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EFFECTS OF CALCIUM AND MANGANESE IONS ON MECHANICAL PROPERTIES OF INTACT AND SKINNED MUSCLES FROM THE GUINEA-PIG STOMACH

BY TAKEO ITOH, HIROSI KURIYAMA AND TAMAKI NANJO

From the Department of Pharmacology, Faculty of Medicine, Kyushu University, Fukuoka 812, Japan

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SUMMARY

1. To investigate the mechanism of generation of contractions in tissues from the guinea-pig stomach, the effects of caffeine, procaine, acetylcholine (ACh), diltiazem or $MnCl_2$ on the contraction evoked from small bundles of intact or skinned muscles (50 μm in width and 250–300 μm in length) were observed.

2. All these agents except for ACh blocked the spontaneously generated contraction. Diltiazem (1×10^{-4} M) had no effect and $MnCl_2$ (3 mM) slightly reduced and caffeine enhanced the tonic contraction evoked in Na-free solution, whereas procaine relaxed the tissue. On the other hand, in the isotonic $[K]_o$ solution, diltiazem, $MnCl_2$ and procaine relaxed the tissue, while caffeine enhanced the tonic contraction.

3. Under pre-treatment with Ca-free solution (2 mM-EGTA-containing solution) after depletion of the stored Ca, application of 2·5 mM-Ca and subsequently applied 5 mM-caffeine produced contractions (Ca- and caffeine-induced contractions, respectively). In polarized (5·9 mM-K_o) and depolarized (128 mM-K_o) muscles, the various agents simultaneously applied with 2·5 mM-Ca modified the amplitude of the Ca-induced and the resulting caffeine-induced contractions. Thus, at least three different Ca influxes required to evoke the Ca- or caffeine-induced contraction were identified; diltiazem-sensitive Ca influx, diltiazem-insensitive but Mn-sensitive Ca influx and Mn-insensitive Ca influx.

4. The Ca- and caffeine-induced contractions in Ca-free and 15·5 mM-Na-containing solutions were gradually reduced in amplitude, in proportion to the time of exposure. However, amplitude of the caffeine-induced contractions was inhibited to a greater extent and the duration of the contracts was less prolonged than the case of the Ca-induced contraction.

5. In saponin-treated skinned muscles, the minimum concentration of Ca required to produce the contraction was 1×10^{-7} M, and the maximum contraction was evoked by application of 1×10^{-5} M-Ca. The effects of Na-free solution on the Ca accumulation and release to and from the storage site were also observed in these skinned muscles. The removal of Na from the cell seems to accelerate the Ca leakage, and depletes the stored Ca. In addition, Na-free solution inhibits to some extent the accumulation of Ca in the store site.

6. In skinned muscles, Mn (over 2×10^{-9} M) significantly enhanced the Ca-induced

contraction and the pCa–tension relationship shifted to the left and upper directions. Mn seemed to possess the property of activating the contractile proteins, as determined from the pMn–tension relationship, and this agent may also inhibit leakage of Ca from the store sites. However, in relation to the latter two actions, the possible effects of Ca contaminations in the solution would have to be ruled out. Under physiological conditions, $MnCl_2$ may act at the level of the myoplasmic membrane and not actually penetrate the cell in this tissue.

INTRODUCTION

The circular muscle of the antrum of the guinea-pig stomach generates spontaneously generated slow potential changes and spikes which are often superimposed on the slow potential changes. The spike generated on the slow potential changes is thought to be due to the influx of Ca with cessation of the spike in Ca-free solution or in the presence of $MnCl_2$. However, there are at least two different components related to the slow potential changes, as determined under conditions of various ionic environments (Magaribuchi, Ohbu, Sakamoto & Yamamoto, 1972; Osa & Kuriyama, 1970; Ohba, Sakamoto & Tomita, 1975, 1977).

The evoked mechanical response could be recorded simultaneously with generations of slow potential changes and, when the spike was superimposed on the slow potential change, the additional contraction superimposed on the contraction induced by the slow potential change (Magaribuchi *et al.* 1972). The mechanical response, therefore, is thought to be generated by the voltage-dependent Ca influx and, in part, by release of the stored Ca in the cell (Kuriyama, Mishima & Suzuki, 1975).

In the guinea-pig mesenteric artery, the influx of Ca during generation of the spike is directly sequestered into the store site, and when the amount of stored Ca reaches an appropriate level activations of the Ca-induced Ca release mechanism occur (Itoh, Kuriyama & Suzuki, 1981*a*). On the other hand, the phasic contractions could be recorded in Na-deficient and Na-free solution, and the minimum reduction in the Na from the solution was below 10 mM (Itoh, Suzuki & Kuriyama, 1981*b*). The K-induced contraction was suppressed by application of diltiazem, a Ca channel blocker (Ca antagonist), but the Na deficient-induced contraction was not affected (Suzuki, Itoh & Kuriyama, 1982). Therefore, the nature of the influx of Ca into the membrane of the muscle is more complicated than would appear.

We investigated the effects of caffeine, procaine, diltiazem and $MnCl_2$ on the mechanical property of circular muscle cells of the guinea-pig stomach. For this purpose, we used very thin muscle strips (50 μm in width and 250–300 μm in length) prepared from the antrum of the guinea-pig stomach. To investigate the available source of Ca contributing to the generation of contraction in the stomach muscle, saponin treated skinned muscles were also used, under the same experimental procedures used in the case of the guinea-pig taenia coli (Saida & Nonomura, 1978; Iino, 1981) and also the guinea-pig mesenteric artery (Itoh *et al.* 1981*a*, *b*).

The effects of $MnCl_2$ on the stomach smooth muscle were also investigated in comparison to the effects of Ca on mechanical properties. Mn is known to inhibit the spike generation and either accelerates or inhibits the contraction in smooth and cardiac muscles (Brading, Bülbring & Tomita, 1969; Imai & Takeda, 1967; Osa, 1974;

Ochi, 1976; Yoshida, Taniyama, Araki & Matsumoto, 1977; Chapman & Ellis, 1977; Ogasawara, Kato & Osa, 1980; Coraboeuf, Gautier & Guiraudou, 1981).

