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MECHANISMS OF RELAXATION INDUCED BY ACTIVATION OF β-ADRENOCEPTORS IN SMOOTH MUSCLE CELLS OF THE GUINEA-PIG MESENTERIC ARTERY

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SUMMARY

Relaxation of smooth muscle cells induced by activation of β-adrenoceptors was investigated in intact and skinned muscles of the guinea-pig mesenteric artery.

1. In concentrations over 10^{-7} M, isoprenaline reduced the resting tone of intact preparations and also the amplitude of K contractions. When Ca was applied after previous superfusion with Ca-free solution, the amount of Ca accumulated into storage sites was increased by isoprenaline in polarized and depolarized ($[K]_o$ 128 mM) muscles. The amount of Ca stored increased even further when procaine and isoprenaline were applied simultaneously during store loading.

2. Isoprenaline increased the concentration of cyclic AMP as determined by radioimmunoassay. Application of isoprenaline at a concentration of 10^{-7} M increased cyclic AMP from $2{\cdot}2 \pm 0{\cdot}3$ to $2{\cdot}8 \pm 0{\cdot}6$ p-mole/mg wet weight and at 10^{-6} M increased it to $4{\cdot}5 \pm 0{\cdot}8$ p-mole/mg wet weight after 5 min incubation ($n = 4$).

3. Application of cyclic AMP (3×10^{-6} M) with cyclic AMP-dependent protein kinase (50 μg/ml.) had no effect on the pCa–tension relationship in the skinned muscles. However, an increased concentration of cyclic AMP ($> 10^{-5}$ M) suppressed the Ca-induced concentration only in the presence of protein kinase. This protein kinase (50 μg/ml.) alone had no effect on the Ca-induced contraction.

4. In skinned fibres, the Ca store could be loaded by applying low concentrations of Ca. If cyclic AMP (3×10^{-6} M) with protein kinase (50 μg/ml.) was applied during the loading procedure, the amount of Ca accumulated by the store increased if the loading solution contained 10^{-6} M-Ca applied for 2 min or less, but if the loading solution was applied for 3 min, or if higher Ca concentrations were used, the presence of cyclic AMP with protein kinase decreased the store size, suggesting that a Ca-induced Ca-release mechanism was also being activated.

5. In skinned muscles, accumulation of Ca into the store site in the presence of cyclic AMP (3×10^{-6} M) with protein kinase (50 μg/ml.) was further accelerated by simultaneous applications of procaine (5 mM), as here the Ca-induced Ca-release mechanism was suppressed.

6. These results indicate that activation of β-adrenoceptors by isoprenaline increases the amount of cyclic AMP in the intact muscles, and leads to an increase in Ca accumulation into the store site. In the skinned muscles, the Ca-induced

Ca-release mechanism is activated by cyclic AMP and the Ca receptor for contraction (leiotonin C or calmodulin) is somewhat suppressed. These effects of exogenously applied cyclic AMP require the presence of protein kinase. The relaxation following β-adrenoceptor activation is more likely to involve Ca extrusion from the cell and accumulation of Ca in internal storage sites than suppression of the binding of calmodulin with the myosin light chain kinase.

INTRODUCTION

Activation of β-adrenoceptors in smooth muscles produces a relaxation of the tissue or a reduction in the amplitude of contraction evoked by various stimulants. These effects could be due to a reduction in cytoplasmic free Ca levels reducing activation of the contractile proteins, or to reactions involving a direct suppression of the contractile apparatus.

There is evidence that a reduction in free Ca could be due to activation by cyclic AMP of Ca extrusion to the extracellular space. This mechanism was suggested by Marshall & Kroeger (1973) as the main cause for the relaxation of rat myometrium by isoprenaline, the Na pump not being involved. However, Scheid, Honeyman & Fay (1979) reported that, in the toad stomach muscle, Ca extrusion is due to activation of a Na–Ca exchange diffusion, as a consequence of a cyclic AMP-dependent increase in Na-K-ATPase. The Na–Ca exchange diffusion would be enhanced by the reduction in $[Na]_i$ and would lead to decrease of free $[Ca]_i$. Bülbring & den Hertog (1980) carried out electrophysiological studies and ion-flux measurements in the guinea-pig taenia coli and obtained evidence indicating that the Na–K pump was not primarily involved but that isoprenaline stimulated an electrogenic Ca-extrusion pump (presumably activation of Ca-ATPase), resulting in hyperpolarization of the membrane.

Reduction in free Ca activity in the cytoplasm could also be due to an increase in the Ca accumulation into storage sites in the cell, mainly sarcoplasmic reticulum (s.r.). If this activation is brought about by cyclic AMP-sensitive Ca-ATPase, the extrusion of Ca across the cell membrane and Ca sequestration by the s.r. would be due to the same mechanisms and would appear simultaneously. The accumulation of Ca into store sites by activation of β-adrenoceptors has also been confirmed using mechanical recordings (Ohashi, Ohga & Saito, 1973; Ohashi, Takewaki & Okada, 1974; Casteels & Raeymaekers, 1979). Mueller & van Breemen (1979) found that the Ca sequestration by the s.r. and relaxation could be observed when Na-K-ATPase was suppressed by pre-treatment with cardiac glycoside. Numerous investigators have used biochemical procedures to observe the β-adrenergic response, measuring the activation of adenyl cyclase and changes in cyclic AMP content of the tissue (Goldberg, Haddox, Nicol, Glass, Stanford, Kuehl & Estensen, 1975; Andersson, Nilsson, Wikberg, Johansson, Mohme-Lundholm & Lundholm, 1975; Weiss, 1977; McNeill, 1977; Andersson & Nilsson, 1977; Fitzpatrick & Szentivanyi, 1977; Diamond, 1978; Bhalla, Webb, Singh & Brock, 1978; Hardman, 1981). Although the role of cyclic AMP in relaxation of visceral smooth muscles has been an area of great interest, a consistent link is lacking between changes in the tissue cyclic AMP and activated ATPase. For instance in vascular smooth muscles, the transport of Ca by microsomes was not increased by applied cyclic AMP (Zelck & Karnstedt, 1977), and the

phosphorylation activated by cyclic AMP and relaxation were not causally related (Hirata & Kuriyama, 1980).

Increase in cyclic AMP mediated by β-receptor activation can also lead to competitive actions between cyclic AMP-dependent protein kinase (protein kinase) and calmodulin on the myosin light chain kinase. This has been elucidated using biochemical procedures and skinned muscles (Cheung, 1970, 1971; Kakiuchi & Yamazaki, 1970; Waisman, Stevens and Wang, 1978; Yagi, Yazawa, Kakiuchi, Oshima & Uenishi, 1978; Hidaka, Yamaki, Totsuka & Asano, 1979; Adelstein, Conti, Hathaway & Klee, 1978; Head, Birnbaum & Kaminer, 1980; Silver & Disalvo, 1979; Sparrow, Mrwa, Hofmann & Rüegg, 1981; Kerrick & Hoar, 1981; Kerrick, Hoar, Cassidy, Bolles & Malencik, 1981; Vallet, Molla & Demaille, 1981). The results in the above papers indicate that the activation of the free catalytic subunit of cyclic AMP-dependent protein kinase is accelerated by increased cyclic AMP, resulting in phosphorylation of the myosin light chain kinase; they also indicate that the binding between calmodulin and myosin light chain phosphokinase is weakened, which causes an increase in the amount of unphosphorylated myosin, resulting in relaxation (Conti & Adelstein, 1980; Adelstein, Pato & Conti, 1980; Adelstein & Eisenberg, 1980).

