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A23187 INCREASES CALCIUM PERMEABILITY OF STORE SITES MORE THAN OF SURFACE MEMBRANES IN THE RABBIT MESENTERIC ARTERY

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SUMMARY

1. The effects of a Ca ionophore, A23187, were investigated on intact and skinned smooth muscle tissues of the rabbit mesenteric artery.

2. A23187 (over 10^{-9} M) inhibited, dose dependently, contractions induced by 10^{-5} M-noradrenaline (NA) or 10 mM-caffeine in Ca^{2+}-free solution containing 2 mM-EGTA. Procaine (3 mM) led to cessation of the caffeine- or NA-induced contractions in the presence or absence of Ca^{2+}. When A23187 (10^{-7} M) was applied, the contractions in the presence of procaine were to some extent restored in Krebs solution.

3. A23187 at a concentration of 10^{-7} M did not modify the resting muscle tone, but this concentration did increase the amplitude of the contraction evoked by 20·2 or 128 mM-K^+ and markedly inhibited the 10^{-7} M-NA or 10 mM-caffeine-induced contraction in Krebs solution. A23187 (10^{-7} M) delayed the onset and rising phase of the 10^{-5} M-NA-induced contraction with inhibition of the oscillatory contractions.

4. High concentrations of A23187 (over 10^{-6} M) produced a large contraction in the presence and a small contraction in the absence of 2·6 mM-Ca^{2+}. These A23187-induced contractions were not inhibited by 10^{-7} M-nisoldipine, a Ca^{2+} antagonist.

5. A23187 (over 10^{-6} M) applied for a long period functionally skinned the muscle tissues. However, the Ca^{2+} sensitivity of the A23187-treated skinned muscles was lower than that of saponin-treated muscles.

6. In saponin-treated skinned muscles, A23187 (below 10^{-6} M) had no effect on the pCa–tension relation. After filling the store, A23187 (over 10^{-7} M) generated a larger contraction than did caffeine in Ca^{2+}-free solution, in the presence or absence of 5 mM-NaN_3. When 10^{-7} M-A23187 was applied once for 5 min, subsequently applied caffeine (20 mM), following application of Ca^{2+}, no longer produced contraction of skinned muscle tissues.

7. The present results indicate that low concentrations of A23187 show a selective Ca^{2+}-releasing action on Ca^{2+} store sites in muscle cells and that high concentrations increase the Ca^{2+} leakage (influx) and the cell membrane is skinned.

INTRODUCTION

In vascular smooth muscle cells, contraction–relaxation cycles are largely dependent on the amount of free Ca^{2+} in the cells, as in the case of skeletal or cardiac muscles, and sources of Ca^{2+} contributing to the activation of contractile proteins can be extra- and intracellular in origin (see for example, Kuriyama, Ito, Suzuki, Kitamura & Itoh, 1982). The sarcoplasmic reticulum (s.r.) plays the main role in excitation–contraction coupling in skeletal muscle (Endo, 1977; Fabiato & Fabiato, 1977). In smooth muscle, however, the relative importance of intracellularly stored Ca^{2+} *versus* extracellular Ca^{2+} in providing Ca^{2+} for contraction is a matter of current debate. Itoh, Kuriyama & Suzuki (1981, 1983) and Itoh, Kajiwara, Kitamura & Kuriyama (1982) have suggested that in vascular smooth muscles, extracellular Ca^{2+} entering the cell as the inward current may first be sequestrated in an internal store leading to activation of a Ca^{2+}-induced Ca^{2+}-release mechanism.

The antibiotic agent, A23187 selectively increases the permeability to divalent cations of biological and artificial membranes and therefore has the properties of an ionophore (Pressman, 1973; Reed & Lardy, 1972). A23187 directly causes contraction of skeletal, cardiac and smooth muscles (Pressman, 1973; Swamy, Ticku, Triggle & Triggle, 1975; Fabiato & Fabiato, 1977; Mandrek & Golenhofen, 1977; Mobley, 1977; Watson, 1978; Ishida & Shibata, 1980, 1982; Haeusler, Richards & Thorens, 1981). However, there is at present no consensus concerning the action of A23187 on smooth muscles. A23187 produces a contraction which is dependent on the external Ca^{2+} in some smooth muscles (Swamy *et al.* 1975; Mandrek & Golenhofen, 1977; Ishida & Shibata, 1980) but does not function solely by increasing the permeability of the cell membrane to Ca^{2+} in vascular smooth muscles (Watson, 1978). Murray, Reed & Fay (1975) reported that in isolated stomach smooth muscle cells of *Bufo marinus*, A23187 produced an initial EGTA-insensitive contraction and a sustained, secondary Ca^{2+}-sensitive contraction. Saida (1981) found that this agent skinned the fibres of guinea-pig taenia coli, after extended application. Furthermore, relaxation is induced by A23187 in some vascular preparations, but this has been shown to be mediated by a vascular relaxing factor released from the endothelium (see for example, Furchgott, 1983). Thus, A23187 seems to possess multiple actions on smooth muscles.

We have attempted to clarify the actions of A23187 on smooth muscle cells of the rabbit mesenteric artery using small pieces of intact or skinned muscle tissue. We found that low concentrations of A23187 selectively increase the Ca^{2+} release from the store site with no change in the resting tension while high concentrations increase the Ca^{2+} influx and lead to a skinning of the muscle tissues. The results are discussed in relation to the Ca^{2+}-induced Ca^{2+} release and to pharmaco-mechanical coupling mechanisms.

METHODS

Male albino rabbits (1·8–2·2 kg) were given sodium pentobarbitone (40 mg/kg I.V.), exsanguinated and the mesenterium of the iliac region was removed and placed in a dissecting chamber filled with Krebs solution. The mesenteric artery was carefully excised and thin circular strips (0·3–0·5 mm in length, 0·05–0·1 mm in width and 0·02–0·03 mm in thickness) were prepared, under a binocular

microscope. To avoid untoward responses, the tunica interna, adventitia and tunica externa were carefully removed.

Solution

The ionic composition of the Krebs solution was as follows (mM): Na^+, 137·4; K^+, 5·9; Mg^{2+}, 1·2; Ca^{2+}, 2·6; HCO_3^-, 15·5; $H_2PO_4^-$, 1·2; Cl^-, 134·4; glucose, 11·5. High-K^+ solution was prepared by replacing NaCl with KCl, isosmotically.

In skinned muscles, the following relaxing solution was used (mM); K methanesulphonate (KMs), 130; Tris maleate, 20; $Mg(Ms)_2$, 5; ATP, 5; and EGTA, 4. Various Ca^{2+} concentrations were prepared by adding appropriate amounts of $Ca(Ms)_2$ to 4 mM-EGTA (Harafuji & Ogawa, 1980; Itoh *et al.* 1981, 1983).

Recordings of mechanical activity

Mechanical activity of intact and skinned muscles was measured by attaching a circular strip to a strain gauge (U-gauge, Shinko Co., Tokyo, Japan) in a chamber with a capacity of 0·9 ml. The solution was changed by perfusing rapidly from one end and siphoning off simultaneously with a water pump from the other end.

Mechanical responses evoked by high external K^+ concentrations in intact preparations were measured in the presence of 10^{-7} M-TTX and 3×10^{-6} M-guanethidine to prevent the release of noradrenaline (NA) from depolarized nerve terminals. In order to estimate the Ca^{2+} release from the intracellular store, the contractions induced by 20 mM-caffeine or 10^{-5} M-NA were measured after rinsing for several minutes in Ca^{2+}-free, 2 mM-EGTA-containing solution.

Skinned muscle preparations were obtained by using saponin (25 μg/ml) for 20 min in relaxing solution (Iino, 1981; Itoh *et al.* 1981, 1983). The tension–pCa relation was obtained by cumulative applications of Ca^{2+}-containing solutions, in a stepwise manner, from low to high concentrations. To measure the amount of Ca^{2+} stored within the skinned muscles, the amplitude of the contraction induced by 20 mM-caffeine was measured in the presence of 0·1 or 0·5 mM-EGTA after application for several minutes of 10^{-6} M-Ca^{2+} buffered with 0·1 or 0·5 mM-EGTA. To rule out mitochondrial functions during investigations of Ca^{2+} mobilization in the skinned muscles, 5 mM-NaN_3 was added to some of the preparations of skinned muscle tissue.