The results are discussed in relation to findings in other visceral smooth muscles, including vascular tissues.

METHODS

Albino guinea-pigs of either sex (300–400 g) were stunned and bled and the stomach was excised and cut in the longitudinal direction along the greater curvature. The contents of the stomach, mucosal layer and longitudinal muscle layer were removed from the circular muscle layer of the antrum in a dissecting chamber filled with Krebs solution. Under an optical microscope, the final strips prepared were 0·05 mm in width and 0·25–0·3 mm in length. To record the spontaneous contraction, strips 0·2 mm in width and 0·25–0·3 mm in length were prepared.

Ionic composition of the Krebs solution was as follows (mM): Na^+, 137·4; K^+, 5·9; Mg^{2+}, 1·2; Ca^{2+}, 2·5; HCO_3^-, 15·5; $H_2PO_4^-$, 1·2; Cl^-, 134·0; glucose, 11·5. The pH of the solution was kept at 7·2 by 97 % O_2 with 3 % CO_2. 128 or 143·6 mM-K (isotonic K) containing solution was prepared by replacing NaCl with KCl and also $NaHCO_3$ and $KHCO_3$, isotonically. The Na-free or Na-deficient solution was prepared by replacing Na with sucrose or choline Cl (containing atropine 4×10^{-6} M) isotonically and the pH of the solution was adjusted by Tris Cl. To prepare the $MnCl_2$-containing solution, the pH of the solution was adjusted by adding NaOH or KOH. The Ca-free solution was prepared by removal of Ca from the solution and 2 mM-EGTA was added throughout the experiments.

Recording the mechanical response from intact muscle tissues

Mechanical responses of the muscle strip were measured by a strain gauge (U-gauge, Shinko Seiki). The linearity of the transducer to the load was previously checked. The tissue was superfused in an organ bath (0·9 ml capacity) filled with Krebs solution. Solutions containing drugs or modified ionic concentrations were added into the bath while the solution already present was sucked off with an aspirator; therefore, only a few seconds were needed to apply the test solution. However, this procedure was accompanied by a large artifact due to the sudden change in the level of the solution in the organ bath. Therefore, before the start of the experiment, recovery of the position of the recording pen to the original level was checked, and the recording system was adjusted appropriately. General principles of the experimental procedures were as follows; the tissue was kept in Ca-free (2 mM-EGTA-containing) solution for 10 min then 2·5 mM-Ca-containing solution was applied for 3–5 min (the Ca-induced contraction). The tissue was again rinsed in Ca-free solution for 2 min, and caffeine was applied for 1 min to evoke the contraction (the caffeine-induced contraction). In some of the experiments, acetylcholine was applied instead of caffeine. Various drugs were applied either simultaeously with 2·5 mM-Ca or with Ca-free solution, just after application of Ca. The amplitude of the Ca- and caffeine-induced contractions was normalized in comparison to findings in the absence of the drugs.

Recordings of the mechanical response from skinned muscle tissues

Skinned muscle preparations were obtained by using saponin, according to the method described by Iino (1981) and Itoh *et al.* (1981*a*, *b*). After a K-induced contraction had been recorded from the intact muscles the bathing solution was replaced with a relaxing solution containing 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. The preparation was left for 20 min in the relaxing solution containing saponin 50 μg/ml (ICN) and was washed again with the same relaxing solution and left until the tension level stabilized at zero level. Immediately before application of a Ca-containing solution, the preparation was superfused again with the relaxing solution. These procedures were also the same as used in experiments on the guinea-pig taenia coli by Saida & Nonomura (1978) and Saida (1981).

To record the pCa–tension relationship (the Ca-contraction), various concentrations of Ca-containing solution were prepared by adding appropriate amounts of $CaCl_2$ to EGTA. The apparent binding constants of EGTA for Ca and Mn were considered to be 10^6 M^{-1} and $10^{7·6}$ M^{-1}, respectively at pH 6·8 and 25 °C (Itoh *et al.* 1981*a*). To record the pMn–tension relationship, 2 mM-EGTA and $MnCl_2$ were added and the pH was adjusted by KOH instead of KCl, isotonically. To obtain a caffeine-induced contraction in the skinned muscles (the caffeine-induced contraction), the concentration of EGTA in the relaxing solution was reduced to 1×10^{-4} M throughout the experiment.

To observe the effects of Na on this caffeine-induced contraction, Na_2 ATP was replaced with K_2 ATP and the Na-free relaxing solution was prepared. To record the effects of Mn on the Ca-induced contraction, 4×10^{-3} M-EGTA plus $MnCl_2$ was added. The pH of the relaxing solution and of the varied Ca solutions was maintained at 6·8 by addition of KOH instead of KCl, isotonically.

The free concentration of Mn in the solution containing EGTA was calculated from the following solution:

$$[Mn]_{free} = \frac{-(K'[EGTA]_t - K'[Mn]_t + 1) + \sqrt{[(K'[EGTA]_t - K'[Mn]_t + 1)^2 + 4K'(Mn)_t]}}{2 \times K'},$$

where K' is the apparent binding constant of EGTA for Mn ($10^{7\cdot6}$ M^{-1}) and t is the total amount of EGTA or Mn in the solution. The amounts of free Mn and EGTA in the presence of 2 or 4 mM-EGTA are shown in Table 1.

TABLE 1. Free Mn and EGTA concentrations in 2 mM (A) and 4 mM-EGTA (B) solutions

Total amount of Mn (M)	Free Mn (M)	Free EGTA (mM)
	(A) 2 mM-EGTA	
1×10^{-3}	$2{\cdot}5 \times 10^{-8}$	~ 1
3×10^{-3}	$1{\cdot}0 \times 10^{-3}$	~ 0
2×10^{-2}	$8{\cdot}0 \times 10^{-3}$	~ 0
	(B) 4 mM-EGTA	
3×10^{-5}	$2{\cdot}02 \times 10^{-10}$	3·97
1×10^{-4}	$6{\cdot}4 \times 10^{-10}$	3·9
3×10^{-4}	$2{\cdot}0 \times 10^{-9}$	3·7

Chemicals used in the experiments were atropine sulphate and acetylcholine Cl (Daiichi), caffeine (Wako), procaine HCl (Sigma), diltiazem (Tanabe), EGTA (Dozin), Na_2 ATP (Kojin) and K_2 ATP (Sigma). The stock solution was freshly prepared just before the experiments.

