+}$ influx (data not shown). Both of these excitatory effects of isoprenaline could be blocked by phentolamine ($10^{-5}$ M), thus confirming an $\alpha$-excitatory component of isoprenaline action at higher concentrations (Dorevitch, 1968). All experiments were therefore done with phentolamine ($10^{-5}$ M) in order to

prevent any $\alpha$-adrenergic action of isoprenaline or of noradrenaline released by high-$K^+$ depolarization.

It is apparent from Fig. 1 that during the initial 2 min, the high-$K^+$ stimulated $Ca^{2+}$ influx is relatively small compared to the cellular exchangeable $Ca^{2+}$ (see later). In order to investigate the effect of isoprenaline during this initial 2 min labelling period, the following modification was carried out. The depolarization-sensitive $Ca^{2+}$ channels were opened by exposure to 145 mM-$K^+$ solution for 5 min before exposing the tissues to $^{45}Ca$. Isoprenaline was present during the last 2 min of the 5 min pre-treatment with high-$K^+$. Thus the isoprenaline effect on $^{45}Ca$ influx was studied at 3 and 4 min exposure to the $\beta$-agonist. Isoprenaline ($10^{-6}$ M) caused a significant inhibition of high-$K^+$ stimulated $Ca^{2+}$ influx at both time points studied (Fig. 2).

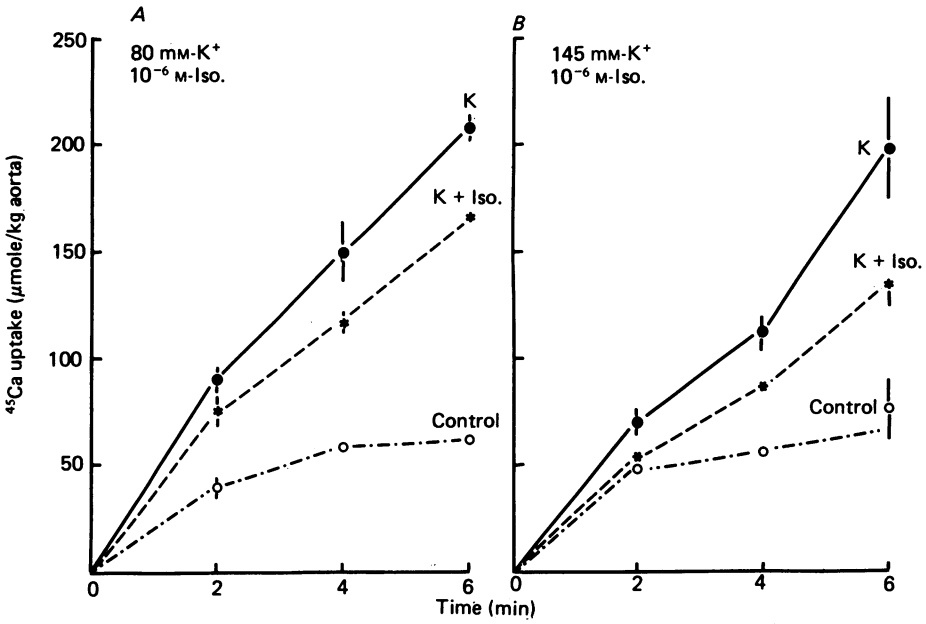


Fig. 1. The effect of isoprenaline (Iso.; $10^{-6}$ M) on high-$K^+$-stimulated $Ca^{2+}$ influx. *A*, 80 mM-$K^+$ (65 mM-$Na^+$), and *B*, 145 mM-$K^+$ (0 $Na^+$). The tissues were exposed to $^{45}Ca$-containing solutions (control or experimental) for 2, 4 and 6 min. Phentolamine ($10^{-5}$ M) was present in all solutions. The data here and in subsequent Figures are given as mean ± S.E. of the mean ($n = 4$–6). The effect of isoprenaline is significant ($P < 0.05$) in both *A* and *B* at 4 and 6 min. ○—·—○, control; ●—●, high-$K^+$; *—*, high-$K^+$ + isoprenaline.