Drugs

Chemicals used were A23187 (free acid; Calbiochemicals, CA, U.S.A.), atropine sulphate (E. Merck, Darmstadt, F.R.G.), caffeine (Wako Pharmac. Co., Tokyo, Japan), saponin (ICN Pharmac. Inc., Cleveland, OH, U.S.A.), noradrenaline HCl (NA; Sigma Chem. Co., St. Louis, MO, U.S.A.), guanethidine HCl (Tokyo Kasei Co., Tokyo, Japan), nisoldipine (Bayer Pharmac. Co. Ltd., Basel, Switzerland), procaine HCl (Sigma Chem. Co., St. Louis), TTX (Sigma Chem. Co., St. Louis), ethyleneglycol-bis-(aminoethylether)-*N,N,N',N'*-tetraacetic acid (EGTA; Dozin Lab., Kumamoto, Japan).

RESULTS

Effects of low concentrations of A23187 ($< 10^{-7}$ M) on contraction

To investigate the effects of A23187 on the Ca^{2+}-induced contraction in depolarized muscles, the tissue was superfused with Ca^{2+}-free, 2 mM-EGTA solution containing 39·2 mM-K^+ (3×10^{-6} M-guanethidine and 10^{-7} M-TTX) for 10 min, and then different concentrations of Ca^{2+} (0–5·2 mM) were applied for 2 min, at 3 min intervals. The amplitude of the Ca^{2+}-induced contraction increased dose dependently between 0·26 and 2·6 mM-Ca^{2+}. The amplitudes of the phasic and tonic contractions evoked by 5·2 mM-Ca^{2+} were less than those evoked by 2·6 mM-Ca^{2+}. With application of A23187 (10^{-7} M) 20 min before application of Ca^{2+}, the phasic contraction to 5·2 mM-Ca^{2+} was markedly enlarged but the effect was less on the contraction evoked by Ca^{2+} at concentrations below 2·6 mM. When concentrations of Ca^{2+} were below 0·32 mM, A23187 (10^{-7} mM) reduced the amplitudes of both phasic and tonic contractions

(Fig. 1). Application of 3×10^{-7} M-nisoldipine completely blocked these contractions, in the presence or absence of 10^{-7} M-A23187.

A23187 (10^{-7} M) slightly increased the amplitude of contractions induced by 20·2 and 128 mM-K^+ in solution containing 2·6 mM-Ca^{2+} (Fig. 2*A*). With application of this agent, the amplitude of the phasic contraction to 128 mM-K^+ was slightly

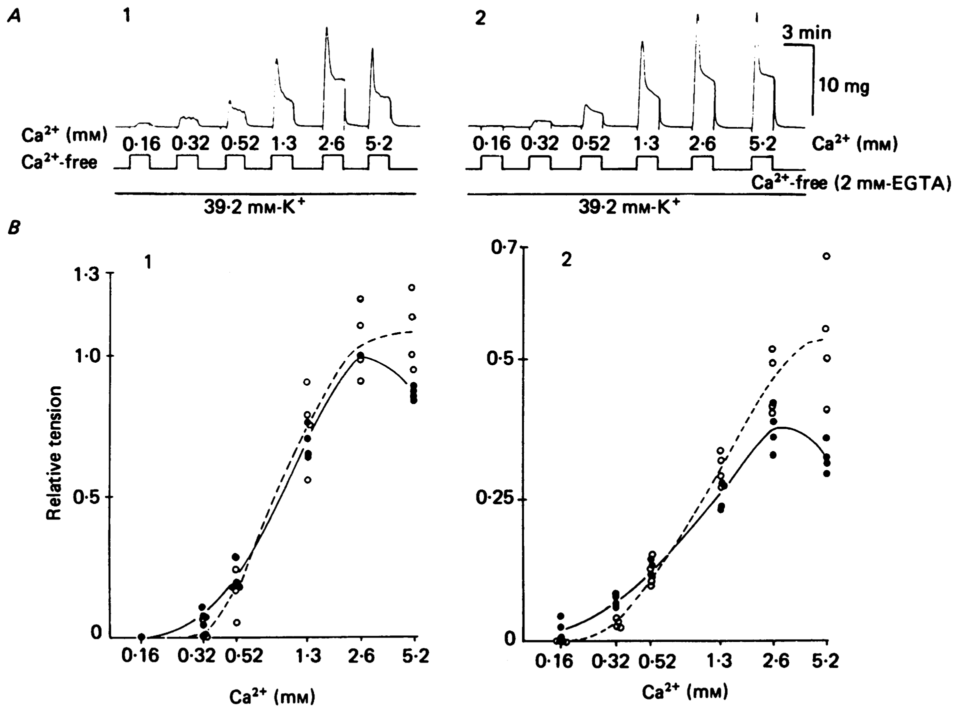

Fig. 1. *A*, effects of A23187 on contractions of rabbit mesenteric artery induced by various concentrations of Ca^{2+} in Ca^{2+}-free, 2 mM-EGTA solution, under conditions of depolarization (39·2 mM-external K^+). 1, control; 2, in the presence of 10^{-7} M-A23187. *B*, effects of A23187 on the relation between Ca^{2+} concentration and mechanical response in depolarized muscles. ●, control; ○, in the presence of 10^{-7} M-A23187. 1, effects of A23187 on the phasic response; 2, effects of A23187 on the tonic response. The amplitude of the phasic contraction was normalized to that with 39·2 mM-K^+ in the presence of 2·6 mM-Ca^{2+}.

enlarged, and relaxation required a longer time to reach the resting level after removal of 128 mM-K^+. The minimum concentration of NA required to produce contraction of the rabbit mesenteric artery was 10^{-7} M, and this contraction appeared with no change in the membrane potential and resistance (Itoh *et al.* 1982). 10^{-5} M-NA depolarized the membrane to the same extent as observed in 20·2 mM-K^+, and the evoked contraction was composed of repetitively generated oscillatory phasic and tonic contractions (Kanmura, Itoh, Suzuki, Ito & Kuriyama, 1983). On treatment with 10^{-7} M-A23187, the contraction induced by 10^{-7} M-NA was blocked, whereas 10^{-5} M-NA evoked a contraction with no oscillation and markedly delayed the onset and rising phase of the contraction (Fig. 2*B*). The amplitude of the 20 mM-caffeine-induced contraction was markedly inhibited (Fig. 2*C*).

These results indicate that 10^{-7} M-A23187 increases the contraction only when voltage-dependent Ca^{2+} influx is activated.

The effects of A23187 on the amount of Ca^{2+} stored in the cells were observed. A23187 was applied throughout the experiment, because low concentrations of this agent required an extended superfusion time to produce a maximum, steady response

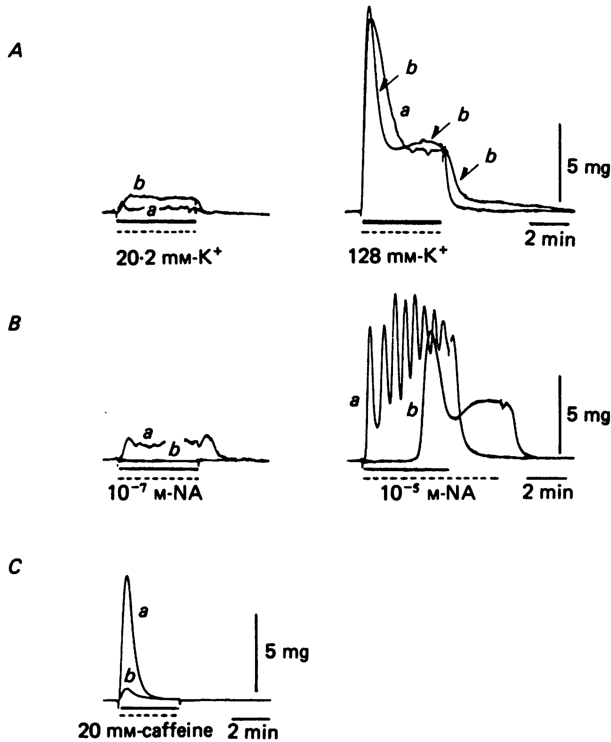

Fig. 2. Effects of A23187 on contractions induced by 20·2 and 128 mM-K^+ (*A*), 10^{-7} and 10^{-5} M-NA (*B*) and 20 mM-caffeine (*C*). *a*, control; *b*, after application of 10^{-7} M-A23187 for 30 min. Continuous and dotted lines indicate addition of K^+, NA or caffeine before and after treatment with A23187, respectively.