Observed experimental values were expressed as the mean ± S.D. Statistical significance was assessed by Student's t test, and probabilities of less than 5% were considered to be significant.

RESULTS

General features of the mechanical responses in intact muscles

Fig. 1*A* shows the effects of various agents on the spontaneously generated contraction. With application of 1×10^{-4} M-diltiazem or 1 mM-$MnCl_2$, the generation of the spontaneously generated contraction ceased (Fig. 1*Aa*, *b*). In the concentration of 1 mM, procaine transiently suppressed the spontaneously generated contraction. With application of 10 mM-procaine, the contraction ceased during application of procaine. When the tissue was rinsed with Krebs solution, a rebound contraction appeared (*Ac*). Application of 0·3 mM-caffeine transiently produced a larger phasic contraction after which the tissue relaxed. However, in the presence of caffeine (1 mM) the contraction was regenerated, albeit with a lower amplitude. Increased concentrations of caffeine generated a transient phasic contraction and relaxed the tissue in the presence of caffeine (*Ad*). The rebound contraction was not consistent. These effects indicate that diltiazem, $MnCl_2$, procaine and caffeine suppress the spontaneously generated contraction in circular smooth muscles of the antrum.

When these agents were applied under conditions of Na-free solution or isotonic 143·6 mM-K_o solution, the responses of tissues differed. Fig. 1*B* shows an example of the effects of these agents on the contraction. In circular smooth muscle cells of the antrum, application of Na-free (substituted by sucrose) solution produced tonic

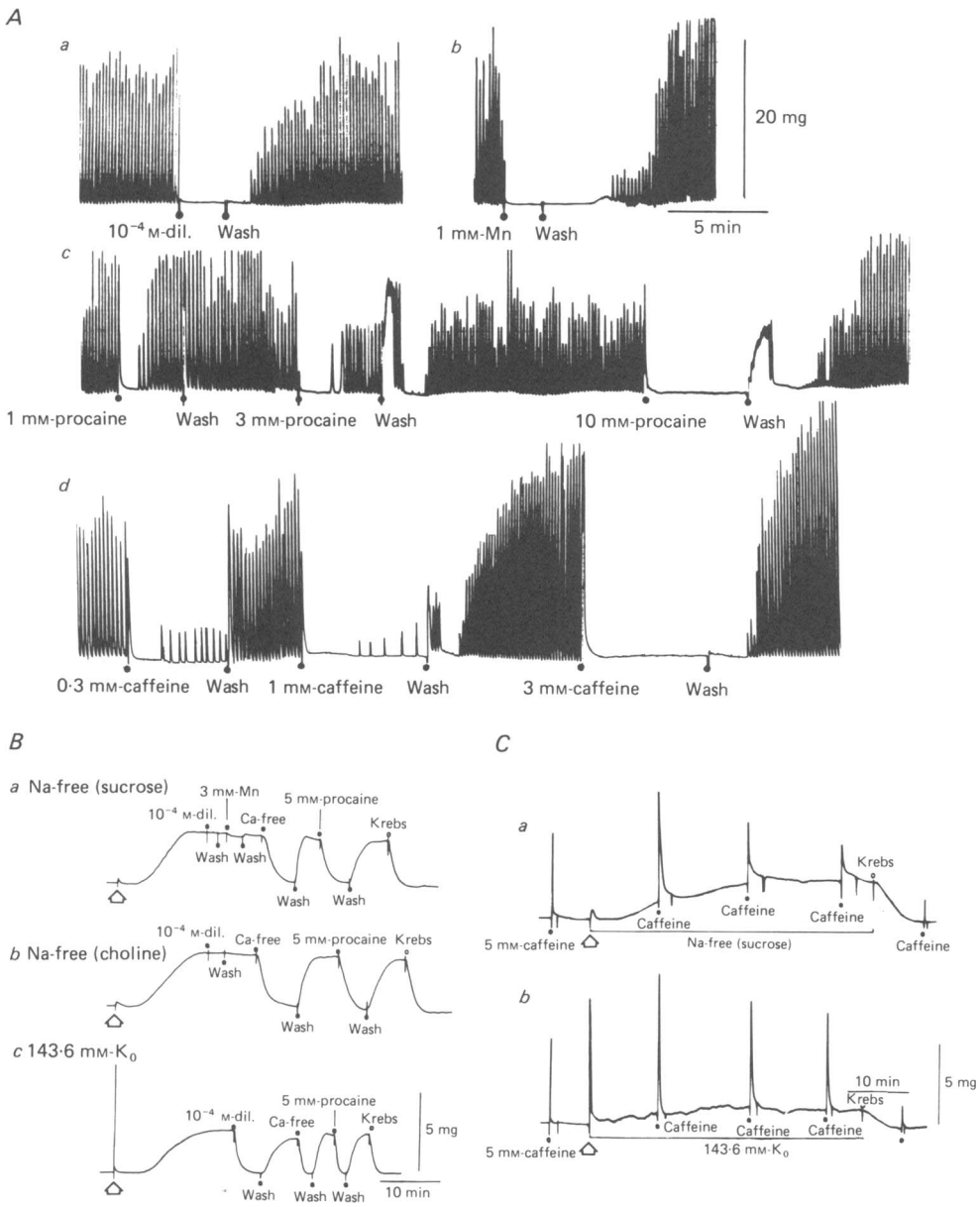

Fig. 1. *A*, effects of diltiazem (dil.), $MnCl_2$ (Mn), procaine and caffeine on the spontaneously generated contraction recorded from circular smooth muscles of the antrum. *B*, effects of diltiazem, $MnCl_2$, procaine on the contraction evoked by Na-free (sucrose) solution (*a*), Na-free (choline) solution (*b*) and 143·6 mM-K_o solution (*c*). *C*, effects of 5 mM-caffeine on the contractions evoked by Na-free (sucrose) solution (*a*) and 143·6 mM-K_o solution (*b*). In *B* and *C* dots indicate application and removal of chemical agents. Arrow indicates application of Na-free solution or 143·6 mM-K_o solution.