Thus, activation of β-adrenoceptors either reduces the amplitude of contraction or relaxes the tissue through extrusion of Ca, sequestration of Ca by the s.r. suppression of Ca binding by the contractile proteins or a combination of these effects.

Application of cyclic AMP together with protein kinase (or catalytic subunits of protein kinase) has been shown to increase Ca accumulation into the s.r. in skinned cardiac muscles (Endo & Kitazawa, 1978). Furthermore, in skinned smooth muscles treated with Triton X-100, application of cyclic AMP-dependent protein kinase modulates the sensitivity of Ca receptors of the contractile protein (Sparrow *et al.* 1981).

In a previous paper (Itoh, Kuriyama & Suzuki, 1981) we have shown that it is possible to prepare chemically skinned smooth muscles of the guinea-pig mesenteric artery, which can be used to investigate pCa–tension relationships, and in which internal Ca stores can be preserved. These stores can be filled by exposure of the tissues to low levels of free Ca and the Ca can be released to cause contraction by applying caffeine. Caffeine has been shown to induce release of Ca from the s.r. in striated muscle (Endo, 1977) probably by activating a Ca-induced Ca-release mechanism, and there is evidence that such a mechanism also exists in the mesenteric artery smooth muscle (Itoh *et al.* 1981). In order to elucidate the mechanism by which isoprenaline causes relaxation in this tissue, we have used both intact and chemically skinned muscles. In intact muscles we have investigated the effects of isoprenaline on the K contraction, on the accumulation of Ca into internal stores and on the cyclic AMP content, whereas in skinned preparations we have investigated the effects of cyclic AMP and protein kinase on the ability of the contractile machinery to be activated by Ca, and on the accumulation of Ca into the internal store. Since a Ca-induced Ca-release mechanism may also be activated by increased accumulation of Ca into the store sites, procaine, which is known to inactivate this mechanism, was used in many of the experiments.

The results are discussed in relation to the relaxation induced by isoprenaline at physiological concentrations.

METHODS

Guinea-pigs of either sex were stunned and bled. The dissected mesenteric artery from the region of the jejunum was about 10 mm in length and the diameter was about 0·1–0·12 mm. Connective tissue and membranous material were removed under a binocular microscope.

The modified Krebs solution was composed of Na^+ 137·4 mM, K^+ 5·9 mM, Mg^{2+} 1·2 mM, Ca^{2+} 2·5 mM, Cl^- 134·0 mM, $H_2PO_4^-$ 1·2 mM, HCO_3^- 15·5 mM and glucose 11·5 mM. The solution was aerated by 97 % O_2 with 3 % CO_2.

For tension recordings from normal (intact) and chemically skinned muscle cells, the vessel was carefully teased apart using jewellers' forceps, opened along the longitudinal direction, and a circularly cut strip of 0·1 mm in width and 0·3 mm in length was prepared.

To observe the effects of isoprenaline on the Ca accumulation into the store site in intact muscle, or the effects of cyclic AMP on Ca accumulation into the store site in skinned muscle, the contraction evoked by caffeine was used as an indicator. The procedure in each case was to fill the store by exposing the tissue to Ca of varying concentrations for different times, and then, after a 2 min wash in Ca-free solution, to release the store with caffeine and measure the contraction. Since enhanced Ca accumulation may lead to activation of a Ca-induced Ca-release mechanism, procaine (which is known to prevent this) was also used during the store filling. As procaine will also block the caffeine contraction, it is necessary that it should be washed from the tissue before caffeine application. Preliminary experiments suggest that procaine is completely washed out of the intact and skinned preparations during the 2 min wash in Ca-free solution.

The experimental procedures for the chemically skinned muscles were the same as described (Saida & Nonomura, 1978; Itoh *et al.* 1981); briefly, the tension was recorded using a strain gauge transducer (U-gauge, Shinko Co.), and to prepare the skinned muscles saponin (50 μg/ml.) was superfused for 20 min. The relaxing solution for the skinned muscles was composed of 130 mM-KCl, 20 mM-Tris maleate, 5 mM-$MgCl_2$, 5 mM-ATP (10 mM Na as Na_2 ATP) and 4 mM-EGTA at pH 6·8. Various Ca concentrations were prepared by adding appropriate amounts of $CaCl_2$ to EGTA. The apparent binding constant of EGTA for Ca was considered to be 10^6 M^{-1} at pH 6·8 at 25 °C. The pH of the procaine and caffeine containing solution was kept at 6·8 by adding KOH instead of KCl isotonically. Since skinned muscle may lose soluble protein kinase, in order to maintain the ability of cyclic AMP to activate Ca pumping into the store, cyclic-AMP-dependent protein kinase (50 μg/ml.) was added to the solution (1×10^{-6} g cyclic-AMP-dependent protein kinase will bind to approximately $0{\cdot}17 \times 10^{-12}$ mole cyclic AMP which is at a concentration of $1{\cdot}6 \times 10^{-7}$ M (Sigma), as has been determined in skinned cardiac muscle cells (Endo & Kitazawa, 1978). This protein kinase is, however, also known to phosphorylate the myosin light chain kinase (see Discussion).

To measure the cyclic AMP before and after application of isoprenaline, tissues weighing 15–20 mg (15 mg in most preparations), were mounted in an organ bath without any load. Tissues kept at 37 °C for 60 min in the bubbled Krebs solution (97 % O_2 and 3 % CO_2) served as control. Isoprenaline was applied to the experimental tissues during the last 5 min of the 60 min incubation. After incubation, the tissues were frozen with liquid nitrogen and homogenized with 6 % trichloroacetic acid. The level of cyclic AMP of the extracts was measured using a radioimmunoassay Kit (Yamasa Shoyu: Honma, Satoh, Takezawa & Ui, 1977).

To record the membrane potential from smooth muscles, intracellular glass capillary microelectrodes filled with 3 M-KCl were used. The resistance of the electrodes was 50–80 MΩ.

The following drugs were used: caffeine (Wako), procaine HCl (Sigma), ethyleneglycol-bis (β-aminoethylether)-*N*-*N*′-tetra acetic acid (EGTA; Dozin), adenosine 3′,5′ cyclic monophosphate (cyclic AMP; Sigma), cyclic-AMP-dependent protein kinase (protein kinase; Sigma P5511), isoprenaline HCl (Tokyo Kasei) and phentolamine (Ciba-Geigy). Stock solutions of drugs were freshly prepared just before each experiment.