(10^{-10} M-A23187 required about 120 min; 10^{-9} M about 90 min; 10^{-8} M about 60 min; 10^{-7} M 20–40 min). After Ca^{2+} stored in cells had been completely depleted by repetitive applications of 20 mM-caffeine in solution containing Ca^{2+}-free, 2 mM-EGTA, 2·6 mM Ca^{2+} was applied for 20 min. The amount of Ca^{2+} stored in cells was then estimated from the size of the contraction evoked by 10^{-5} M-NA or 20 mM-caffeine after 5 min superfusion with Ca^{2+}-free, 2 mM-EGTA solution (Fig. 3*A*). During the application of 2·6 mM-Ca^{2+}, contraction was not evoked in the presence or absence of A23187 (below 10^{-7} M). The amplitude of NA- or caffeine-induced contraction was inhibited by 10^{-8} M-A23187, dose dependently. The concentration required for 50% inhibition (IC_{50}) values for the A23187-induced inhibition were 7×10^{-10} M for the NA-induced contraction and 8×10^{-10} M for the

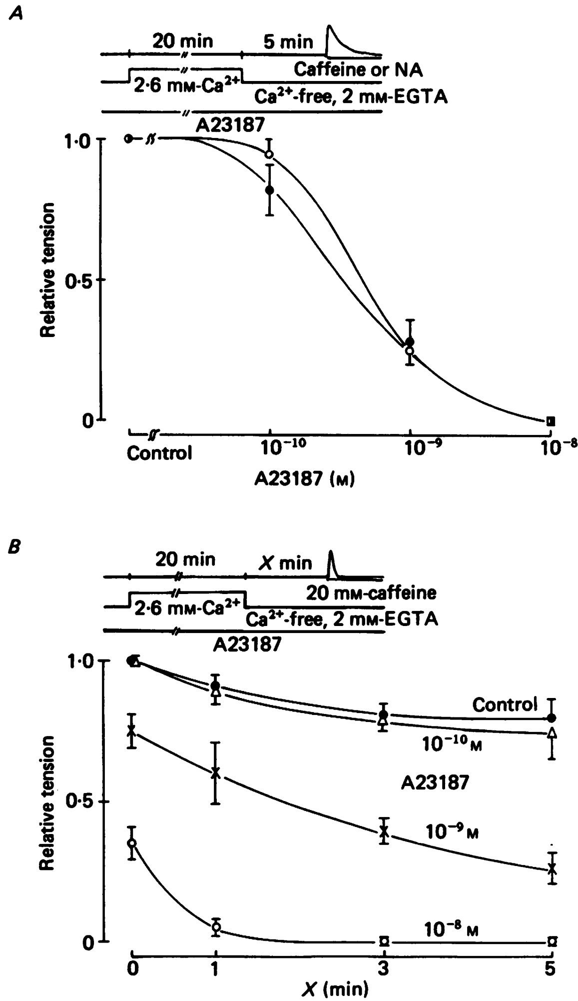

Fig. 3. *A*, effects of A23187 on the NA- (●) or caffeine-induced (○) contraction evoked in Ca^{2+}-free, 2 mM-EGTA solution. The experimental protocol is illustrated. The amplitude of contraction was normalized to the amplitude evoked by 10^{-5} M-NA or 20 mM-caffeine in the absence of A23187. A23187 (10^{-10}–10^{-8} M) was present throughout each experiment. Each value indicates mean ± S.D. or 2 × S.D. ($n = 5$). *B*, effects of A23187 on the contraction induced by caffeine at various times after introducing Ca^{2+}-free, 2 mM-EGTA solution. The experimental protocol is illustrated. X = incubation time. A23187 was present throughout each experiment. The amplitude of contraction is relative to that evoked by 20 mM-caffeine just after 20 min incubation in 2·6 mM-Ca^{2+} in the absence of A23187. Vertical bars indicate S.D. or 2 × S.D. ($n = 5$).

caffeine-induced one. These values were not significantly different ($P > 0·1$) (Fig. 3*A*).

The effects of A23187 on the stored Ca^{2+} were also estimated from the amplitude of 20 mM-caffeine-induced contraction after various incubation times in Ca^{2+}-free solution (Fig. 3*B*). The amplitude of contraction was found to decrease in proportion

to the incubation time, e.g. after 5 min exposure in Ca^{2+}-free solution, it was reduced to $0{\cdot}80 \pm 0{\cdot}07$ times the control ($n = 5$). With applications of A23187 (10^{-9}–10^{-8} M), the amplitudes of the caffeine-induced contractions were further and more rapidly reduced dose dependently. These results indicate that in concentrations over 10^{-9} M, A23187 reduces the amount of Ca^{2+} stored within the cells.

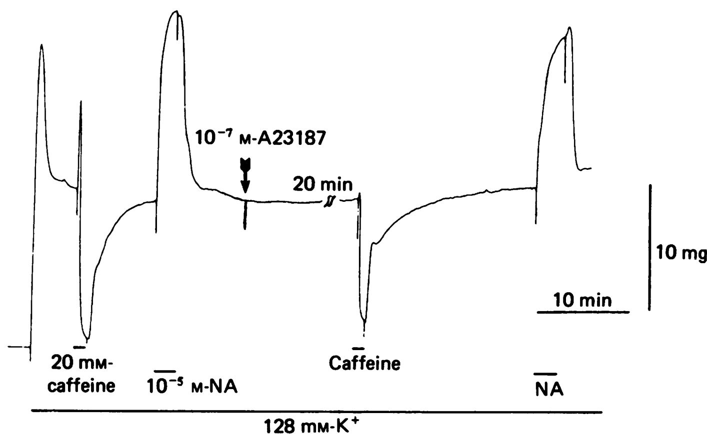

Fig. 4. Effects of A23187 on the NA- and caffeine-induced contractions evoked during 128 mM-K^+-induced tonic contraction. 20 mM-caffeine or 10^{-5} M-NA was applied during the tonic response to 128 mM-K^+ before and after application of 10^{-7} M-A23187.

Application of caffeine (20 mM) or NA (10^{-5} M) during treatment with 128 mM-K^+ produced a contraction superimposed on the tonic response to 128 mM-K^+ which reached a larger value than that evoked in Krebs (5·9 mM-K^+) solution. In the presence of 128 mM-K^+, the caffeine-induced contraction was followed by a marked relaxation of the tissue, but NA produced a sustained contraction (Fig. 4). When 10^{-7} M-A23187 was applied during the tonic response to 128 mM-K^+, the tonic response was unchanged, but the caffeine-induced contraction was markedly inhibited with no change in the subsequent relaxation. On the other hand, A23187 (10^{-7} M) only partly inhibited the amplitude and delayed the rate of rise of the NA-induced contraction (Fig. 4). Caffeine has been shown to enhance to a greater extent than NA-induced Ca^{2+} extrusion from cells following release from the store sites (Itoh *et al.* 1983). Therefore, these results indicate that 10^{-7} M-A23187 reduces the caffeine-activated release of Ca^{2+} from store sites presumably due to reduction in the amount of Ca^{2+} stored, but does not inhibit the enhanced Ca^{2+} extrusion from cells.