contraction with the latency of several min (*Ba*). Diltiazem (1×10^{-4} M) had no effect on the mechanical response, yet $MnCl_2$ (3 mM) only slightly and procaine (5 mM) completely relaxed the tissue. This Na-free-induced contraction ceased with removal of Ca (2 mM-EGTA added). When Na in Krebs solution was replaced with choline (in the presence of atropine 4×10^{-6} M), much the same amplitude of the tonic contraction and drug actions as seen with Na-freee (sucrose solution) were observed. The tonic contraction was also generated by application of 143·6 mM-K_0 with the latency of several min. Diltiazem (1×10^{-4} M) and procaine (5 mM) relaxed the tissue completely (*Bc*). With application of Ca-free (2 mM-EGTA) solution, a tonic contraction was evoked in the presence of Na-free and 143·6 mM-K_0 and the tissue relaxed completely. This means that the tonic contraction is mainly due to the influx of Ca.

Caffeine produced a transient phasic contraction in the spontaneously active tissue (Fig. 1*C*). In the case of Na-free and 143·6 mM-K_0-induced contractions, caffeine enlarged the amplitude of contraction to a greater extent than that induced in Krebs solution, yet with prolonged treatment with either Na-free or isotonic $[K]_0$, the amplitude was gradually reduced and the duration was prolonged. Even more marked inhibitions on the amplitude and prolongation of the duration were observed by treatment with caffeine in the presence of Na-free solution were those that induced by the isotonic $[K]_0$ solution, i.e. the response of the polarized and depolarized muscles in the absence of Na differed with the various chemical agents.

Effects of Ca and caffeine on the mechanical response of intact muscles

When the tissue was superfused with Ca-free EGTA-containing (2 mM) solution for over 1·0 min, an excess concentration of K_0 did not evoke a contraction in the circular muscle cells of antrum. When caffeine (5 mM) was repetitively applied, the contraction was evoked only once. Hereafter to deplete the stored Ca, caffeine (5 mM) was applied once in the Ca-free solution throughout the following experiments. With application of 2·5 mM-Ca in Ca-free solution, the contraction was evoked and the subsequently applied caffeine in doses of 5·9 mM and 128 mM-K_0 produced the contraction.

In smooth muscle cells of the antrum, application of 2·5 mM-Ca in Ca-free EGTA-containing solution produced a larger contraction in 5·9 mM-K_0 solution (polarized muscle) than that produced by 128 mM-K_0 (depolarized muscle). The reversed sequences were observed on the subsequently generated 5 mM-caffeine-induced contraction (Fig. 2 and Table 2). These contractions evoked by 2·5 mM-Ca in the polarized muscle of the antrum could never be evoked in the guinea-pig mesenteric artery (Itoh *et al.* 1981*a*). Fig. 3 shows the effects of the various concentrations of K_0 solution on the Ca-induced and caffeine-induced contractions in the presence of Ca-free EGTA solution. When the membrane was depolarized from −54 mV in 5·9 mM-K_0 to −6 mV in 128 mM-K_0 (Mishima & Kuriyama, 1976), the amplitude of the Ca-induced contraction was reduced to $0{\cdot}22 \pm 0{\cdot}06$ times the control (5·9 mM-K_0), yet the caffeine-induced contraction increased to $1{\cdot}58 \pm 0{\cdot}05$ times the control ($n = 5$). This means that, despite a reduction in the amplitude of the Ca-induced contraction in excess K_0 solution, the amount of Ca stored in the cells increased.

To determine whether or not the amount of stored Ca could be estimated from the

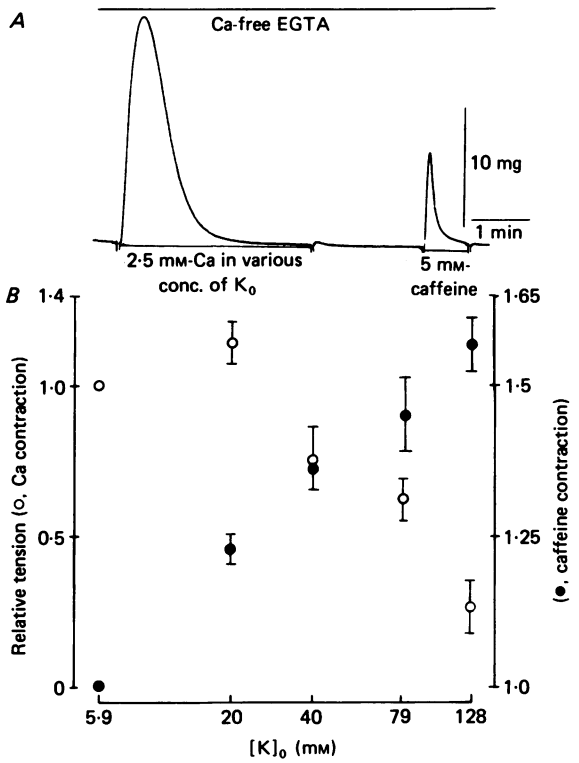

Fig. 2. Effects of various concentrations of K_0 on the Ca- and caffeine-induced contractions in Ca-free 2 mM-EGTA-containing solution. *A*, experimental procedures. In Ca-free solution, 2·5 mM-Ca and various concentrations of K_0 (from 5·9 to 128 mM) were applied for 3·5 min (Ca-induced contraction), the tissue was rinsed with Ca-free solution again for 2 min and 5 mM-caffeine was applied (caffeine-induced contraction). *B*, amplitudes of Ca and caffeine-induced contractions against the various concentrations of K_0. The amplitude of 5·9 mM-K_0 with 2·5 mM-induced contraction was registered as a relative tension of 1·0 (○) and that of 5 mM-caffeine-induced contraction under the above treatment was also normalized as 1·0 (●). Vertical bars indicate 2 × S.D. (n = 5–7).