The high-$K^+$-induced contraction and stimulated net $Ca^{2+}$ uptake in rabbit aorta are due to the entry of $Ca^{2+}$ from the extracellular space (van Breemen, Farinas, Gerba & McNaughton, 1972; Deth & van Breemen, 1974). Due to its inhibitory effect on $Ca^{2+}$ influx, isoprenaline would also be expected to inhibit net $Ca^{2+}$ uptake stimulated by high-$K^+$. The results of such an experiment are shown in Table 1. Isoprenaline caused a significant inhibition of high-$K^+$-induced net $Ca^{2+}$ uptake at 4 and 6 min.

The following experiment was carried out to correlate the time course of inhibition of $Ca^{2+}$ influx with the time course of relaxation produced by isoprenaline. The use of high-$K^+$-depolarized muscle offers the advantage that the onset of relaxation

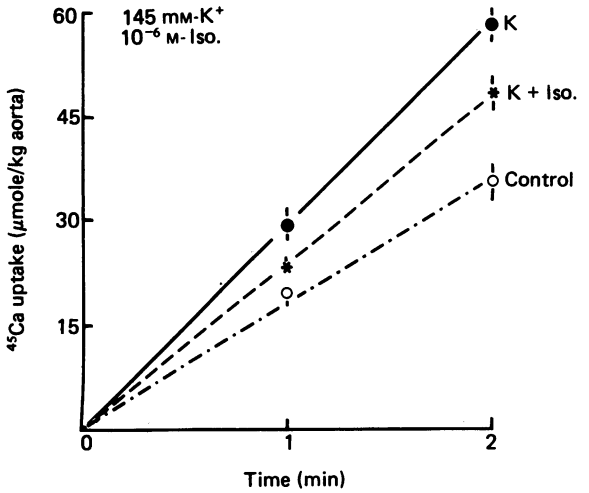


Fig. 2. The effect of isoprenaline ($10^{-6}$ M) on 145 mM-$K^+$-stimulated $Ca^{2+}$ influx. O-·-O, control; ●—●, high-$K^+$; *– –*, high-$K^+$ + isoprenaline. The control curve represents exposure to $^{45}Ca$ for 1 or 2 min. The tissues were exposed to 145 mM-$K^+$ for 5 min before exposing them to $^{45}Ca$ + high-$K^+$. Isoprenaline was present during the last 2 min of the 5 min pre-treatment with non-labelled 145 mM-$K^+$ and during the 2 min with 145 mM-$K^+$ + $^{45}Ca$. The effect of isoprenaline is significant ($P < 0.05$) at both time points.

TABLE 1. The effect of isoprenaline ($10^{-6}$ M) on high-$K^+$-induced net $Ca^{2+}$ uptake

| Treatment | Net $Ca^{2+}$ uptake (μmole/kg aorta) | |
|---|---|---|
| Control (PSS) | 192 ± 10 (5) | |
| 145 mM-$K^+$, 4 min | 340 ± 20 (5) | $P \leq 0.05$ |
| 145 mM-$K^+$ + Iso. $10^{-6}$ M, 4 min | 290 ± 10 (5) | |
| 145 mM-$K^+$, 6 min | 388 ± 11 (5) | $P \leq 0.05$ |
| 145 mM-$K^+$, Iso. 6 min | 324 ± 6 (5) | |
| 145 mM-$K^+$, 8 min | 438 ± 22 (5) | |
| 145 mM-$K^+$, Iso. 8 min | 424 ± 16 (5) | |

The tissues were labelled with $^{45}Ca$ for 2–3 hr and then exposed to $^{45}Ca$-containing high-$K^+$ or high-$K^+$ + isoprenaline for 4, 6 and 8 min. Phentolamine ($10^{-5}$ M) was present throughout. The values are shown as mean ± S.E. of the mean with the number of measurements shown in parentheses. Isoprenaline caused significant ($P < 0.05$) inhibition at 4 and 6 min.