As procaine inhibits the high-K^+-, NA- and caffeine-induced contractions due to inhibition of the Ca^{2+}-induced Ca^{2+}-release mechanism in vascular smooth muscle (Itoh *et al.* 1981; Kuriyama *et al.* 1982), we studied the effect of this local anaesthetic agent on the contraction induced by 128 mM-K^+ in the presence or absence of 10^{-7} M-A23187. Procaine (1–10 mM) applied 5 min before and during application of 128 mM-K^+ inhibited the 128 mM-K^+-induced phasic and tonic responses, dose dependently (Fig. 5*A a*). With application of 10^{-7} M-A23817, the inhibition by procaine was reduced (Fig. 5*A b*). Fig. 5*A c* shows the relation between concentrations

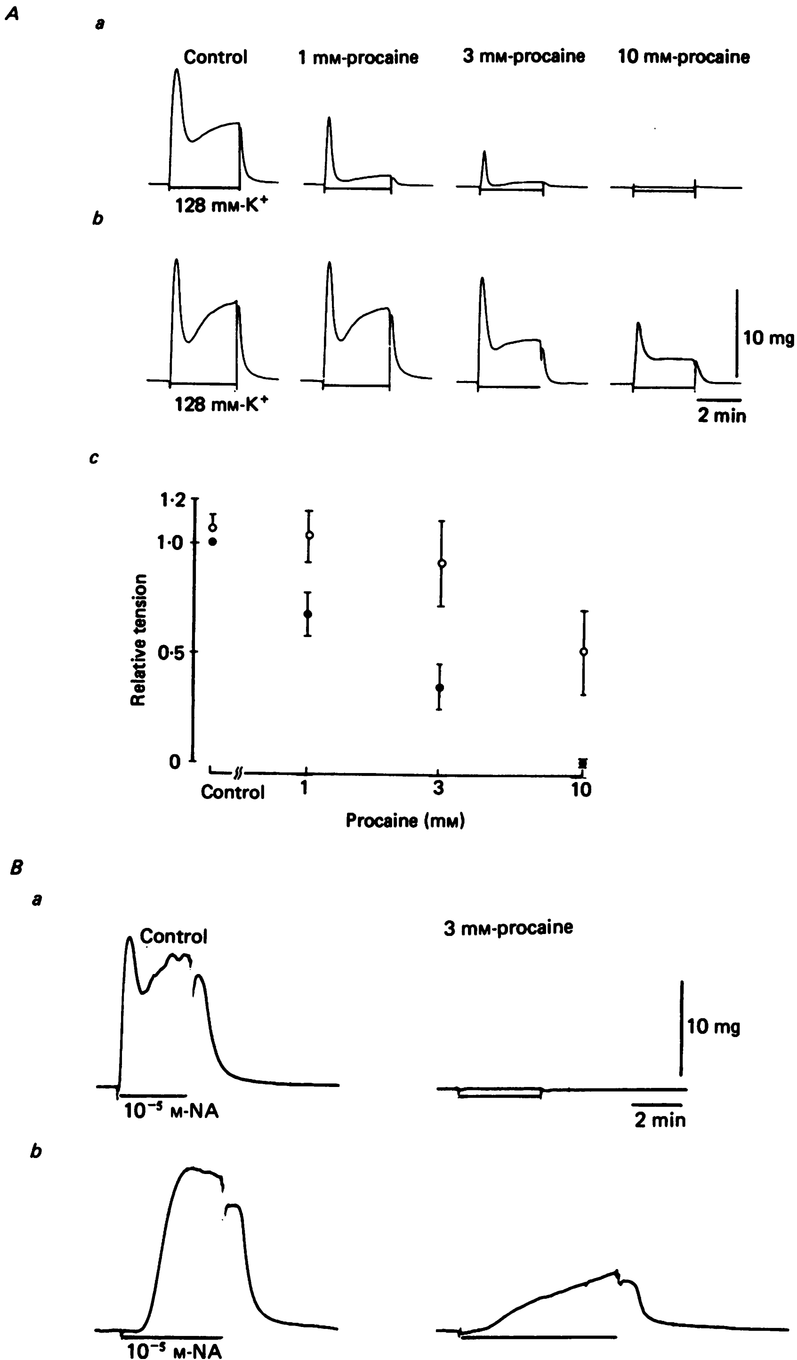

Fig. 5. *A*, effects of procaine on the 128 mM-K^+-induced contraction in the presence (*b*) or absence (*a*) of 10^{-7} M-A23187. Concentrations of procaine varied from 1 to 10 mM. The contractions are shown relative to that produced by 128 mM-K^+ in the absence of procaine and A23187. *c*, shows the effects of procaine on the 128 mM-K^+-induced phasic contraction ○, in the absence of A23187 and ●, in the presence of 10^{-7} M-A23187. Vertical bars indicate $2 \times$ S.D. ($n = 3$). *B*, effects of 3 mM-procaine on 10^{-5} M-NA-induced contraction before (*a*) and following (*b*) application of 10^{-7} M-A23187 for 20 min.

of procaine and the 128 mM-K^+-induced phasic contraction, in the presence or absence of 10^{-7} M-A23187. The IC_{50} for the inhibitory action of procaine was 1·9 mM in the control and 10 mM in the presence of 10^{-7} M-A23187.

Fig. 5*B* shows the action of procaine on 10^{-5} M-NA-induced contraction in the presence and absence of 10^{-7} M-A23187. Application of 3 mM-procaine completely inhibited the NA-induced contraction in the absence of A23187, but with application of A23187, NA still produced contraction but the onset was delayed and the development was slow.

The effects of high concentrations of A23187 (over 10^{-6} M) on mechanical responses in intact muscles

The minimum concentration of A23187 required to produce a transient phasic and small sustained contraction in Krebs solution was 10^{-6} M (3×10^{-6} M-guanethidine and 10^{-7} M-TTX present throughout; Fig. 6*A*). The amplitude of the A23187-induced contractions increased with dose. The maximum amplitude of contraction was evoked by application of 10^{-5} M-A23187 ($0{\cdot}49 \pm 0{\cdot}24$ times the 128 mM-K^+-induced contraction; $n = 14$; Fig. 6*B*).

In Ca^{2+}-free, 2 mM-EGTA solution, A23187 (3×10^{-6} M) produced a small transient contraction after 2 min superfusion, and when 2·6 mM-Ca^{2+} was re-added, A23187 produced a large phasic contraction (Fig. 7*A*).

If A23187 (10^{-6} M) and Ca^{2+} were removed following application for 20 min, subsequently applied 2·6 mM-Ca^{2+} produced contraction (Fig. 7*B b* and *c*). Nisoldipine (10^{-7} M) had no effect on this Ca^{2+} contraction (Fig. 7*B b*), but procaine (10 mM) slightly inhibited the contraction (Fig. 7*B c*). This concentration of procaine abolished the K^+-, Na- and caffeine-induced contractions, in the absence of A23187 (Itoh *et al.* 1981). Following 20 min superfusion with 10^{-6} M-A23187, 10^{-5} M-Ca^{2+} did not produce a contraction in the presence of ATP-containing relaxing solution (see Methods) as shown in Fig. 7*B c*. This means that 20 min superfusion of 10^{-6} M-A23187 does not skin the muscle fibre, but releases Ca^{2+} from store sites.

A23187-prepared skinned muscles and the effects of A23187 on the saponin-treated skinned muscles

Before application of A23187 or saponin, the amplitude of contraction evoked by 128 mM-K^+ was recorded. After application of 10^{-7} M-A23187 for 90 min, 10^{-4} M-Ca^{2+} did not produce contraction (Fig. 8*A*). However, after treatment with 10^{-6} M-A23187 for 60 min or more, Ca^{2+} in concentrations over 10^{-6} M produced contraction, dose dependently, and application of 10^{-5} M-Ca^{2+} produced a contraction larger than the 128 mM-K^+-induced one (Fig. 8*B* and *D*; see also Fig. 7*B c* for 20 min superfusion). This suggests that application of A23187 in concentrations over 10^{-6} M for more than 60 min skinned the muscle tissue.