amplitude of the caffeine-induced contraction, caffeine or ACh was applied after incubation with Ca in the polarized and depolarized muscles of the antrum. As shown in Fig. 3, in the polarized muscle, application of 1×10^{-5} M-ACh after incubation of 2·5 mM-Ca produced a larger contraction than that evoked by 5 mM-caffeine (*Aa vs. Ab*). However, the subsequently applied 10 mM-caffeine still produced a small contraction in the case of the ACh-induced contraction (*Aa*) but subsequently applied 1×10^{-5} M-ACh after generation of the 5 mM-caffeine-induced contraction did not produce the contraction (*Ab*). In the depolarized muscle, much the same responses were recorded (*Ba*). On the other hand, application of 1×10^{-5} M-ACh after generation of the 5 mM-caffeine-induced contraction did not produce a contraction (*Bb*). In the polarized and depolarized muscle, duration of the caffeine-induced contraction was shorter than that in the ACh-induced contraction generated under the above experimental conditions. The ACh-induced contraction was abolished with application of 4×10^{-6} M-atropine, in both the polarized and depolarized muscles. These

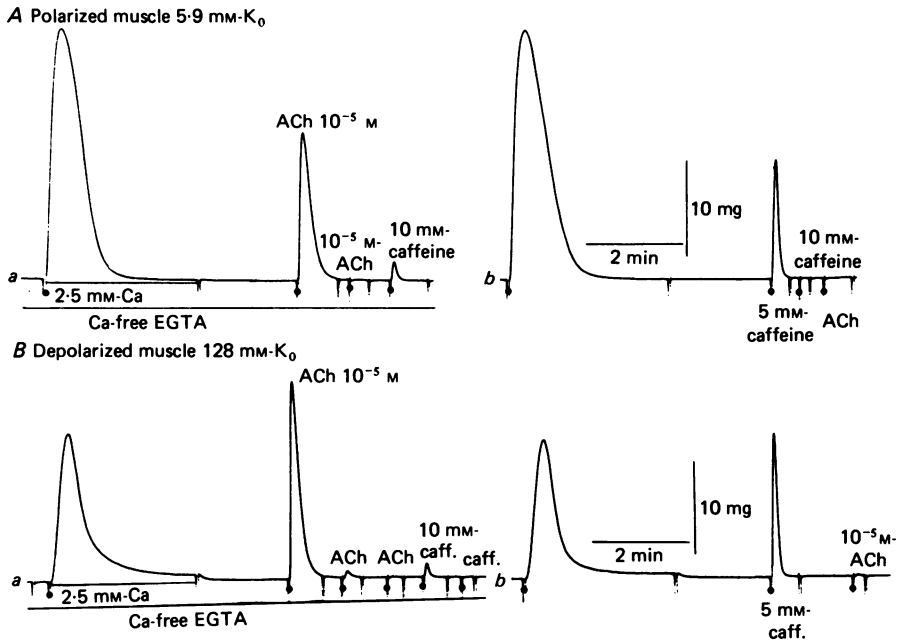

Fig. 3. Responses of smooth muscle cells of the antrum to applications of acetylcholine and caffeine after pre-loading of 2·5 mM-Ca in 5·9 mM-K_0 (polarized muscle: *A*) and in 128 mM-K_0 (depolarized muscle: *B*). *A*, after 2·5 mM-Ca was applied in 5·9 mM-K with Ca-free 2 mM-EGTA-containing solution for 3 min, acetylcholine (1×10^{-5} M) was applied following 2 min superfusion with Ca-free 2 mM-EGTA-containing solution (*a*) or 5 mM-caffeine (*b*). After application of caffeine or acetylcholine, 10 mM-caffeine or 1×10^{-5} M-acetylcholine was then applied successively. *B*, the same procedures were used for the tissue under Ca-free 2 mM-EGTA and 128 mM-K_0-containing solutions. Dots in the Figure indicate application of various agents in Ca-free 2 mM-EGTA-containing solution.

results indicate that even when the amplitude of the caffeine-induced contraction is smaller than that evoked by ACh, caffeine potently releases Ca from the caffeine-sensitive Ca store sites.

Effects of various agents on the Ca- and caffeine-induced contractions

Fig. 4 shows the effects of caffeine and procaine on the Ca-induced and caffeine-induced contractions evoked from circular smooth muscles of the antrum. In the polarized muscles, application of 2·5 mM-Ca with 1 mM-caffeine increased the amplitude of contraction, but reduced the amplitude of the subsequently generated caffeine-induced contraction, while with application of procaine plus 2·5 mM-Ca, the amplitude of the Ca-induced contraction was decreased yet that of the caffeine-induced contraction was increased. When procaine (10 mM) was applied under conditions of Ca-free solution following treatment with 2·5 mM-Ca, as shown in Fig. 4*Ae*, 5 mM-caffeine did not generate the contraction (Table 2). When 2·5 mM-Ca and 5 mM-caffeine were applied to the depolarized muscle, the amplitude of the Ca-induced contraction was increased, while that of the caffeine-induced contraction was markedly suppressed. On the other hand, when 5 mM-procaine was applied with 2·5 mM-Ca, the Ca-induced contraction all but ceased and the amplitude of caffeine-induced contraction remained