induced by the agonist can be determined much more precisely and thus temporal correlations can be made accurately. As shown in Fig. 3*A*, the muscle ring was exposed to high-$K^+$ for 10 min at which time the muscle exhibited a sustained contracture. Isoprenaline ($10^{-6}$ M) was then added; the maximum relaxation occurred at around 3 min and the tension reached control in about 6–7 min with isoprenaline still present in the bath. Fig. 3*B* shows the corresponding data on the time course of changes in $Ca^{2+}$ influx occurring during high-$K^+$ contraction and isoprenaline-induced relaxation. The $Ca^{2+}$ influx was measured under this condition using a 3 min pulse labelling with $^{45}Ca$ since isoprenaline produced maximum relaxation at 3 min. In Fig. 3*B* the [$K^+$] was raised to 145 mM at $t = 0$. $^{45}Ca$ was added for 3 min only during the time courses indicated by the horizontal bars. The tissues were then removed from the experimental solutions for analysis of cellular $^{45}Ca$ as described in

the Methods section. Isoprenaline was added to one set of aortic rings at $t = 10$ min. High-$K^+$ caused an increase in $Ca^{2+}$ influx, which stabilized at around 11 min. When isoprenaline was added there was a significant decrease in $Ca^{2+}$ influx during the first 6 min of exposure. As was the case with contraction, $Ca^{2+}$ influx was transiently inhibited, returning to control levels after 6 min.

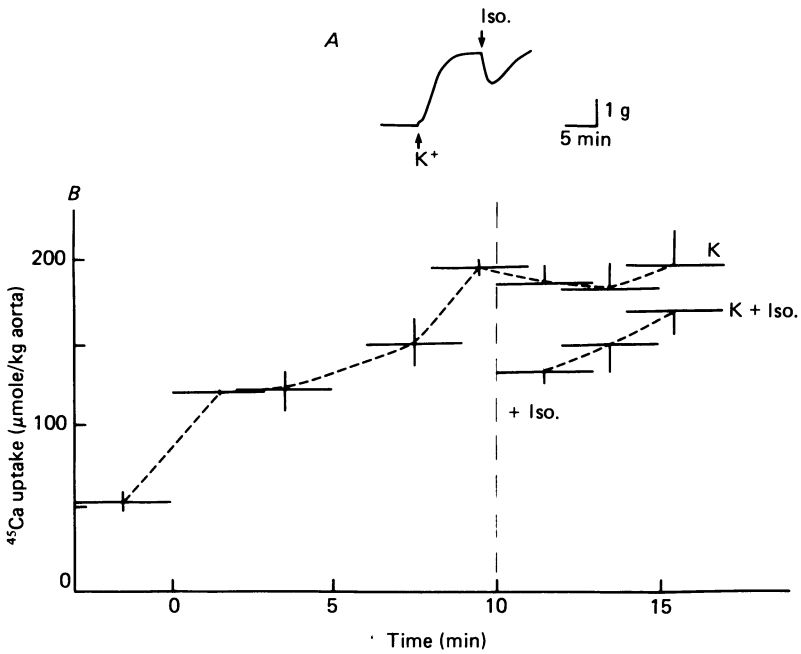


Fig. 3. The effect of isoprenaline ($10^{-6}$ M) on 145 mM-$K^+$ induced contraction (*A*) and stimulated $Ca^{2+}$ influx (*B*). $Ca^{2+}$ influx was measured during 3 min exposures to $^{45}Ca$ which are indicated by the horizontal bars. $K^+$ was raised to 145 mM at $t = 0$. Isoprenaline was added at 10 min after high-$K^+$. The inhibitory effect of isoprenaline on $Ca^{2+}$ influx was significant ($P < 0.05$) during the first 3 min of exposure after the agonist addition.

*Effect of isoprenaline on $^{45}Ca$ efflux*

The efflux was carried out under high-$K^+$ depolarization conditions in order to correlate the data obtained with $^{45}Ca$ influx and relaxation experiments. As discussed above a decrease in cellular $Ca^{2+}$ could also be brought about by stimulation of Ca extrusion. However, as illustrated by the experimental curve of Fig. 4 (stars) addition of isoprenaline did not stimulate $^{45}Ca$ efflux but instead appeared to depress it. This latter effect is consistent with the earlier observation of an inhibition in the rate of $Ca^{2+}$ entry (see Discussion).