The sensitivity to Ca^{2+} of muscles skinned by treatment with A23187 in concentrations over 10^{-7} M was compared with those prepared by saponin treatment (25 μg/ml applied for 20 min). Ca^{2+} produced contraction of saponin-treated skinned muscles, dose dependently, and 3×10^{-6} M-Ca^{2+} evoked a maximum contraction which exceeded the peak amplitude of the 128 mM-K^+-induced contraction recorded in intact muscles. Fig. 8*D* shows the pCa–tension relation obtained from skinned

muscles prepared by 90 min application of 10^{-6} M-A23187 and by 20 min application of saponin (25 μg/ml). To compare these amplitudes, the Ca^{2+}-induced contractions were normalized to the contraction evoked by 10^{-4} M-Ca^{2+}. These results indicate that skinned muscles prepared by A23187 had a lower Ca^{2+} sensitivity (median effective dose, $ED_{50} = 2 \times 10^{-6}$ M) than those prepared by saponin ($ED_{50} = 3{\cdot}4 \times 10^{-7}$ M).

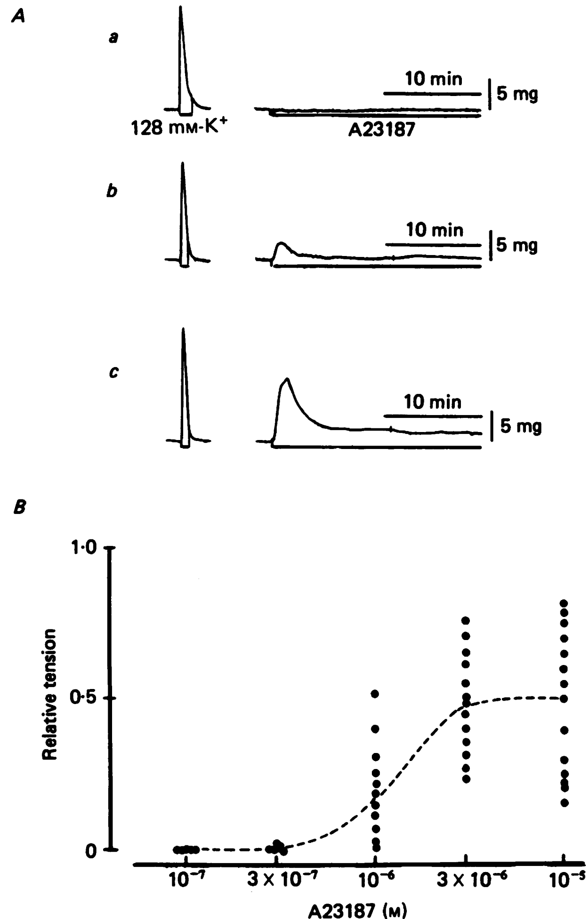

Fig. 6. *A*, the mechanical responses of the rabbit mesenteric artery evoked by 128 mM-K^+ and *a*, 3×10^{-7} M-, *b*, 10^{-6} M-, and *c*, 3×10^{-6} M-A23187. *B*, dose–response relation of the A23187-induced contraction. The amplitude of the 128 mM-K^+-induced contraction was registered as a relative tension of 1·0. The dotted line connects the mean values. At 10^{-5} M-A23187, the contraction developed was $0{\cdot}49 \pm 0{\cdot}24$, $n = 14$.

Fig. 9 shows the effect of 10^{-6} M-A23187 on the Ca^{2+}-induced contraction in saponin-treated skinned muscle. After the contraction evoked by 3×10^{-7} M-Ca^{2+} or 10^{-6} M-Ca^{2+} had reached a steady amplitude, 10^{-6} M-A23187 was applied for 2–3 min. A23187 (10^{-6} M) had no effect on the Ca^{2+}-induced contraction at any concentration (Fig. 9*A* and *B*). The observations are summarized in Fig. 9*C*. A23187 (10^{-6} M) did not modify the pCa–tension relation measured in the presence of various concentrations

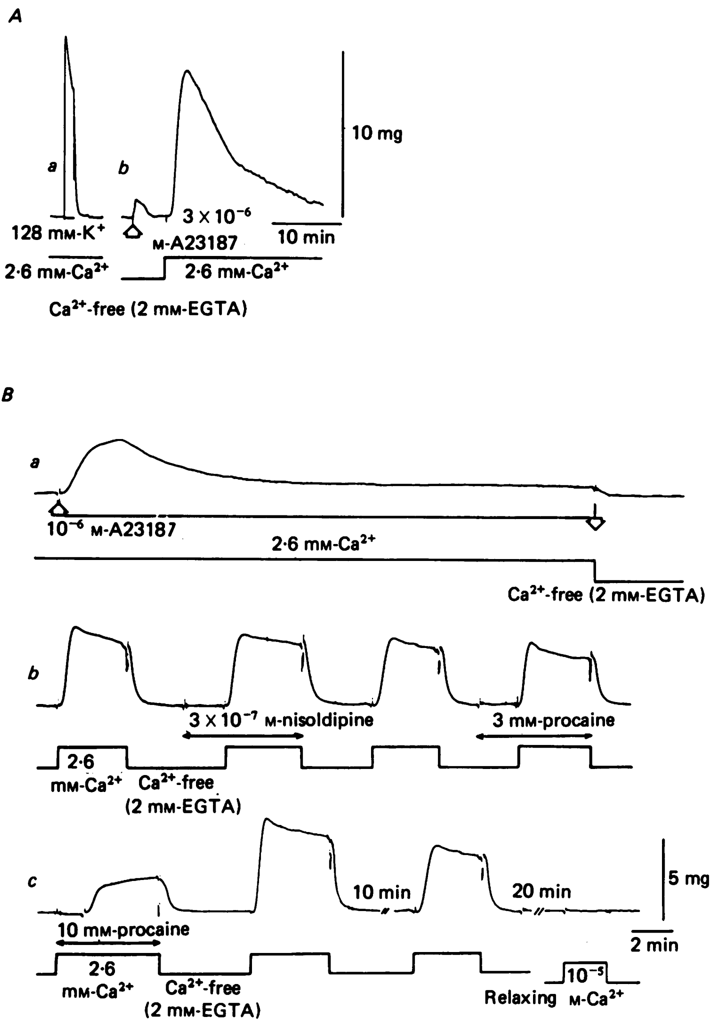

Fig. 7. *A*, effects of Ca^{2+} on the A23187-induced contraction. *a*, the 128 mM-K^+-induced contraction; *b*, effects of A23187 in the absence (containing 2 mM-EGTA) or presence of 2·6 mM-Ca^{2+}. *B*, effects of nisoldipine and procaine on the 2·6 mM-Ca^{2+}-induced contraction in Ca^{2+}-free, 2 mM-EGTA solution after treatment with 10^{-6} M-A23187. *a*, in Krebs solution, application of 10^{-6} M-A23187 produced a transient phasic and sustained contraction in the presence of 2·6 mM-Ca^{2+}. *b* and *c*, after removal of 10^{-6} M-A23187 and Ca^{2+} for 20 min, 2·6 mM-Ca^{2+} produced contraction. The contraction was not inhibited by 3×10^{-7} M-nisoldipine but was slightly inhibited by 3 mM-procaine and definitely inhibited by 10 mM-procaine. After removal of procaine, a transient enhancement of the Ca^{2+}-induced contraction occurred. When the solution was replaced with relaxing solution, 10^{-5} M-Ca^{2+} did not produce the contraction. This means that the muscle fibre is not skinned by 20 min exposure to 10^{-6} M-A23187.