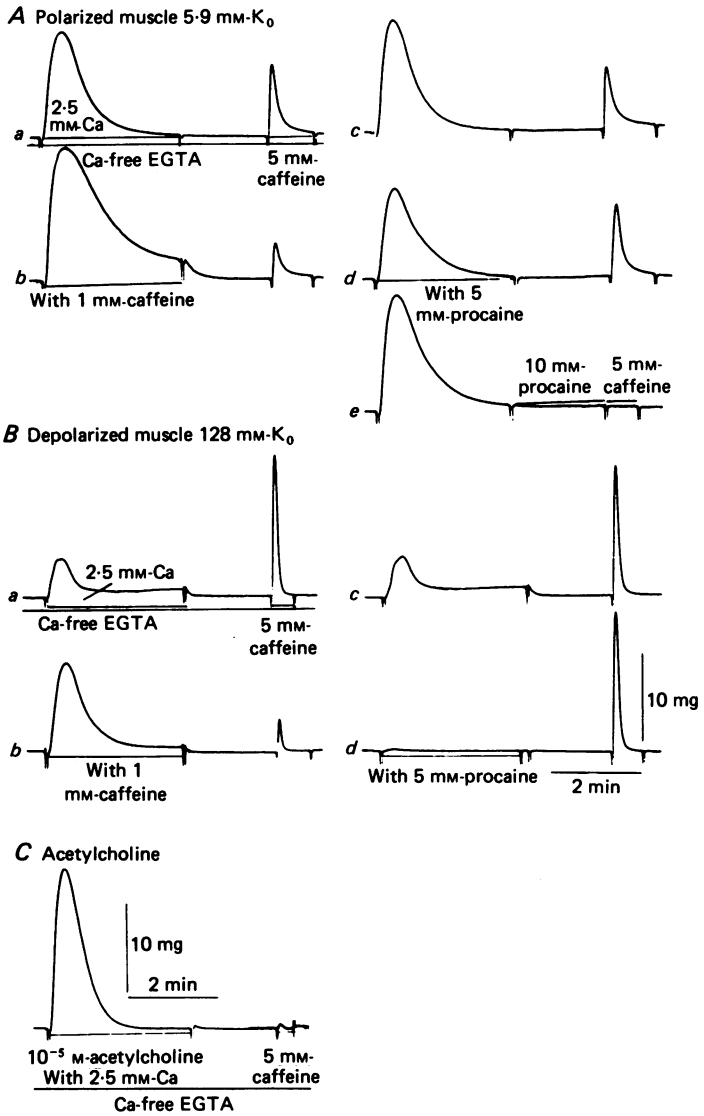

Fig. 4. Effects of caffeine and procaine on the Ca and caffeine-induced contraction in polarized and depolarized tissues of smooth muscle cells of the antrum of the guinea-pig stomach. The experimental procedures were the same as those described in Fig. 3. Either 1 mM-caffeine or 5 mM-procaine was applied simultaneously with 2·5 mM-Ca (*b* and *d*), and also 10 mM-procaine was applied following pre-treatment with 2·5 mM-Ca in polarized muscle (*Ae*). *Aa* and *c*, *Ba* and *c* are control experiments in polarized and depolarized muscles, respectively. *C*, simultaneous applications of 2·5 mM-Ca and 1×10^{-5} M-acetylcholine for generation of the contraction and subsequently generated contraction by application of 5 mM-caffeine.

unchanged. With simultaneous applications of 1×10^{-5} M-ACh and 2·5 mM-Ca_o the Ca-induced contraction was enhanced and the caffeine-induced contraction was reduced (Fig. 4*C* and also Table 2).

When 1×10^{-4} M-diltiazem was applied with 2·5 mM-Ca to the polarized muscle (Fig. 5*Ab*), the amplitude of the Ca-induced contraction and subsequently generated

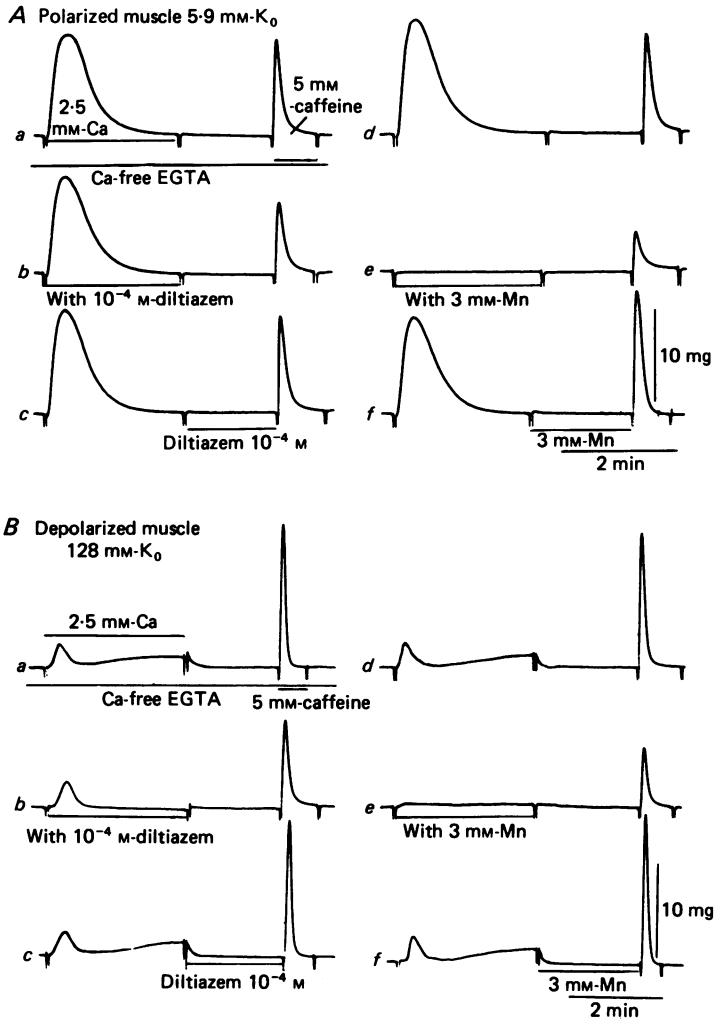

Fig. 5. Effects of diltiazem (1×10^{-4} M) and $MnCl_2$ (3 mM) on the Ca (2·5 mM)-induced and caffeine (5 mM)-induced contractions in muscle cells of the antrum. The experimental procedures were the same as described in Figs. 3 and 4. *a* and *d* in *A* and *B* were control experiments in polarized (*A*) and depolarized (*B*) muscles. *b* and *c*, application of diltiazem; *e* and *f*, application of $MnCl_2$.

caffeine-induced contraction were little affected. When 1×10^{-4} M-diltiazem was applied in Ca-free solution following treatment with Ca (Fig. 5*Ac*), the amplitude of the subsequently generated caffeine-induced contraction was not affected. On the other hand, when 3 mM-$MnCl_2$ was applied with 2·5 mM-Ca to the polarized muscle, contraction was not evoked (Fig. 5*Ae*) but the subsequently applied caffeine in Ca-free

TABLE 2. Effects of various agents on amplitudes of Ca- and caffeine-induced contractions in Ca-free 5·9 and 128 mM-K_o solutions (n = 4–5).