*Involvement of cyclic AMP in the $Ca^{2+}$ influx inhibitory effect of isoprenaline*

The second objective of this study was to investigate the involvement of cyclic AMP in the responses stimulated by the $\beta$-adrenergic agonist. This was examined by the use of several criteria originally established by Sutherland & Robison (1966). First, the effect of a phosphodiesterase (PDE) inhibitor was examined. Ro 20-1724 is a

rather specific PDE inhibitor and has been shown previously in smooth muscle to increase cyclic AMP levels (van Breemen, 1977; Meisheri & McNeill, 1979). Fig. 5*A* shows the effects of 1 mM-Ro 20-1724 on high-$K^+$ contraction of rabbit aorta. Like isoprenaline, Ro 20-1724 relaxed the aortic ring. A 5 min pre-treatment with Ro 20-1724 produced a potentiation of isoprenaline-induced relaxation. Ro 20-1724 (1 mM) alone caused a reduction in stimulated $Ca^{2+}$ influx when added at 10 min after high-$K^+$ stimulation (Fig. 5*B*). Furthermore, as was the case with relaxation, pre-treatment with Ro 20-1724 caused potentiation of isoprenaline-induced inhibition of $Ca^{2+}$ influx. The experimental protocol for 3 min $^{45}Ca$ influx was similar to that described earlier for Fig. 3*B*.

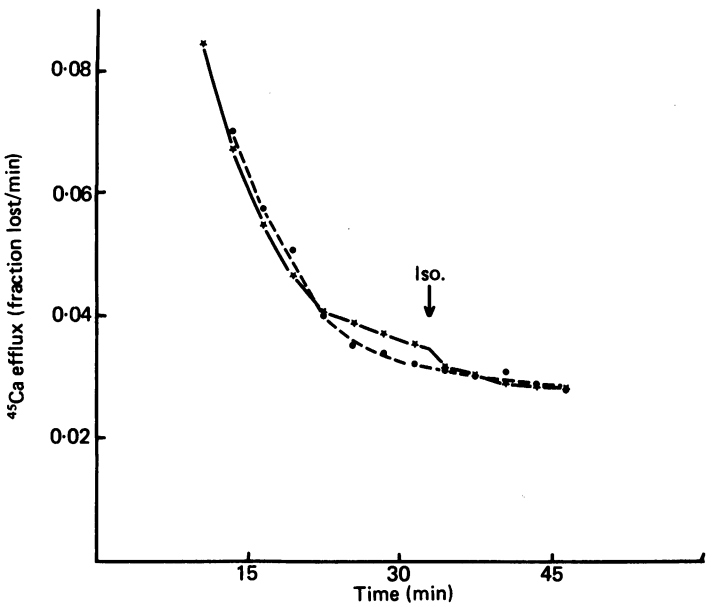


Fig. 4. The effect of isoprenaline ($10^{-6}$ M) on $^{45}Ca$ efflux in rabbit aorta. The tissues were labelled with $^{45}Ca$ and subsequently washed in a 145 mM-$K^+$ solution (with $10^{-5}$ M-phentolamine). $10^{-6}$ M-isoprenaline was added at 33 min after the start of efflux to the experimental tissues (stars). Each point is the mean of four determinations. The standard error of each point was less than 5% of the mean.

In order to complete the correlation, cyclic AMP measurements were also carried out under similar conditions. The data are given in Table 2. Isoprenaline and Ro 20-1724 each caused a significant increase in cyclic AMP levels under high-$K^+$-depolarizing conditions. The increase in cyclic AMP levels by isoprenaline was observed at a time prior to maximum relaxation obtained with the agonist. Pre-treatment with Ro 20-1724 produced a potentiating effect on the isoprenaline-induced increase in cyclic levels.

For the next criterion it was decided to use an agent that does not increase cyclic AMP levels but causes inhibition of $Ca^{2+}$ influx. Such an agent would not be expected to cause potentiation of isoprenaline-induced $Ca^{2+}$ influx inhibition and relaxation. D600 at a submaximal dose of $10^{-7}$ M was used based on our previous observation