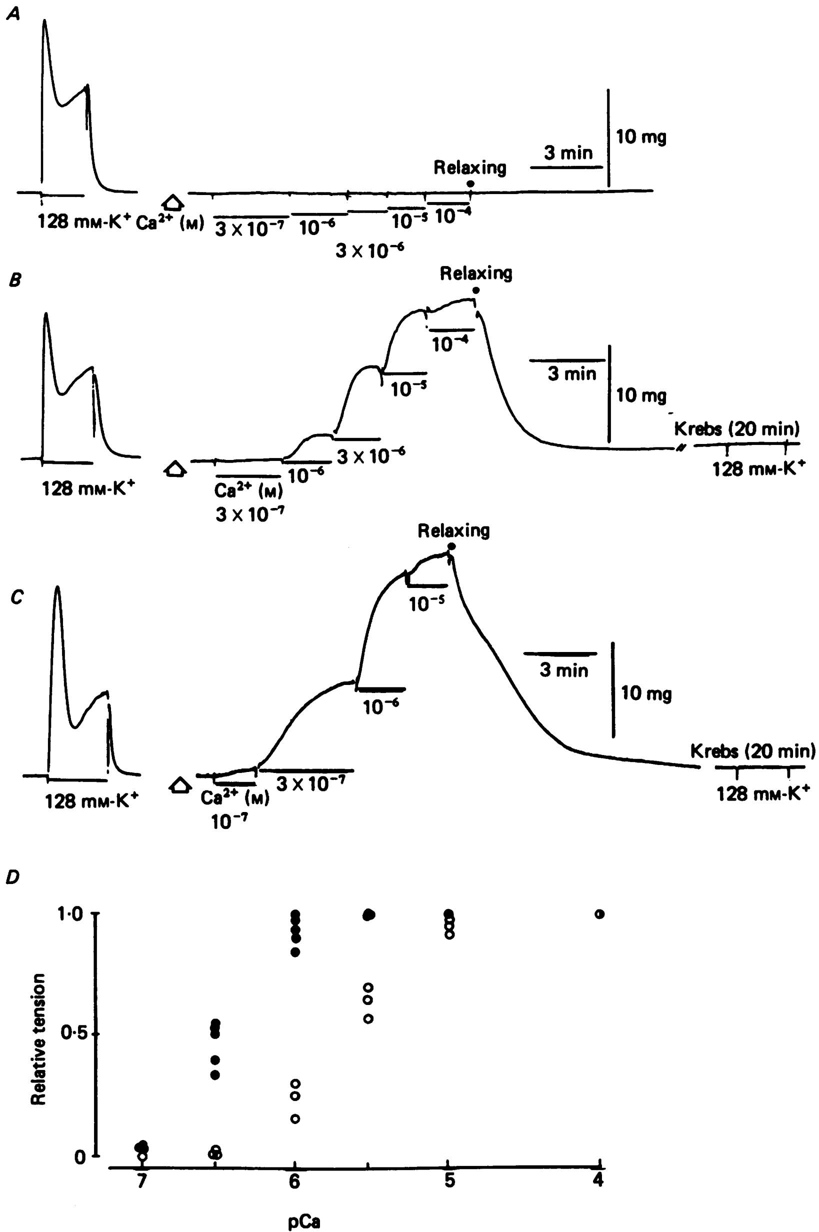

Fig. 8. Skinning effects of A23187 and saponin on the rabbit mesenteric artery. *A*, after treatment with 10^{-7} M-A23187 in the relaxing solution for 90 min (arrow), addition of Ca^{2+} up to 10^{-4} M did not induce contraction. *B*, on application of 10^{-6} M-A23187 for 90 min (arrow), the smooth muscle cells were skinned. The minimum of free Ca^{2+} concentration for contraction was 10^{-6} M. After skinning, application of 128 mM-K^+ did not produce contraction. *C*, after treatment with saponin (25 μg/ml) for 20 min (arrow), the tissue was skinned. The threshold concentration of Ca^{2+} required to produce contraction was 10^{-7} M. After skinning, the 128 mM-K^+-induced contraction did not occur. *D*, the pCa–tension relation in the saponin- (25 μg/ml for 20 min; ●, $n = 5$) and A23187- (10^{-6} M for 90 min; ○, $n = 3$) treated rabbit mesenteric artery. Various concentrations of Ca^{2+} were applied cumulatively. The relative tension measured at pCa 4 was recorded as 1·0.

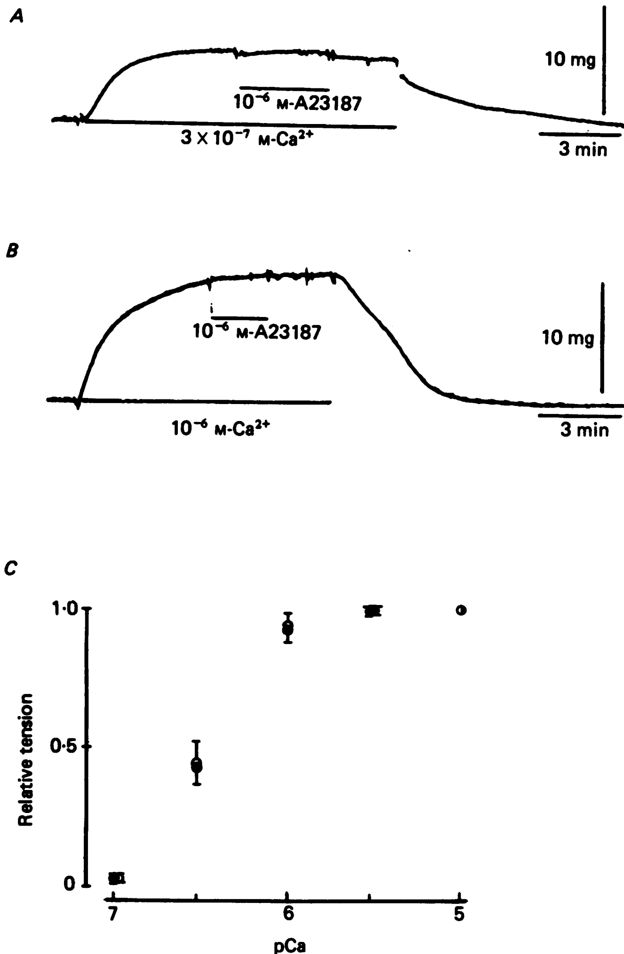

Fig. 9. Effects of A23187 on the Ca^{2+}-induced contraction of saponin-treated skinned muscles. *A* and *B*, after skinning the tissue with saponin, 3×10^{-7} M-Ca^{2+} (*A*) or 10^{-6} M-Ca^{2+} (*B*) was applied. A23187 (10^{-6} M) was applied after the Ca^{2+}-induced contraction had reached a steady amplitude. *C*, the effects of 10^{-6} M-A23187 (○) on the pCa–tension relation observed in saponin-treated skinned muscles (●, control). Vertical bars indicate ± S.D., $n = 3$–5.

of Ca^{2+} (10^{-7}–10^{-4} M). Therefore, this agent does not modify the properties of the contractile proteins.

Effects of A23187 on the Ca^{2+} store site, as estimated from the caffeine-induced contraction in skinned muscle

To observe the effects of A23187 on Ca^{2+} accumulation into and Ca^{2+} release from store sites in saponin-treated skinned muscles, the following procedures were used: after skinning the tissue, 10^{-6} M-Ca^{2+} was applied for several minutes, the tissue was rinsed with Ca^{2+}-free, 10^{-4} M-EGTA solution for 3 min, and 20 mM-caffeine was subsequently applied. The amplitude of the caffeine-induced contraction depended

on the incubation time in Ca^{2+}. Fig. 10*A* shows the relation between caffeine-induced contraction and the duration of Ca^{2+} incubation. The amplitude of the caffeine-induced contraction after 2 min of 10^{-6} M-Ca^{2+} treatment was registered as 1·0. NaN_3 (5 mM) did not modify the caffeine-induced contractions, for any time of Ca^{2+} incubation, indicating that mitochondria in skinned muscles have no causal relation to the caffeine-induced contraction. When A23187 was used instead of caffeine, with the same experimental procedures, 10^{-8} M-A23187 evoked a contraction. Fig. 10*B* shows the actual recordings of the caffeine- and A23187-induced contractions in skinned muscles. With applications of 10^{-8}–10^{-7} M-A23187 following incubation in 10^{-6} M-Ca^{2+}, small contractions were evoked. The amplitude of the 10^{-6} M-A23187-induced contraction was $1·72 \pm 0·18$ times that of the caffeine-induced contraction in the absence of NaN_3 (Fig. 10*B*) or $1·68 \pm 0·16$ times in the presence of NaN_3. Futhermore, when 10^{-6} M-A23187 was applied once, 20 mM-caffeine or 10^{-6} M-A23187 no longer evoked contraction (Fig. 10*B b*). Application of 10 mM-procaine did not prevent the generation of the A23187-induced contraction, but did abolish the caffeine-induced contraction.

These results indicate that in the presence of A23187, the caffeine-sensitive Ca^{2+} stored in cells (mainly s.r.) is released. However, the Ca^{2+}-release mechanism differs between caffeine and A23187.