RESULTS

Effects of isoprenaline on the polarized and depolarized muscles

The membrane potential of smooth muscle cells of the guinea-pig mesenteric artery was $-67{\cdot}8 \pm 2{\cdot}3$ mV ($n = 20$) and application of 128 mM-K markedly depolarized the

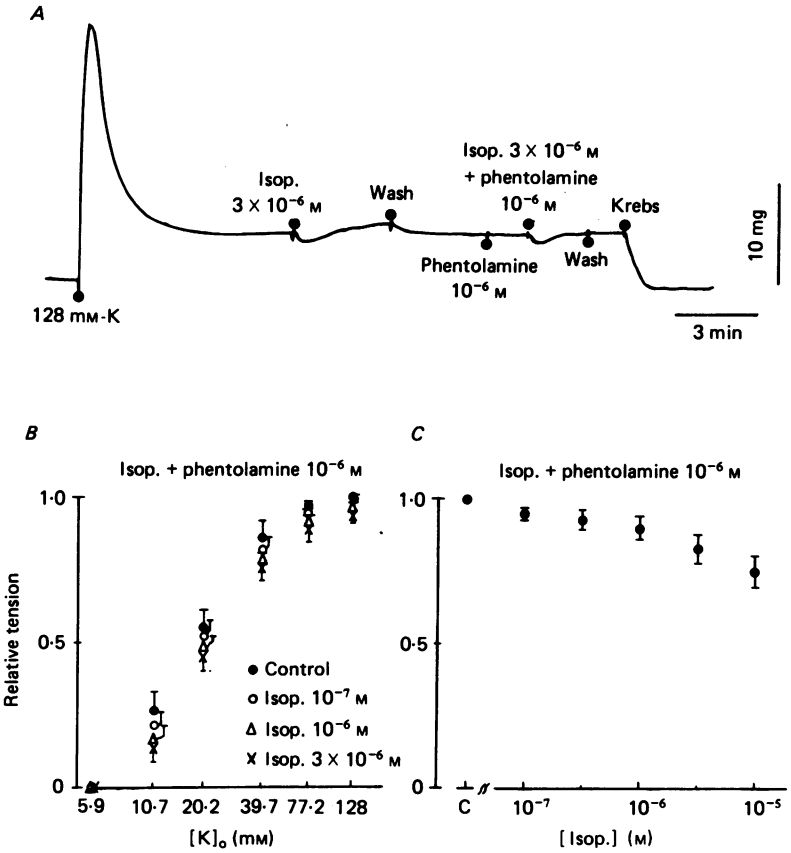

Fig. 1. Effects of isoprenaline on the K-induced contraction. *A*, effects of isoprenaline (isop.), and isoprenaline with phentolamine on the tonic response of 128 mM-K-induced contraction. *B*, effects of isoprenaline ($+10^{-6}$ M-phentolamine) on the phasic response of K-induced contraction. The concentration of isoprenaline was varied from 10^{-7} to 3×10^{-6} M, and $[K]_o$ was varied from 5·9 to 128 mM. *C*, effects of different concentrations of isoprenaline (10^{-7}–10^{-5} M) on the phasic contraction evoked by 39·7 mM-K. Phentolamine (10^{-6} M) was applied throughout the experiment. In *B* and in *C*, the amplitude of K-induced contractions evoked by 128 mM-K and 39·7 mM-K were normalized. $n = 5$, and bars represent mean ± S.D.

membrane ($-8{\cdot}1 \pm 0{\cdot}6$ mV, $n = 20$). The depolarization was sustained during application of excess K concentration for over 20 min. In normal Krebs solution isoprenaline at a concentration below 10^{-6} M did not alter either the membrane potential or the membrane resistance. Increased concentrations of isoprenaline ($> 10^{-5}$ M) slightly hyperpolarized the membrane ($-68{\cdot}1 \pm 2{\cdot}4$ mV to $-70{\cdot}9 \pm 1{\cdot}7$ mV, $n = 15$) and these findings confirmed the observations made by Takata (1980). A reduction in the resting muscle tone was observed on application of isoprenaline at concentrations over 10^{-7} M. The magnitude of relaxation depended on the extent to which the tissues were stretched in the organ bath.

Application of 128 mM-K produced a contraction with phasic and tonic components. Fig. 1 shows the effects of isoprenaline (3×10^{-6} M) applied during the tonic

component. The tissue was transiently relaxed and subsequently the amplitude of the tonic response was enlarged. This secondary enlargement was abolished if isoprenaline was applied in the presence of 10^{-6} M-phentolamine (Fig. 1*A*). Therefore, 10^{-6} M-phentolamine was applied throughout these experiments. Fig. 1*B* shows the effects of isoprenaline on the phasic contraction evoked by various external concentrations of K. 10^{-7} M isoprenaline slightly reduced the contraction and a clear reduction was observed with 3×10^{-6} M-isoprenaline. Fig. 1*C* shows the effects of different concentrations of isoprenaline on the contraction evoked by 39·7 mM-K. Application of 10^{-7} M-isoprenaline reduced the amplitude of the phasic response only slightly but 10^{-5} M reduced the amplitude to 75% of the control.

Effects of isoprenaline on the caffeine-induced contraction in polarized and depolarized muscles

Fig. 2*A* shows the effects of isoprenaline on the caffeine-induced contraction in polarized muscle ($K = 5{\cdot}9$ mM). The tissue was superfused with Ca-free solution (containing 2 mM-EGTA) for 5 min, then 2·5 mM-Ca was applied for various durations, and subsequently 5 mM-caffeine was applied after 2 min pre-incubation in Ca-free solution. The amplitude of the caffeine-induced contraction increased proportionally with the duration of incubation in 2·5 mM-Ca, up to 5 min. The size of contraction following 10 min superfusion with Ca was normalized as 1·0. When Ca was applied simultaneously with 3×10^{-6} M-isoprenaline, the amplitude of the caffeine contraction was consistently increased following any duration of exposure to Ca (up to 5 min). Fig. 2*B* shows the effects of isoprenaline and various concentrations of procaine on the caffeine-induced contraction. The duration of incubation in 2·5 mM-Ca was fixed at 5 min. Ca with different concentrations of procaine (0·1–10 mM) was applied in the presence or absence of isoprenaline. After pre-treatment with 0·1 mM-procaine, the amplitude of the caffeine-induced contraction was slightly enhanced, and after 3 mM-procaine the amplitude was enhanced to $1{\cdot}75 \pm 0{\cdot}12$ times that of the control. However, when isoprenaline was applied simultaneously with Ca and procaine, the amplitude of the caffeine contraction was enhanced to $2{\cdot}3 \pm 0{\cdot}21$ times that of the control.

Fig. 3 shows the results of experiments on depolarized muscles. The tissues were superfused with Ca-free (containing 2 mM-EGTA) high-K (128 mM) solution for 5 min, and then 2·5 mM-Ca was applied for various durations with or without isoprenaline or procaine. After a 2 min wash with Ca-free high-K solution a caffeine contraction was evoked to assess the store size. In control experiments, the size of the caffeine contraction was almost its maximum after 1–2 min exposure to Ca. When isoprenaline was added with Ca, the caffeine contraction reached its maximum after only 30 sec Ca exposure, and after 3 min the caffeine contraction was somewhat smaller than without isoprenaline (see Fig. 3*A* and *C*). Thus the stimulating effect of isoprenaline in these conditions can only be seen for short Ca applications. Fig. 3*B* shows the effects of different isoprenaline concentrations applied during a 30 sec Ca loading period, on the caffeine contraction. In the presence of 3×10^{-6} M-isoprenaline the amplitude was increased to $1{\cdot}32 \pm 0{\cdot}09$ times that of the control.