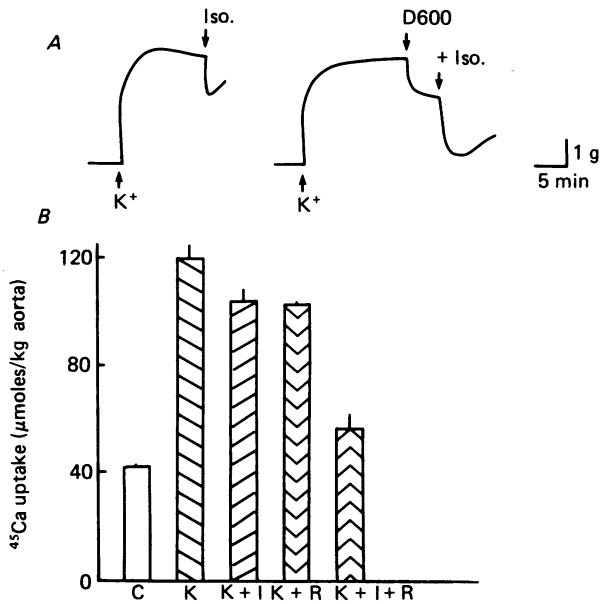


Fig. 5. The effect of Ro 20-1724 (1 mM) on isoprenaline ($10^{-6}$ M)-induced relaxation (*A*) and inhibition of $Ca^{2+}$ influx (*B*) in 145 mM-$K^+$-depolarized rabbit aorta. $^{45}Ca$ influx as measured after a 3 min exposure to $^{45}Ca$. The histogram indicates values for $^{45}Ca$ influx under the following conditions: (C) PSS; (K) 145 mM-$K^+$; (K+I) 145 mM-$K^+$ + $10^{-6}$ M-isoprenaline; (K+R) 145 mM-$K^+$ + 1 mM-Ro 20-1724; (K+I+R) 145 mM-$K^+$ + $10^{-6}$ M-isoprenaline + 1 mM-Ro 20-1724. Isoprenaline, Ro 20-1724 or their combination were added after a 10 min pre-incubation in 145 mM-$K^+$. Isoprenaline and Ro 20-1724 each alone caused a significant ($P < 0.05$) inhibition of high-$K^+$-stimulated $Ca^{2+}$ influx. The $Ca^{2+}$ influx inhibition by isoprenaline in the presence of Ro 20-1724 is significantly ($P < 0.05$) greater than that caused by isoprenaline in the absence of Ro 20-1724.

TABLE 2. Cyclic AMP in rabbit aorta

| Treatment | Cyclic AMP levels (p-mole/mg tissue wet wt.) | Average increase over control |
|---|---|---|
| 145 mM-$K^+$, 10 min (control) | 0·727 ± 0·100 (8) | — |
| 145 mM-$K^+$, 10 min + Iso. $10^{-6}$ M, last 2·5 min | 1·001 ± 0·111 (8)* | 0·274 |
| 145 mM-$K^+$, 10 min + Ro 20-1724, 1 mM, last 5 min | 1·086 ± 0·108 (7)* | 0·359 |
| 145 mM-$K^+$, 5 min + $K^+$ with Ro, 5 min + $K^+$ with Ro and Iso. 2·5 min | 1·875 ± 0·289 (8)** | 1·147 |

The effect of isoprenaline (Iso.; $10^{-6}$ M), Ro 20-1724 (Ro; 1 mM) and Iso. + Ro 20-1724 together on cyclic AMP levels in 145 mM-$K^+$-depolarized rabbit aorta. The values are given as mean ± S.E. of the mean with *n* in parentheses. Iso. and Ro each alone caused a significant ($P < 0.05$) increase in cyclic AMP levels over control. The increase in cyclic AMP levels by Iso. + Ro over control is significantly greater ($P < 0.05$) than the addition of increase in cyclic AMP over control obtained with each agent alone.