	(1)	(2)	(3)
5·9 mM-K			
Control	1·0	1·0	1·0
Procaine	0·76 ± 0·05	1·11 ± 0·02	0
Caffeine	1·28 ± 0·05	0·46 ± 0·03	—
Diltiazem	0·94 ± 0·04	0·77 ± 0·11	0·99 ± 0·02
$MnCl_2$	0	0·55 ± 0·05	1·40 ± 0·23
Acetylcholine	1·38 ± 0·01	0·05 ± 0·02	0·03 ± 0·02
128 mM-K			
Control	1·0	1·0	1·0
	(0·22 ± 0·06)	(1·58 ± 0·05)	(1·58 ± 0·05)
Procaine	0·03 ± 0·01	1·0 ± 0·06	0
	(0·01 ± 0·00)	(1·58 ± 0·10)	(0)
Caffeine	1·89 ± 0·08	0·10 ± 0·05	—
	(0·42 ± 0·02)	(0·16 ± 0·08)	
Diltiazem	0·98 ± 0·02	0·39 ± 0·07	0·96 ± 0·06
	(0·22 ± 0·00)	(0·62 ± 0·11)	(1·52 ± 0·05)
$MnCl_2$	0	0·20 ± 0·05	1·32 ± 0·11
	(0)	(0·32 ± 0·08)	(2·08 ± 0·17)

Column 1, amplitudes of Ca-induced contraction evoked by applications of various agents with 2·5 mM-Ca.

Column 2, amplitudes of caffeine-induced contraction evoked by 5 mM-caffeine after pre-loading the Ca in the cell by application of 2·5 mM-Ca with drug followed by Ca-free EGTA-containing solution.

Column 3, amplitudes of caffeine-induced contraction evoked by 5 mM-caffeine. After application of 2·5 mM-Ca the tissue was rinsed again with Ca-free EGTA-containing solution with various agents for 2 min and caffeine was applied.

Number in parentheses indicates the relative amplitude of Ca and caffeine-induced contraction in 128 mM-K_o as relative to those obtained in 5·9 mM-K_o control solution.

solution produced the contraction, with a small amplitude. Application of 3 mM-$MnCl_2$ in Ca-free solution following treatment with 2·5 mM-Ca increased the amplitude of the caffeine-induced contraction (Fig. 5*Af*). In depolarized muscles, application of 1×10^{-4} M-diltiazem with 2·5 mM-Ca did not modify the amplitude of the phasic component but did inhibit the tonic component of the Ca-induced contraction (Fig. 5*Bb*). The amplitude of the subsequently generated caffeine-induced contraction in the Ca-free solution was reduced. When diltiazem was applied in Ca-free solution following pre-treatment with 2·5 mM-Ca (Fig. 5*Bc*), the amplitude of the subsequently generated caffeine-induced contraction was little affected.

By application of 3 mM-$Mn\text{-}Cl_2$ with 2·5 mM-Ca in Ca-free solution (Fig. 5*Be*), contraction was not evoked and the amplitude of the caffeine-induced contraction was inhibited. Application of 3 mM-$MnCl_2$ in Ca-free solution following pre-treatment with 2·5 mM-Ca (Fig. 5*Bf*) produced a larger amplitude of the caffeine-induced contraction than was seen in the control. The effects of various agents on the Ca and caffeine-induced contractions in polarized and depolarized muscles are summarized in Table 2.

Effects of Na on the Ca and caffeine-induced contractions

When the amplitudes of caffeine-induced contractions in polarized (5·9 mM-K_o) and depolarized (128 mM-K_o) muscles were compared, the amplitude was consistently larger in the depolarized tissue, regardless of procaine applied with 2·5 mM-Ca (Table 2, column 2). Diminution of the membrane potential and Na-free condition may increase the amount of stored Ca. To confirm the above postulation, the effects of Na-free or Na-deficient solution on the mechanical responses were investigated.