The fact that isoprenaline only enhances the caffeine contraction when the store is loaded by short exposures to Ca, suggests that a Ca-induced Ca-release process may

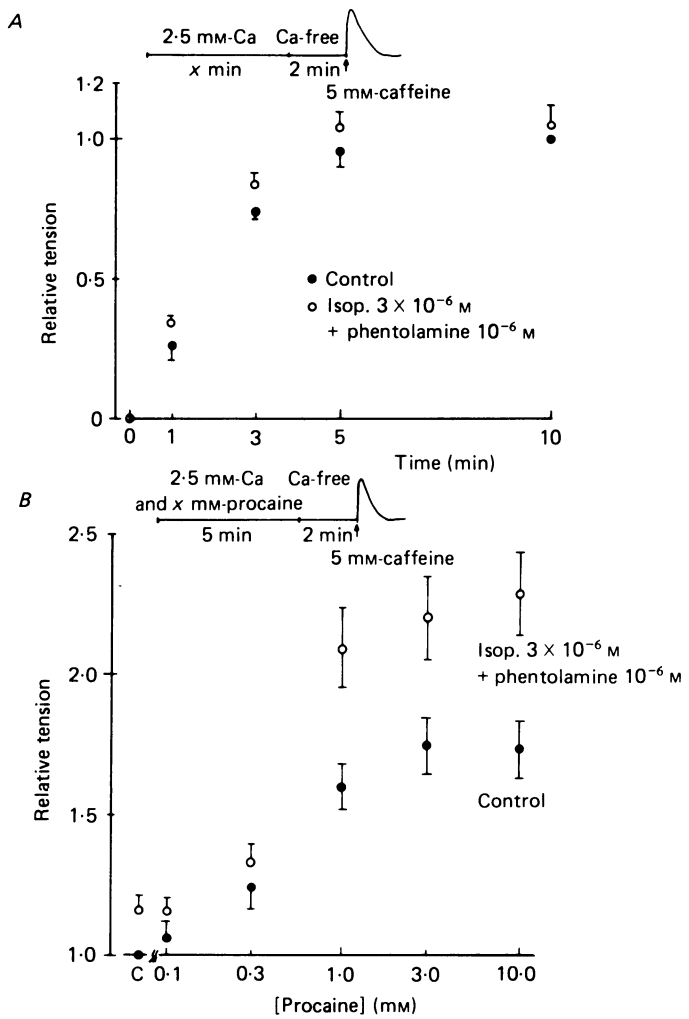

Fig. 2. Effects of isoprenaline applied during pre-incubation with Ca on the caffeine-induced contraction in Ca-free solution. Phentolamine (10^{-6} M) was applied simultaneously with isoprenaline (3×10^{-6} M). The tissues were initially bathed in Krebs solution, followed by 5 min exposure to Ca-free solution before the experiment started. *A*, 2·5 mM-Ca was superfused for various durations (*x* min), and 5 mM-caffeine was applied following 2 min superfusion in Ca-free EGTA (2 mM)-containing solution. Schematic procedures are inserted in the Figure. ●, control; ○, in the presence of 3×10^{-6} M-isoprenaline, 10 min exposure in 2·5 mM-Ca is normalized as 1·0. *B*, 3×10^{-6} M-isoprenaline (10^{-6} M-phentolamine) was applied during exposure (5 min) to 2·5 mM-Ca with various concentrations of procaine. Caffeine was applied after 2 min wash in Ca-free solution. ●, 2·5 mM-Ca with various concentrations of procaine (i.e. control); ○, 2·5 mM-Ca, 3×10^{-6} M isoprenaline and various concentrations of procaine were applied simultaneously. $n = 5$–6; bars are mean ± S.D. In *A*, the caffeine contraction evoked after 10 min superfusion with 2·5 mM-Ca, and in *B* after 5 min superfusion with 2·5 mM-Ca, is normalized as the control (1·0).

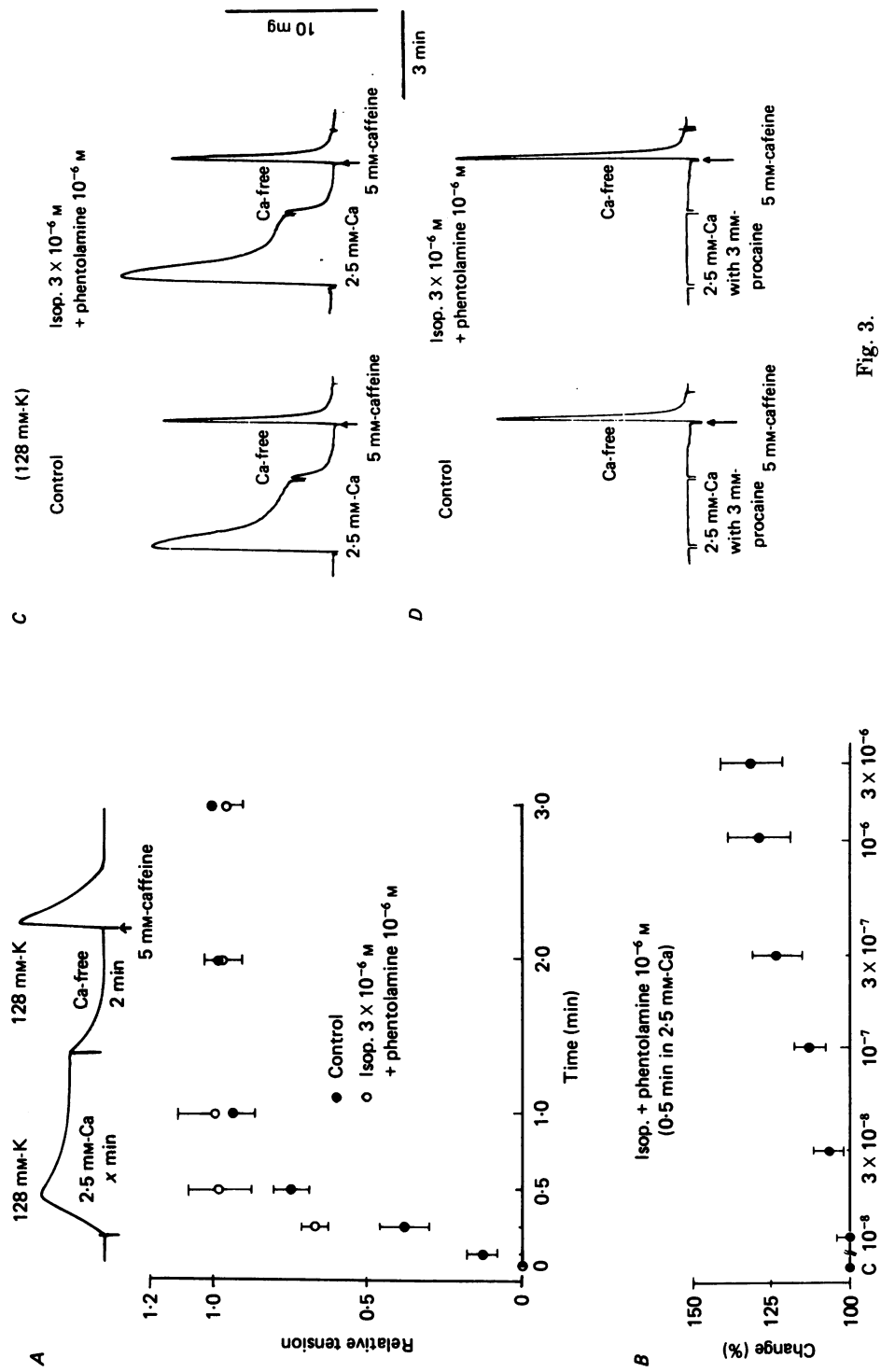
A
128 mM-K
2·5 mM-Ca
x min
128 mM-K
Ca-free
2 min
5 mM-caffeine
Relative tension
1·2
1·0
0·5
0
Control
Isop. 3 × 10⁻⁶ M
+ phentolamine 10⁻⁶ M
0
0·5
1·0
2·0
3·0
Time (min)
B
Isop. + phentolamine 10⁻⁶ M
(0·5 min in 2·5 mM-Ca)
Change (%)
150
125
100
C 10⁻⁸
3 × 10⁻⁸
10⁻⁷
3 × 10⁻⁷
10⁻⁶
3 × 10⁻⁶
[Isop.] (M)
C
(128 mM-K)
Control
Isop. 3 × 10⁻⁶ M
+ phentolamine 10⁻⁶ M
Ca-free
2·5 mM-Ca
5 mM-caffeine
10 mg
3 min
D
Control
Isop. 3 × 10⁻⁶ M
+ phentolamine 10⁻⁶ M
Ca-free
2·5 mM-Ca
with 3 mM-
procaine
5 mM-caffeine