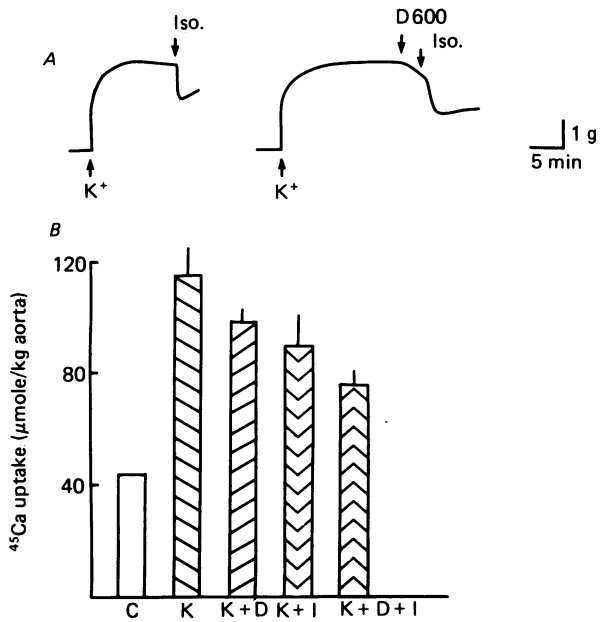


Fig. 6. The effect of a submaximal dose of D600 ($10^{-7}$ M) on isoprenaline ($10^{-6}$ M)-induced relaxation (*A*) and inhibition of $Ca^{2+}$ influx (*B*) in 145 mM-$K^+$-depolarized rabbit aorta. $Ca^{2+}$ influx was measured after a 3 min exposure to $^{45}Ca$. The histogram indicates values for $^{45}Ca$ influx under the following conditions: (C) PSS; (K) 145 mM-$K^+$; (K+D) 145 mM-$K^+$ + $10^{-7}$ M-D600; (K+I) 145 mM-$K^+$ + $10^{-6}$ M-isoprenaline; (K+D+I) 145 mM-$K^+$ + $10^{-7}$ M-D600 + $10^{-6}$ M-isoprenaline. Isoprenaline, D600 or their combination were added after a 10 min pre-incubation in 145 mM-$K^+$. Isoprenaline and D600 each alone caused a significant ($P < 0.05$) inhibition of high-$K^+$-stimulated $Ca^{2+}$ influx. The $Ca^{2+}$ influx inhibition by isoprenaline in the presence of D600 is not significantly greater ($P > 0.05$) than that caused by isoprenaline in the absence of D600.

(Meisheri *et al.* 1981). Exposure to $10^{-7}$ M-D600 for 5 min caused a partial relaxation of high-$K^+$-induced contraction (Fig. 6*A*). D600 also caused partial inhibition of $Ca^{2+}$ influx (Fig. 6*B*). However, unlike Ro 20-1724, D600 did not potentiate isoprenaline-induced relaxation or inhibition of $Ca^{2+}$ influx. In Fig. 6*A*, the decline of relaxation does not occur in the record with D600 and isoprenaline, presumably because the slower acting D600 is still effective. The experimental protocol for 3 min $^{45}Ca$ influx was similar to that described earlier for Fig. 3*B*.

The final criterion involved the use of dibutyryl cyclic AMP, a lipid soluble analogue of cyclic AMP. Dibutyryl cyclic AMP (1 mM) caused a slow relaxation of high-$K^+$-induced contraction in rabbit aorta (Fig. 7*A*). The relaxation, in comparison with isoprenaline, is much slower, presumably due to the time required for the entry into the cell. The corresponding data on $Ca^{2+}$ influx indicates that dibutyryl cyclic AMP also caused a significant attenuation of high-$K^+$-stimulated $Ca^{2+}$ influx (Fig. 7*B*).

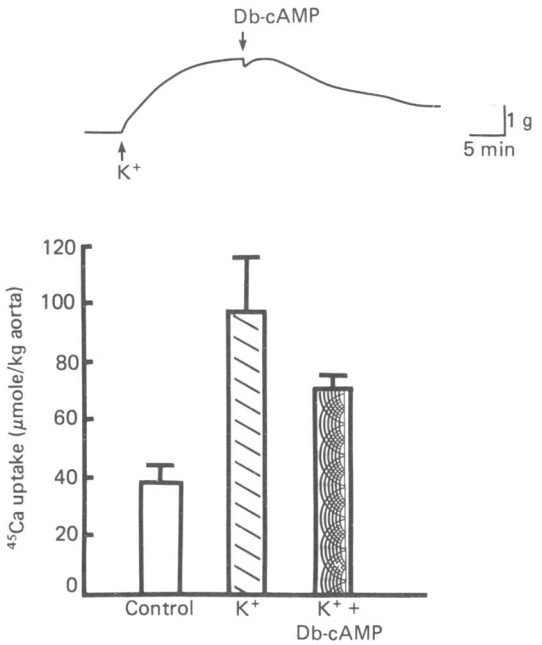


Fig. 7. The effect of dibutyryl cyclic AMP (1 mM) on 145 mM-$K^+$-induced contraction (*A*) and stimulated $Ca^{2+}$ influx (*B*). $Ca^{2+}$ influx was measured after a 3 min exposure to $^{45}Ca$ as described for Fig. 3*B* in the text. The histogram indicates values for $^{45}Ca$ influx under the following conditions: (control) PSS; ($K^+$) 145 mM-$K^+$; ($K^+$ + Db-cAMP) 145 mM-$K^+$ + 1 mM-dibutyryl cyclic AMP. In this case the tissues were treated for 10 min with 145 mM-$K^+$ and for 15 min with 145 mM-$K^+$ and 1 mM-dibutyryl cyclic AMP. $^{45}Ca$ was present during the last 3 min. Dibutyryl cyclic AMP caused a significant ($P < 0.05$) inhibition of high-$K^+$-stimulated $Ca^{2+}$ influx.