DISCUSSION

In the rabbit mesenteric artery, exogenously applied low concentrations of NA (10^{-7} M) produced contraction with no change in membrane potential and resistance; pharmaco-mechanical coupling plays a role in generation of this contraction (Somlyo & Somlyo, 1968). In high concentrations (over 10^{-6} M), NA evoked membrane depolarization with an oscillatory contraction. This oscillatory contraction ceased in Ca^{2+}-free, 2 mM-EGTA solution but the NA-induced phasic contraction could still be evoked. Thus, NA produces contraction as a result of release of Ca^{2+} from the intracellular store, and the oscillatory nature of the contractions probably results from an interplay between increases in the influx and release of Ca^{2+} from store sites (Deth & Van Breemen, 1974; Haeusler *et al.* 1981; Itoh *et al.* 1983; Kanmura *et al.* 1983), possibly involving a Ca^{2+}-induced Ca^{2+}-release mechanism (Itoh *et al.* 1983).

The K^+-induced contraction was composed of phasic and tonic components and this contraction ceased in Ca^{2+}-free or procaine-containing solution. This suggests that the contraction was probably due both to activation of the voltage-dependent influx of Ca^{2+} and the resulting Ca^{2+}-induced Ca^{2+}-release mechanism (Itoh *et al.* 1981). Caffeine (20 mM) could produce contraction in the presence or absence of Ca^{2+} and this contraction was blocked by procaine, suggesting that caffeine may release the Ca^{2+} from store sites in the same manner as that induced by the Ca^{2+}-induced Ca^{2+}-release mechanism (Itoh *et al.* 1981).

It is uncertain whether or not the NA-sensitive Ca^{2+} store is the same as the caffeine-sensitive one. Some investigators have reported that the Ca^{2+} store is the same, as estimated from measurements of Ca^{2+} flux and tension (Deth & Casteels, 1977; Deth & Lynch, 1981; Itoh *et al.* 1983); Haeusler *et al.* (1981) and Saida & Van Breemen (1984) have suggested that these stores are not completely the same. All

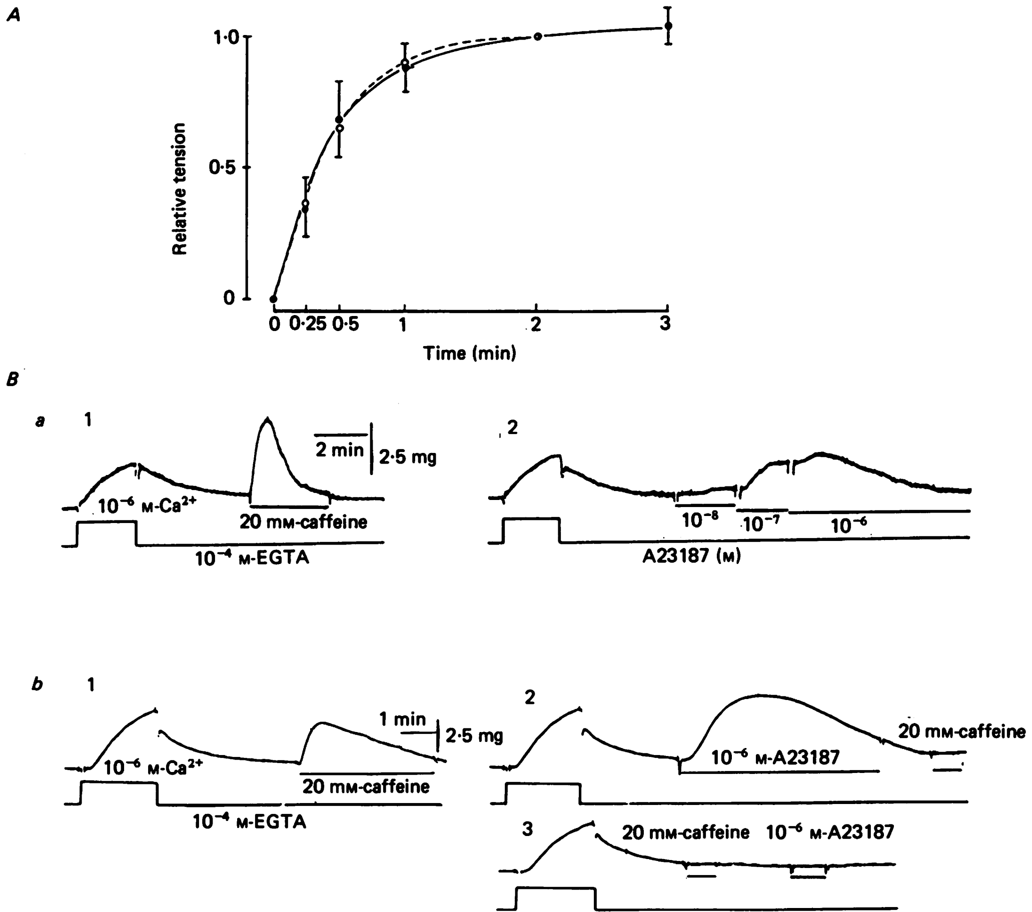

Fig. 10. Effects of A23187 on the Ca^{2+} store site (s.r.) in saponin-treated skinned muscles. *A*, effects of 5 mM-NaN_3 on the Ca^{2+} uptake into store sites in skinned muscles. After application of 10^{-6} M-Ca^{2+} for various times in the presence (○) or absence (●) of 5 mM-NaN_3, the muscle was put into 10^{-4} M-EGTA-containing solution and after 3 min, 20 mM-caffeine was applied. The amplitude of the caffeine-induced contractions is relative to the contraction evoked after application of 10^{-6} M-Ca^{2+} for 2 min in the absence of 5 mM-NaN_3. Vertical bars indicated S.D. or 2 × S.D., $n = 3$–5. *B*, the caffeine- and A23187-induced contraction evoked in saponin-treated skinned muscles. *a*, after 10^{-6} M-Ca^{2+}, 10^{-4} M-EGTA solution was applied for 2 min, the tissue was rinsed with 10^{-4} M-EGTA-containing solution, and after 3 min, 20 mM-caffeine (1) or different concentrations (10^{-8}–10^{-6} M) of A23187 were successively applied (2). *b*, with the same procedure as described in *a*, the caffeine- and A23187-induced contractions were recorded. 1, control (tissue was rinsed with Ca^{2+}-free solution for 5 min and 20 mM-caffeine was subsequently applied). 2, 10^{-6} M-A23187 was applied instead of caffeine. 3, after A23187 was applied in 2, caffeine and A23187 were successively applied after accumulation of Ca^{2+} into the store sites as shown in 1. The Ca^{2+}-induced contraction was generated but neither caffeine nor A23187 produced the contraction. *b*, 1–3 were recorded from the same tissue.

these investigators, however, proposed that the Ca^{2+} release from the store by caffeine is due to a similar mechanism to that of the NA-induced Ca^{2+} mobilization.

The ionophore A23187 is expected to make the plasma membrane and the membrane of the intracellular store site hyperpermeable to Ca^{2+}. Consistent with this, in intact tissues in normal solution, A23187 (over 10^{-6} M) caused a contraction which was not mediated via release of NA from perivascular nerves, since throughout the experiments 10^{-7} M-TTX and 3×10^{-6} M-guanethidine were present, or by increased Ca^{2+} sensitivity of the contractile proteins, as A23187 had no effect on the pCa–tension relation in saponin-treated skinned muscles. On the other hand, low concentrations (below 10^{-7} M) of A23187 did not change the resting tension, but inhibited 10^{-7} M-NA- and 10 mM-caffeine-induced contractions. Futhermore, A23187 (10^{-7} M) prolonged the time required to initiate and reduced the rate of rise of the 10^{-5} M-NA-induced contraction, and inhibited the oscillatory component. These effects of low concentrations of A23187 may be due to an ability to selectively act on the Ca^{2+} store site rather than the plasma membrane and increase the Ca^{2+} leakage from the store (Pressman, 1973; Reed & Lardy, 1972). We have shown that 10^{-7} M-A23187 can release Ca^{2+} from the s.r. in saponin-treated skinned muscles, and the long latency and slow development required for the generation of the 10^{-5} M-NA-induced contractions in 10^{-7} M-A23187 may be because in these conditions the contraction will be dependent on the influx of Ca^{2+}, the stored Ca^{2+} having been depleted during the previous exposure to A23187.