Fig. 3.

be operating after longer exposure times. In Fig. 3*C* a slight reduction by isoprenaline in the caffeine contraction is seen after a 3 min Ca loading period, in combination with an increase in the Ca contraction. In Fig. 3*D* this procedure was repeated with procaine present during the loading period. The procaine abolishes the Ca contraction, but the size of the subsequent caffeine contraction is enhanced, and addition of isoprenaline reveals a further enhancement of the caffeine contraction.

Effects of isoprenaline on cyclic AMP contents

The tissues were incubated in Krebs solution for 60 min. During the last 5 min two different concentrations of isoprenaline (10^{-7} and 10^{-6} M) were added to the Krebs solution. In control tissues, the cyclic AMP content was $2{\cdot}2 \pm 0{\cdot}3$ p-mole/mg wet weight; this increased to $2{\cdot}8 \pm 0{\cdot}6$ p-mole/mg wet weight in the presence of 10^{-7} M-isoprenaline and to $4{\cdot}5 \pm 0{\cdot}8$ p-mole/mg wet weight in the presence of 10^{-6} M-isoprenaline ($n = 4$). However, the measured value will only be a rough indication of the increase in the amount of cyclic AMP induced by isoprenaline as it is difficult to dissect only muscle tissue of the mesenteric artery, and the measured samples included some mesentery and small segments of lymph and venous tissues.

The following experiments were done under the assumption that isoprenaline increases the amount of cyclic AMP in the guinea-pig mesenteric artery.

Effects of cyclic AMP and protein kinase on the Ca contraction and the caffeine-induced contraction in skinned muscles

Before skinning the muscle cells, the amplitude of contraction evoked by 128 mM-K in the intact muscles was registered. The tissue was then rinsed with the relaxing solution and saponin (50 μg/ml.) was applied for about 20 min. One criterion for the completion of skinning was that the maximum amplitude of the contraction produced by Ca was as large or larger than that of the 128 mM-K-induced contraction in the intact tissue. Fig. 4*A* shows an experiment in which cyclic AMP (3×10^{-6} M) with protein kinase (50 μg/ml.) applied before and also during application of free Ca (10^{-5} M), and in Fig. 4*B*, cyclic AMP and protein kinase were applied repeatedly during the maintained Ca-induced contraction. Cyclic AMP with protein kinase had no marked effect on the development of the Ca contraction, but if they were applied during a pre-established Ca contraction, the amplitude was slightly reduced.

Fig. 3. Effects of isoprenaline (isop.) or isoprenaline with procaine on the caffeine-induced contraction in Ca-free EGTA (2 mM) and 128 mM-K containing solution. *A*, experimental procedures were the same as those described in Fig. 2. ●, control; ○, isoprenaline (3×10^{-6} M) and phentolamine (10^{-6} M) were applied during application of 2·5 mM-Ca. $n = 4$–5; mean ± S.D. The amplitude of caffeine-induced contraction observed after application of 2·5 mM-Ca for 3 min was taken as 1·0. *B*, effects of various concentrations of isoprenaline applied simultaneously with 2·5 mM-Ca for 0·5 min. The amplitude of caffeine-induced contraction in the absence of isoprenaline was normalized as 100% (capital C). *C*, contractions evoked by 2·5 mM-Ca or 2·5 mM-Ca with isoprenaline were recorded, and subsequently caffeine-induced contractions after 2 min wash in Ca-free solution were recorded. *D*, procaine (3 mM) was applied simultaneously with 2·5 mM-Ca in the presence or absence of isoprenaline. Subsequently, following 2 min wash, 5 mM-caffeine was applied.

Fig. 5*A* shows the effects of cyclic AMP with protein kinase on the pCa–tension relationship. To evoke the contractions, various concentrations of free Ca were applied cumulatively from lower to higher concentrations or *vice versa*. The minimum concentration of free Ca required to produce a contraction was just above 10^{-7} M and the maximum amplitude of conctraction was evoked by free Ca at a concentration

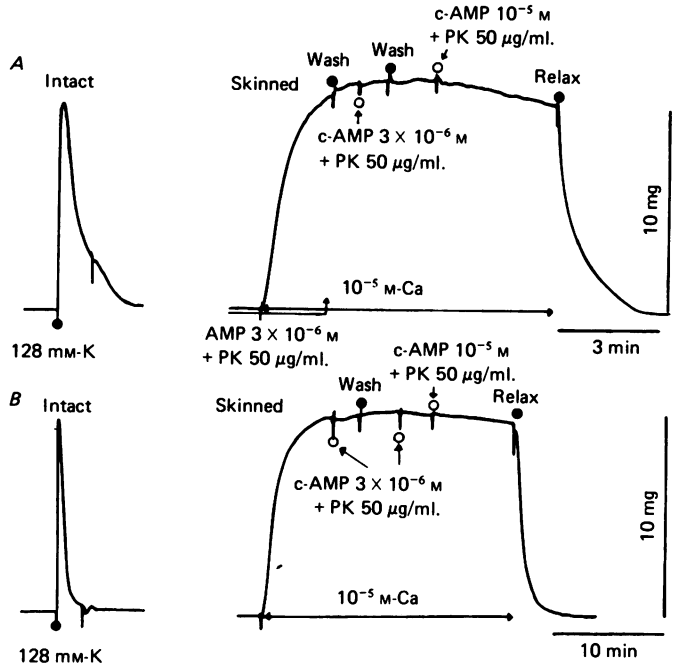

Fig. 4. Effects of cyclic AMP (c-AMP) and cyclic AMP-dependent protein kinase (PK) on the contraction evoked by free Ca (10^{-5} M) in skinned muscles. In *A* and *B*, before skinning the tissue, 128 mM-K was applied to the tissue as a control (●), and after skinning by saponin, 10^{-5} M free Ca was applied. *A* and *B*: two different concentrations of c-AMP (3×10^{-6} M and 10^{-5} M) with c-AMP-dependent protein kinase (50 μg/ml.) were applied before and during the Ca contraction as indicated. ○, application of c-AMP; ●, wash in skinned muscles. Relax: application of the relaxing solution (see Methods).