## DISCUSSION

Several mechanisms have been implicated in the inhibitory action of catecholamines on smooth muscle mediated by $\beta$-receptor activation. Uncertainty concerning the specific effects of $\beta$-agonists on $Ca^{2+}$ distribution in smooth muscle arises from the fact that there are multiple mechanisms by which the $\beta$-agonists can alter $Ca^{2+}$ distribution to produce relaxation. As pointed out earlier, isoprenaline could affect influx, efflux or intracellular sequestration of $Ca^{2+}$. The ability to delineate an effect on a particular $Ca^{2+}$ pathway resides in the precision of the $Ca^{2+}$ flux measurement technique. The changes in net $Ca^{2+}$ content do not accurately reflect changes in unidirectional movement of $Ca^{2+}$ across the cell membrane. On the other hand, the technique of pulse labelling, i.e. short term exposure to $^{45}Ca$, allows relative measurements of unidirectional $Ca^{2+}$ influx with minimal back-flux complications. The results of Fig. 2 show that isoprenaline-induced inhibition of $^{45}Ca$ uptake in high-$K^+$ could be demonstrated as early as 1 min, at which time the $^{45}Ca$ labelling process was less than 10% completed. The assumption that this $\beta$-adrenergic receptor-mediated reduction of initial $^{45}Ca$ uptake was due to inhibition of $Ca^{2+}$ influx was further strengthened by the results presented in Fig. 4. Addition of isoprenaline to high-$K^+$-depolarized rabbit aorta did not stimulate the rate of $^{45}Ca$ efflux but

instead slightly decreased it. This result is in agreement with the above-discussed inhibition of $Ca^{2+}$ influx, which might inhibit cellular $^{40}Ca$–$^{45}Ca$ exchange during the washout experiment (van Breemen, Wuytack & Casteels, 1975). Thus it appears that isoprenaline causes an inhibition of the sarcolemmal $Ca^{2+}$ pathway that is activated by high-$K^+$ depolarization. This inhibitory effect on stimulated $Ca^{2+}$ influx can contribute to the relaxation of depolarized aorta by the β-adrenergic agonist.

In a recent study on guinea-pig taenia coli, isoprenaline was observed not to affect stimulated $^{45}Ca$ influx in a low-$Na^+$ (18 mM) solution (Bülbring & den Hertog, 1980). It has been observed that in guinea-pig taenia coli the low-$Na^+$ solution stimulates $Ca^{2+}$ uptake which, unlike high-$K^+$-stimulated $Ca^{2+}$ uptake, was not blocked by D600 (Aaronson & van Breemen, 1981). Presumably, β-receptor activation affects the influx of $Ca^{2+}$ through one membrane pathway but not the other. Consistent with this concept we have also observed that isoprenaline did not inhibit $Ca^{2+}$ influx stimulated by α-adrenergic receptor activation of the rabbit aortic smooth muscle (Meisheri & van Breemen, 1981). In the dog saphenous vein isoprenaline ($5 \times 10^{-9}$ to $5 \times 10^{-6}$ M) caused dose-dependent relaxation of contractions induced by acetylcholine, norepinephrine, and high-$K^+$ (Collis & Shepherd, 1979). Isoprenaline inhibited the contractile response to acetylcholine to a greater extent than the responses by noradrenaline and high-$K^+$. It was suggested, based on these contraction experiments, that the β-receptor-induced relaxation was primarily due to the inhibition of stimulated $Ca^{2+}$ influx.