Although A23187 (10^{-7} M) does not appear to increase Ca^{2+} influx in normal conditions, it enlarged the 128 mM-K^{+}-induced contraction (both phasic and tonic components) and delayed the relaxation after removal of high K^{+}. Under these conditions the Ca^{2+} stores will be depleted. A23187 appears to be able to enhance the contractile response to activation of voltage-dependent Ca^{2+} channels, possibly because all the Ca^{2+} entering is available to the contractile machinery, none being taken up by the Ca^{2+} stores.

In skinned muscles, A23187 had a more potent action than caffeine on the Ca^{2+} release from internal stores. Although several biochemical results have indicated that A23187 (10^{-7} M) releases Ca^{2+} from mitochondria as well as from s.r. (Caswell & Pressman, 1972; Reed & Lardy, 1972; Vaghy, Johnson, Matlib, Wang & Schwartz, 1982), the enhanced response to A23187 is probably not due to mitochondrial release, since it also occurs in the presence of NaN_3. In the saponin-treated skinned muscles in the rabbit mesenteric artery, Haeusler *et al.* (1981) found that high concentrations of X-537A or A23187 did not prevent the subsequently generated NA-induced contraction. Discrepancies arising from these experiments remain to be clarified. In mechanically skinned heart cells, 10^{-5} M-A23187 showed a more potent release of Ca^{2+} from the s.r. than caffeine (Fabiato & Fabiato, 1977). Our observations support the above data on cardiac muscles.

Skinned muscles prepared by A23187 had a lower Ca^{2+} sensitivity than those prepared by saponin. These discrepancies may be raised by differences in the amount of leakage of the soluble calmodulin from cells, due to different exposure times to prepare the skinned muscles by these agents (90 min in 10^{-6} M-A23187 and 20 min in saponin (25 μg/ml)).

In conclusion, low concentrations of the Ca^{2+} ionophore, A23187 (below 10^{-7} M)

preferentially accelerate the release or leakage of Ca^{2+} from the intracellular store site (s.r.), so that the Ca^{2+}-induced Ca^{2+}-release mechanism probably plays no role in the generation of contractions. Application of high concentrations of A23187 increases the Ca^{2+} influx and skins the muscle cell.

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REFERENCES

CASWELL, A. H. & PRESSMAN, B. C. (1972). Kinetics of transport of divalent cations across sarcoplasmic reticulum vesicles induced by ionophores. *Biomedical and Biophysical Research Communications* **49**, 292–298.

DETH, R. C. & CASTEELS, R. (1977). A study of releasable Ca fractions in smooth muscle cells of the rabbit aorta. *Journal of General Physiology* **69**, 401–416.

DETH, R. C. & LYNCH, C. J. (1981). Mobilization of a common source of smooth muscle Ca^{2+} by norepinephrine and methylxanthines. *American Journal of Physiology* **240**, 239–247.

DETH, R. C. & VAN BREEMEN, C. (1974). Relative contributions of Ca^{2+} influx and cellular Ca^{2+} release during drug-induced activation of the rabbit aorta. *Pflügers Archiv* **348**, 13–22.

ENDO, M. (1977). Calcium release from the sarcoplasmic reticulum. *Physiological Reviews* **57**, 71–108.

FABIATO, A. & FABIATO, F. (1977). Calcium release from the sarcoplasmic reticulum. *Circulation Research* **40**, 119–129.

FURCHGOTT, R. F. (1983). Role of endothelium in responses of vascular smooth muscle. *Circulation Research* **53**, 557–573.

HAEUSLER, G., RICHARDS, J. G. & THORENS, S. (1981). Noradrenaline contractions in rabbit mesenteric arteries skinned with saponin. *Journal of Physiology* **321**, 537–556.

HARAFUJI, H. & OGAWA, Y. (1980). Re-examination of the apparent binding constant of ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N'*,*N'*-tetraacetic acid with calcium around neutral pH. *Journal of Biochemistry* **87**, 1305–1312.

IINO, M. (1981). Tension reponses of chemically skinned fibre bundles of the guinea-pig taenia caeci under varied ionic environments. *Journal of Physiology* **320**, 449–467.

ISHIDA, Y. & SHIBATA, S. (1980). Characteristic contractile response to the calcium ionophore, A23187, in guinea-pig vas deferens. *British Journal of Pharmacology* **71**, 581–583.

ISHIDA, Y. & SHIBATA, S. (1982). Relaxing and metabolic inhibitory action of X537A (Lasalocid) on the taenia of the guinea-pig caecum. *Journal of Physiology* **333**, 293–304.

ITOH, T., KAJIWARA, M., KITAMURA, K. & KURIYAMA, H. (1982). Roles of stored calcium on the mechanical response evoked in smooth muscle cells of the porcine coronary artery. *Journal of Physiology* **322**, 107–125.

ITOH, T., KURIYAMA, H. & SUZUKI, H. (1981). Excitation–contraction coupling in smooth muscle cells of the guinea-pig mesenteric artery. *Journal of Physiology* **321**, 513–535.

ITOH, T., KURIYAMA, H. & SUZUKI, H. (1983). Differences and similarities in the noradrenaline- and caffeine-induced mechanical responses in the rabbit mesenteric artery. *Journal of Physiology* **337**, 606–629.

KANMURA, Y., ITOH, T., SUZUKI, H., ITO, Y. & KURIYAMA, H. (1983). Effects of nifedipine on smooth muscle cells of the rabbit mesenteric artery. *Journal of Pharmacology and Experimental Therapeutics* **226**, 238–248.

KURIYAMA, H., ITO, Y., SUZUKI, H., KITAMURA, K. & ITOH, T. (1982). Factors modifying contraction–relaxation cycle in vascular smooth muscles. *American Journal of Physiology* **243**, H641–662.

MANDREK, K. & GOLENHOFEN, K. (1977). Activation of gastro-intestinal smooth muscle induced by the calcium ionophore A23187. *Pflügers Archiv* **371**, 119–124.

MOBLEY, B. A. (1977). Calcium ionophores and tension production in skinned frog muscle fibers. *European Journal of Pharmacology* **45**, 101–104.

MURRAY, J. J., REED, P. W. & FAY, F. S. (1975). Contraction of isolated smooth muscle cells by ionophore A23187. *Proceedings of the National Academy of Sciences of the U.S.A.* **72**, 4459–4463.

PRESSMAN, B. C. (1973). Properties of ionophores with broad range cation selectivity. *Federation Proceedings* **32**, 1698–1703.

REED, P. W. & LARDY, H. A. (1972). A23187: a divalent cation ionophore. *Journal of Biological Chemistry* **247**, 6970–6977.

SAIDA, K. (1981). A method of skinning smooth muscle fibers with A23187. *Biomedical Research* **2**, 134–142.

SAIDA, K. & VAN BREEMEN, C. (1984). Characteristics of the norepinephrine-sensitive Ca^{2+} store in vascular smooth muscle. *Blood Vessels* **21**, 43–52.

SOMLYO, A. V. & SOMLYO, A. P. (1968). Electromechanical and pharmacomechanical coupling in vascular smooth muscle. *Journal of Pharmacology and Experimental Therepeutics* **159**, 129–145.

SWAMY, V. C., TICKU, M., TRIGGLE, C. R. & TRIGGLE, D. J. (1975). The action of the ionophores, X537A and A23187, on smooth muscle. *Canadian Journal of Physiology and Pharmacology* **53**, 1108–1114.

VAGHY, P. L., JOHNSON, J. D., MATLIB, M. A., WANG, T. & SCHWARTZ, A. (1982). Selective inhibition of Na^{+}-induced Ca^{2+} release from heart mitochondria by diltiazem and certain other Ca^{2+} antagonist drugs. *Journal of Biological Chemistry* **257**, 6000–6002.

WATSON, E. L. (1978). Effects of ionophores A23187 and X537A on vascular smooth muscle activity. *European Journal of Pharmacology* **52**, 171–178.