of 10^{-5} M, as observed by Itoh *et al.* (1981). The pCa–tension relationship was not affected by the presence of 3×10^{-6} M-cyclic AMP with 50 μg protein kinase/ml. (Fig. 5*A*). Higher concentration of cyclic AMP ($> 10^{-5}$ M) with 50 μg protein kinase/ml. consistently suppressed the pCa–tension relationship (Fig. 5*B*). Application of cyclic AMP ($< 10^{-4}$ M) or protein kinase (50 μg/ml.) alone did not modify the amplitude of the Ca-induced contraction, but in the presence of 50 μg protein kinase/ml., concentrations of cyclic AMP of 10^{-5} M or more reduced the amplitude of contraction. Application of 10^{-6} M-isoprenaline with different Ca concentrations had no effect on the pCa–tension relationship in skinned muscles.

Although caffeine does not affect the pCa–tension relationship, it does induce tension in skinned muscles (Itoh *et al.* 1981).

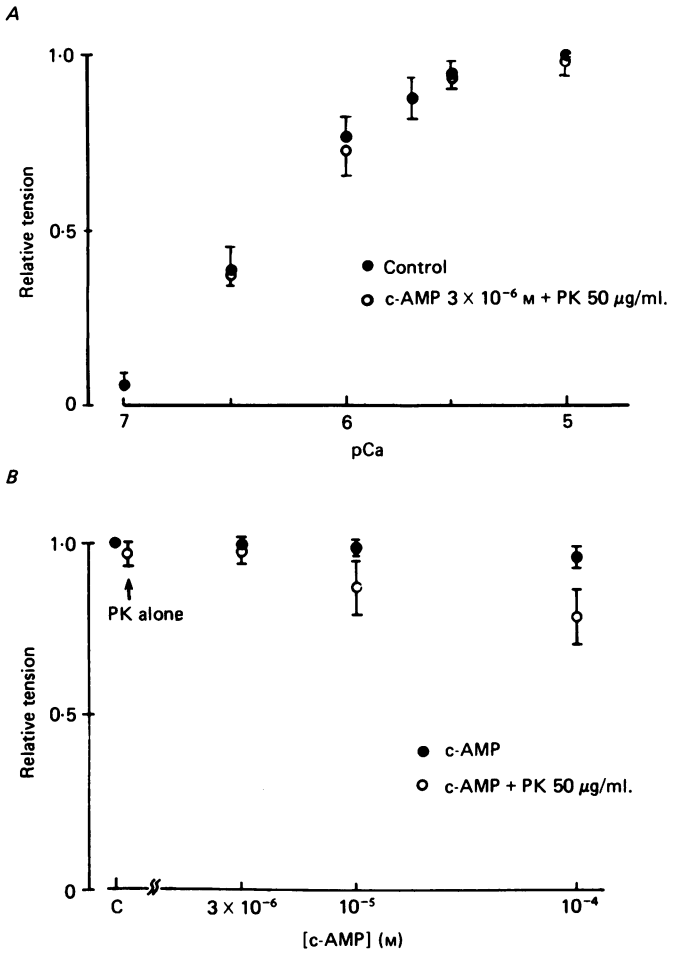

Fig. 5. Effects of c-AMP and c-AMP-dependent protein kinase (PK) on the Ca-induced contraction in skinned muscles. *A*, pCa–tension relationship observed in the presence (○) or absence (●; control) of c-AMP with protein kinase. Various concentrations of free Ca were applied cumulatively. (n = 3–5; mean ± S.D). The amplitude of 10^{-5} M-Ca-induced contraction is registered as a relative tension of 1·0. *B*, effects of c-AMP with (○) or without (●) c-AMP-dependent protein kinase on the Ca-induced contraction (10^{-5} M-free Ca). PK alone; application of 50 μg protein kinase/ml. (n = 3–5; mean ± S.D.). The amplitude of the 10^{-5} M-Ca-induced contraction is normalized as 1·0.

To study the effects of cyclic AMP on the caffeine-induced contraction in skinned muscles, under the conditions when the Ca stores would be filled (Itoh *et al.* 1981), 10^{-6} M-Ca with 10^{-4} M-EGTA was applied for 2 min (procedure 1), and a subsequent contraction was evoked with 5 mM-caffeine (procedure 3) which was applied after a 2 min wash in Ca-free solution (containing 10^{-4} M-EGTA) (procedure 2). A schematic illustration is shown in Fig. 6*A*. When 3×10^{-6} M-cyclic AMP with 50 μg protein kinase/ml. was applied during procedure 1, the amplitude of the caffeine-induced contraction was 113·4 ± 6·4 % (n = 6) of the control. Application of cyclic-AMP with

protein kinase during procedure 2 or 3 did not affect the amplitude of the caffeine-induced contraction ($96{\cdot}6 \pm 2{\cdot}2\,\%, n = 6$ or $103{\cdot}4 \pm 3{\cdot}2\,\%, n = 6$, respectively). These results indicate that application of cyclic AMP with protein kinase only affects the amplitude of the caffeine-induced contraction when applied during the filling of the Ca store and not during release of Ca from the storage site.