The involvement of cyclic AMP in the inhibitory effect of isoprenaline on high-$K^+$-induced contraction and $Ca^{2+}$ influx is suggested, based on the following observations: (1) isoprenaline increased cyclic AMP levels, inhibited $Ca^{2+}$ influx, and caused relaxation; (2) the increase in cyclic AMP levels by isoprenaline was observed before the maximum inhibition of $Ca^{2+}$ influx and relaxation; (3) Ro 20-1724, a PDE inhibitor, also produced the above three responses similar to isoprenaline; (4) pre-treatment with Ro 20-1724 potentiated the above three responses (cyclic AMP elevation, $Ca^{2+}$ influx inhibition, and relaxation) by isoprenaline; (5) pre-treatment with D600, which inhibits $Ca^{2+}$ influx but does not increase cyclic AMP levels, did not potentiate isoprenaline-induced $Ca^{2+}$ influx inhibition and relaxation, and (6) dibutyryl cyclic AMP, a lipid soluble analogue of cyclic AMP, produced $Ca^{2+}$ influx inhibition as well as relaxation. Although its actions were much slower than those of isoprenaline, these observations provide support for the role of cyclic AMP in the responses to β-stimulation, although a causal relationship between increases in cyclic AMP levels and β-responses cannot be conclusively established (Diamond, 1978; Kramer & Hardman, 1980).

The mechanism by which isoprenaline can produce inhibition of $Ca^{2+}$ influx via a cyclic AMP pathway remains unknown. It has been suggested that stimulation of the electrogenic $Na^+$–$K^+$ pump by cyclic AMP in smooth muscle causes hyperpolarization and thus reduces excitability of the cell membrane (Webb & Bohr, 1981). The results of the present study exclude such a mechanism. It is unlikely that the $Na^+$–$K^+$ pump remained active during exposure of the tissue to 145 mM-$K^+$/0 $Na^+$ solution due to the loss of $Na^+$ gradient (Mueller & van Breemen, 1979). However, since the tissue was completely depolarized with high-$K^+$, isoprenaline would be unlikely to cause hyperpolarization even if the electrogenic $Na^+$ pump were active.

It has been demonstrated in several smooth muscles that the relaxant action of isoprenaline in high-$K^+$-depolarized solution is not associated with a change in membrane potential (Johansson, Johnsson, Axelsson & Wahlstrom, 1967; Somlyo & Somlyo, 1968; Meisheri, McNeill & Marshall, 1979). Experimental manipulations that abolish $Na^+$–$K^+$ pump activity (ouabain or 0 $K^+$ treatment) reduce but do not abolish isoprenaline-induced relaxation or hyperpolarization responses (Marshall & Kroeger, 1973; Marshall, 1977; Bülbring & den Hertog, 1980). However, the contribution of the electrogenic $Na^+$ pumping to isoprenaline-induced relaxation in normally polarized smooth muscle cannot be excluded.

Another suggested mechanism by which stimulation of the $Na^+$–$K^+$ pump could contribute to $\beta$-adrenergic-mediated relaxation in smooth muscle is enhancement of the $Na^+$–$Ca^{2+}$ exchange mechanism (Scheid, Honeyman & Fay, 1979). Again, the use of 145 mM-$K^+$/0 $Na^+$ solution in the present study excludes such a mechanism. The exposure of the tissue to 145 mM-$K^+$/0 $Na^+$ depletes extracellular $Na^+$ and reverses the $Na^+$ gradient and thus would be unable to cause $Ca^{2+}$ extrusion via the hypothetical $Na^+$–$Ca^{2+}$ exchange mechanism (Mueller & van Breemen, 1979; Mueller, Meisheri & van Breemen, 1981).

In summary, isoprenaline-induced relaxation of high-$K^+$-depolarized aortic smooth muscle was found to be associated with inhibition of stimulated $Ca^{2+}$ influx. The involvement of cyclic AMP in these actions of $\beta$-adrenergic stimulation is indicated but the mechanism by which this may occur remains obscure. A possibility that there is a direct inhibitory effect of cyclic AMP on the depolarization-sensitive $Ca^{2+}$ channels mediated by cyclic AMP-dependent protein kinase-induced phosphorylation needs to be tested. The results do not rule out involvement of additional mechanisms such as intracellular sequestration of $Ca^{2+}$ and extrusion of $Ca^{2+}$ in decreasing intracellular free $Ca^{2+}$ concentration during $\beta$-adrenergic receptor mediated relaxation. No evidence for a role of the $Na^+$–$K^+$ pump or $Na^+$–$Ca^{2+}$ exchange mechanism in this mode of relaxation was obtained in the present study.

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