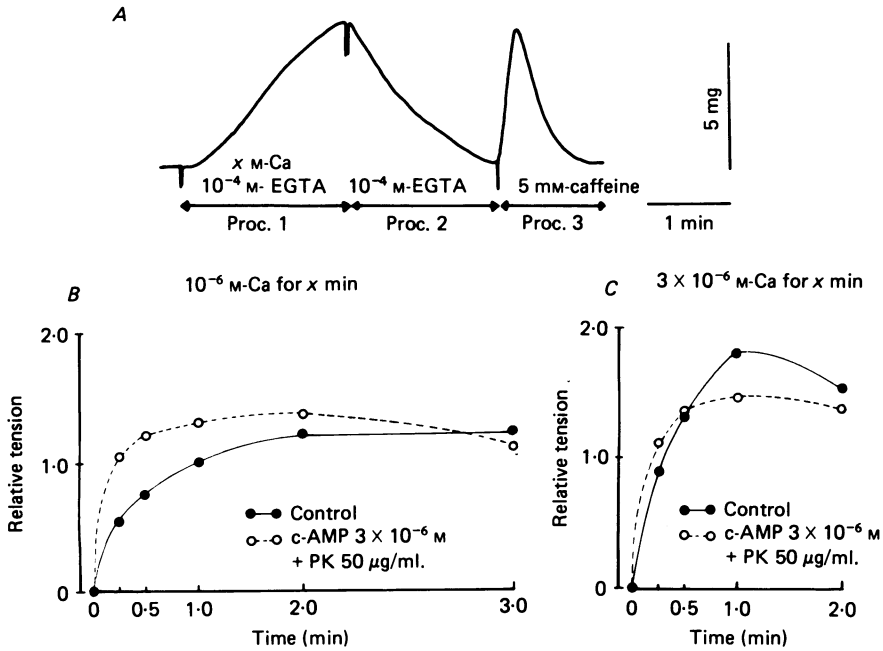

Fig. 6. Effects of incubation time (x min) in different Ca concentrations on the caffeine-induced contraction in skinned muscles. *A*, schematic procedure shown on top. Procedure 1: after skinning the tissue, 10^{-6} M-Ca with 10^{-4} M-EGTA was applied (2 min). Proc. 2: the tissue was again rinsed with Ca-free EGTA (10^{-4} M) solution (2 min). Proc. 3: 5 mM-caffeine was applied. *B* and *C*, effects on the caffeine-induced contraction of 10^{-6} M-Ca (*B*) and 3×10^{-6} M-Ca (*C*) applied for various durations (proc. 1) in the presence (○) or absence of c-AMP with protein kinase (control; ●), respectively. The amplitude of the caffeine-induced contraction recorded during proc. 3 after pre-incubation in 10^{-6} M-Ca for 1 min was normalized as 1·0.

To investigate the filling of the store further, the size of the caffeine contraction was measured after loading the store for various lengths of time in 10^{-6} M-Ca and 3×10^{-6} M-Ca in the presence and absence of cyclic AMP and protein kinase. In Fig. 6*B*, when the store was filled using 10^{-6} M-Ca, it can be seen that cyclic AMP and protein kinase only increased the store size if Ca was applied for 2 min or less. After 3 min these agents actually reduce the size of the caffeine contraction. When the store was filled with 3×10^{-6} mCa (Fig. 6*C*) the enhancing effect of cyclic AMP and protein kinase was only seen with Ca applications of less than half a minute; with longer exposures these agents reduced the size of the caffeine contraction compared with the control. These results could be explained if, with enhanced uptake of Ca into the

Ca store, a Ca-induced Ca-release mechanism were activated, resulting in leakage of Ca from the store, as reflected by the decrease in the size of the caffeine contraction.

Application of cyclic AMP alone is also able to enhance Ca uptake into the store. The caffeine contraction after filling the store for 30 sec or 1 min with 10^{-6} M-Ca is larger if 3×10^{-6} M-cyclic AMP is present during filling. The enhancement, however, is not as great as when cyclic AMP and protein kinase are applied together during filling (Table 1).

TABLE 1. Effects of cyclic AMP (c-AMP) and c-AMP-dependent protein kinase (PK) on the caffeine-induced contraction. c-AMP (3×10^{-6} M) or c-AMP with c-AMP-dependent protein kinase (50 μg/ml.) were applied during procedure 1. The amplitude of caffeine-induced contraction (5 mM) after pre-incubation in 10^{-6} M-Ca for 60 sec was normalized as 100% ($n = 5$; mean ± S.D.).

	% change (mean ± S.D.)		
Application of drugs	Control	c-AMP	c-AMP + PK
10^{-6} M-Ca for 30 sec	78·8 ± 0·9	83·4 ± 3·6*	94·4 ± 2·7**
10^{-6} M-Ca for 60 sec	100	111·1 ± 3·9	117·2 ± 4·3†

* $P < 0.025$ *vs.* 10^{-6} M-Ca for 30 sec.
** $P < 0.001$ *vs.* 10^{-6} M-Ca for 30 sec and 10^{-6} M-Ca with c-AMP for 30 sec.
† $P < 0.05$ *vs.* 10^{-6} M-Ca with c-AMP for 60 sec.

TABLE 2. Effects of procaine, or c-AMP + protein kinase (PK) with and without procaine on the caffeine-induced contraction in skinned muscles. During procedure 1, 10^{-6} M-Ca for 3 min or 3×10^{-6} M-Ca for 2 min was applied with c-AMP (3×10^{-6} M) and c-AMP dependent protein kinase (50 μg/ml.), or procaine (5 mM) or both. The caffeine-induced contraction (5 mM) recorded after application of 10^{-6} M-Ca for 3 min or 3×10^{-6} M-Ca for 2 min was registered as the control (100%). $n = 3$–5; mean ± S.D.

	% change (control: 100) (mean ± S.D.)		
Application of drugs	c-AMP + PK	Procaine	c-AMP + PK + procaine
10^{-6} M-Ca for 3 min	91·8 ± 3·6	117·8 ± 4·8*	129·0 ± 3·2**
3×10^{-6} M-Ca for 2 min	92·7 ± 6·1	122·2 ± 2·8†	124·8 ± 2·7††

* $P < 0.001$ *vs.* 10^{-6} M-Ca for 3 min control.
** $P < 0.001$ and $P < 0.005$ *vs.* 10^{-6} M-Ca for 3 min control and 10^{-6} M-Ca with procaine for 3 min, respectively.
† $P < 0.001$ *vs.* 3×10^{-6} M-Ca for 2 min control.
†† $P < 0.001$ *vs.* 3×10^{-6} M-Ca for 2 min control.

If enhanced uptake into the store does trigger a Ca-induced Ca-release mechanism, the size of the caffeine contraction may not give a valid estimate of the degree of enhancement produced by cyclic-AMP and protein kinase. Procaine is thought to suppress Ca-induced Ca release, and this has been shown in the guinea-pig mesenteric artery (Itoh *et al.* 1981); the effects of cyclic AMP with protein kinase were therefore investigated in the presence of procaine. Ca (10^{-6} M for 3 min, or 3×10^{-6} M for 2 min) was applied to skinned muscles in the presence of cyclic AMP with protein kinase, or in the presence of procaine (5 mM) or in the presence of both. After a 2 min wash in Ca-free solution, the size of the caffeine contraction was measured. The results are shown in Table 2. Procaine alone enhances the size of the caffeine contraction, and

also brings to light a further increase caused by addition of cyclic AMP and protein kinase, although these substances in the absence of procaine actually reduce the store size with these Ca exposure times.

The results shown in Tables 1 and 2 indicate that incubation with Ca and cyclic AMP accelerates the amount of Ca stored and activates a Ca-induced Ca-release mechanism. The concentration of cyclic AMP required to increase the Ca accumulation in the store site is lower than that required to suppress the Ca receptor of the contractile protein in smooth muscles of the mesenteric artery. Furthermore, accumulation of Ca into the store site by cyclic AMP is accelerated by application of protein kinase.

DISCUSSION

In the guinea-pig mesenteric artery, isoprenaline reduces the amplitude of contraction evoked by excess external K and relaxes the muscle tone. The underlying mechanism postulated from the present experiments made on the intact and skinned muscles is as follows: isoprenaline activates adenyl cyclase and increases the concentration of cyclic AMP, which results in an increased accumulation of Ca in the store site, mainly s.r., and may also slightly suppress activation of the light chain phosphokinase of myosin through activation of catalytic subunits of cyclic AMP-dependent protein kinase. These over-all actions, including an increased Ca extrusion across the cell membrane, produce relaxation of the tissues.

An indication of the amount of Ca taken up into intracellular stored sites, when Ca is applied for a short period to a tissue bathed in Ca-free solution, was obtained by measuring the size of contraction produced by caffeine (which is known to act in these conditions by releasing Ca from store sites). The presence of isoprenaline during the Ca-loading period increased the size of the caffeine contraction in Ca-free solution both in polarized and in depolarized muscle. During the filling procedure Ca release may be induced by the Ca uptake itself, procaine is known to inactivate this mechanism, and in the present experiments the action of isoprenaline was further enhanced by simultaneously applied procaine. In depolarized muscles, prepared by application of 128 mM-K with Ca-free solution, application of Ca produced contraction. However, when Ca was applied simultaneously with procaine (3 mM) the contraction was no longer generated, but the subsequent caffeine contraction was larger than that observed after Ca loading in the absence of procaine. Thus, in the presence of procaine, Ca entering the cell during exposure to excess K does not directly increase the amount of free Ca in the cell, but is sequestered by the Ca store site.

The observations concerning the effect of isoprenaline on Ca accumulation in the store site in intact muscles of the guinea-pig mesenteric artery confirm the observations made on the guinea-pig taenia coli (Ohashi *et al.* 1973, 1974; Casteels & Raeymaekers, 1979).

Application of cyclic AMP and protein kinase increased the Ca accumulation into the store site in skinned muscles, as estimated from the amplitude of caffeine-induced contraction, and this increase was further enhanced by simultaneously applied procaine. However, an increase in the Ca store was only seen if the loading period was short (< 1 min). This was taken to indicate the existence of a Ca-induced

Ca-release mechanism which may be activated by cyclic AMP increasing Ca uptake. It was found that high concentrations of cyclic AMP applied during the filling, or prolongation of the Ca loading to several minutes, reduced the size of the subsequent caffeine contraction. In intact and skinned muscles, procaine did not affect the pCa–tension relationship, but did suppress both the Ca-induced contraction and the Ca-induced Ca-release mechanism.

There is apparently an increase in the ability of microsomes to bind Ca on phosphorylation of a microsomal protein due to either endogenously activated or exogenously applied cyclic AMP-dependent protein kinase, i.e. a reciprocal interaction between Ca and cyclic AMP had been postulated (Kroeger, Teo, Ho & Wang, 1975; Webb & Bhalla, 1976; Thoren & Haeusler, 1978). However, Hirata & Kuriyama (1980) found that in the porcine coronary artery 80% of the total protein kinase binding sites were occupied by endogenous cyclic AMP in the cytosol fraction and 100% in the particulate fraction, even when the concentration of cyclic AMP was markedly reduced in excess K. Although full saturation of cyclic AMP binding to the cytosol fraction was observed on treatment with isoprenaline, the phosphorylation was not enhanced. These observations in the porcine coronary artery differed from findings in the cardiac muscle of the same species, because the phosphorylation was increased in the cardiac muscles. The nature of smooth muscle cells of porcine coronary and mesenteric arteries differed markedly (Itoh *et al.* 1981; Itoh, Kajiwara, Kitamura & Kuriyama, 1982).

The catalytic subunit of cyclic AMP-dependent protein kinase is also known to play an important action in phosphorylation of the subunit of myosin light chain kinase and to inhibit its interaction with the Ca-binding regulatory subunit, calmodulin, in smooth muscles (Adelstein *et al.* 1978; Krebs & Beavo, 1979; Conti & Adelstein, 1980; Noiman 1980; Sparrow *et al.* 1981; Kerrick & Hoar, 1981; Kerrick *et al.* 1981). In skinned muscles of the guinea-pig taenia coli, application of the catalytic subunit of cyclic AMP-dependent protein kinase shifted the pCa–tension relationship to the right at relatively low but not high concentrations of Ca (Sparrow *et al.* 1981). The amount of catalytic subunit applied in those experiments was very high compared with the amount calculated from applied cyclic AMP-dependent protein kinase in the present experiments. Presumably, differences in the two experiments are in part due to different procedures for preparing the skinned muscle. Because the cyclic AMP-dependent protein kinase and calmodulin are soluble proteins, during the skinning procedure a part of this protein may leak out from the cell. The amount of this catalytic subunit actually required to regulate the physiological function in this vascular tissue has yet to be determined.

Although cyclic AMP in a solution containing protein kinase slightly suppressed the Ca-induced contraction in skinned muscles in our experiments, a higher concentration was required than that needed to increase the accumulation of the Ca into the store site. In the presence of these agents the Ca-induced Ca-release mechanism appears to be enhanced, probably as a consequence of the increase in the Ca accumulation. Therefore, if the Ca-induced Ca-release mechanism is activated to a greater extent in the presence of these agents, there would not be a suppression of the contraction. This means that the β-adrenergic relaxation induced by isoprenaline may also be related to extrusion of Ca to the extracellular space and, in part,

suppression of the myosin light chain kinase. A possible mechanism by which stored Ca can be extruded to the extracellular space has been discussed with regard to the guinea-pig taenia coli (Brading, Burnett & Sneddon, 1980; Brading, 1981). In red cells, the Ca pump was accelerated by the actions of calmodulin (Marx, 1980). Evidence for an activation of an electrogenic Ca pump by isoprenaline has been obtained in rat myometrium (Marshall & Kroeger, 1973) and in the guinea-pig taenia coli (Bülbring & den Hertog, 1980).

Mueller & van Breemen (1979) reported that in the guinea-pig taenia coli the contraction evoked by application of either ouabain or excess K was reduced in amplitude by application of Ro 20-1724, a phosphodiesterase inhibitor, with no change in the total intracellular Ca content. Therefore, they postulated that the β-adrenoceptor-mediated relaxation is at least partially regulated by increase in Ca uptake into the stores and suppression of contractile protein rather than by Ca extrusion.

In the mesenteric artery, application of cyclic AMP with protein kinase to skinned muscles increases the amount of stored Ca, and high concentrations of cyclic AMP may, by increased Ca uptake, activate Ca-induced Ca-release from the store, resulting in depression of the Ca receptor of the contractile protein. The relaxation of the intact muscle by isoprenaline appears to be mainly due to Ca accumulation into stores and Ca extrusion, rather than to depression of the myosin light chain kinase or leiotonin C (Ebashi, Mikawa, Hirata, Toyo-oka & Nonomura, 1977; Mikawa, Nonomura, Hirata, Ebashi and Kakiuchi, 1978).

It must be made clear that the amount of cyclic AMP measured in the mesenteric artery after application of isoprenaline was much smaller than that measured in the non-pregnant hormone-treated myometrium (Harbon, Vesin & Do Khac, 1976) or pregnant rat myometrium (longitudinal and circular muscles; Kishikawa, 1981). In the mesenteric artery, extracellularly applied isoprenaline in intact muscles or cyclic AMP with protein kinase in skinned muscles regulates the free Ca in the cell, however, whether or not these actions directly correlate with the physiological function. Thus there still remains a need to clarify whether the activation of β-adrenoceptor in this tissue *in vivo* plays a physiological role through production of cyclic AMP for regulation of muscle tone.

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