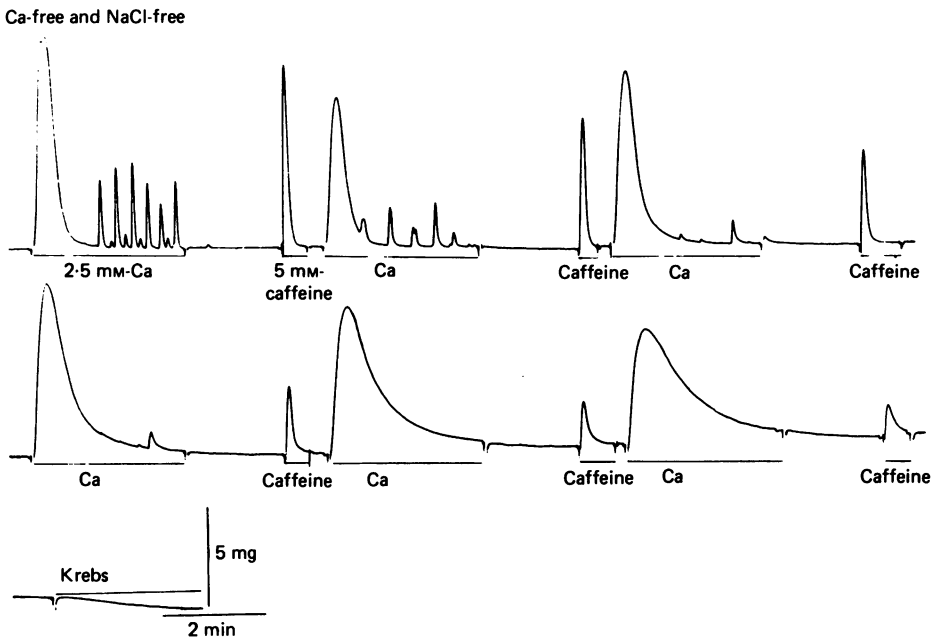

Fig. 6. Effects of NaCl-free and Ca-free solution on the 2·5 mM-Ca-induced and 5 mM-caffeine-induced contractions in muscle cells of the antrum. Experimental procedures were the same in Figs. 3 and 5. The traces are continuous recordings from the same tissue just after superfusion in the NaCl and Ca-free solution. NaCl-free solution was prepared by addition of sucrose instead of NaCl in Krebs solution.

Fig. 6 shows the effect of Ca-free with NaCl-free (15·5 mM-Na as $NaHCO_3$) solution on the Ca and caffeine-induced contractions in polarized muscles of the antrum. Application of 2·5 mM-Ca just after treatment with NaCl-free and Ca-free solution produced a large contraction and often repetitively generated phasic contractions were recorded (Fig. 6). By treatment with 5 mM-caffeine, using the procedure described in Fig. 5, a large contraction was evoked. Successive applications of 2·5 mM-Ca and caffeine gradually reduced both contractions. After 20–30 min, the caffeine-induced contraction was inhibited to a greater extent than was the Ca-induced one. The duration of the Ca-induced contractions was markedly prolonged.

To acquire detailed knowledge of the mechanical properties, the Ca sensitivity of the contractile protein was investigated by observing the pCa–tension relationship in the skinned muscle. To confirm the completion of chemical skinning of the tissue, the tissue producing a larger Ca-induced contraction in the skinned conditions than

K-induced contraction in the intact conditions was selected (see Methods). Fig. 7*A* shows the effects of 128 mM-K_0 in intact muscles and cumulatively applied varied concentrations of Ca on the skinned muscle. Before application of saponin 50 μg/ml, 128 mM-K_0 was applied to the tissue (*a*); after treatment with saponin for 20 min in the relaxing solution, various concentrations of Ca from 10^{-7} to 10^{-4} M were cumulatively applied (*b*). The minimum concentration of Ca required to evoke the

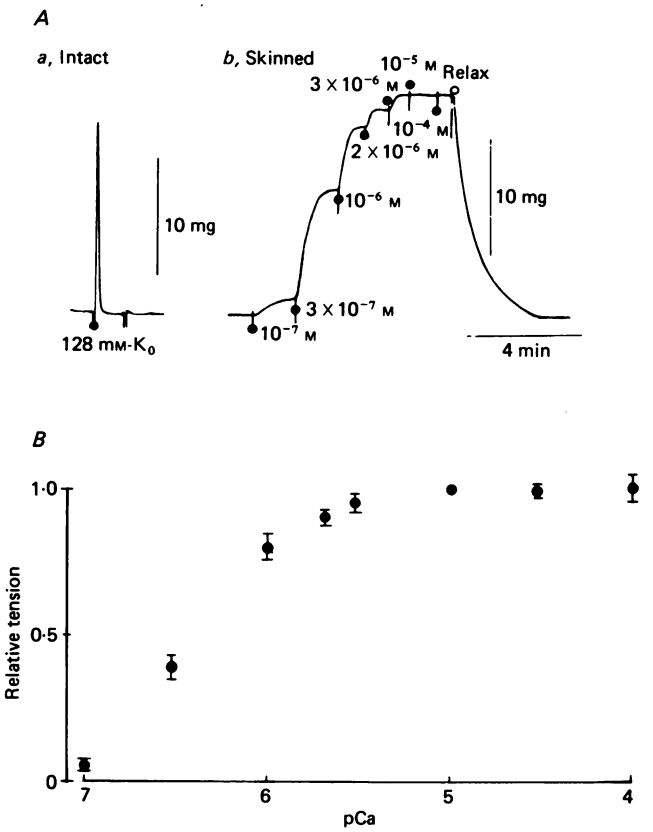

Fig. 7: Effects of various concentrations of Ca on the mechanical responses evoked from skinned muscle cells of the antrum (*Ab*) and the pCa–tension relationship (*B*). The method of skinning is described in Methods. *Aa*, before application of saponin, the tissue was superfused with 128 mM-K_0. *Ab*, after skinning the tissue, 1×10^{-7} to 1×10^{-4} M-Ca was cumulatively applied to the tissue in the relaxing solution. *B*, pCa–tension relationships of skinned circular muscle cells of the antrum. The amplitude of Ca-induced contraction produced by 1×10^{-5} M-Ca in the tissue was normalized as a relative tension of 1·0. Vertical bars indicate 2 × S.D. ($n = 6$).

contraction was 1×10^{-7} M, and 3×10^{-7} M-Ca produced about 40 % of the maximum amplitude of contraction evoked by 10^{-5} M-Ca. Rinsing the tissue with the relaxing solution relaxed it to the resting level. Fig. 7*B* shows the relationship between the contraction and free Ca on the skinned muscles prepared from circular smooth muscle of the antrum. The minimum and maximum contractions were observed at 1×10^{-7} and 1×10^{-5} M-Ca, respectively (the contraction observed in the preserved in the presence of 1×10^{-5} M-Ca was normalized). These results indicate that the minimum

concentration of Ca required to evoke the concentration in skinned muscles is much the same as that observed in the guinea-pig mesenteric artery and the porcine coronary artery (Itoh *et al.* 1981*a*, *b*; Itoh, Kajiwara, Kitamura & Kuriyama, 1982).

Table 3 shows the effects of Na on the Ca and caffeine-induced contractions in the skinned muscles. The Ca was stored in the cell by the procedure described by Itoh *et al.* (1981*a*, *b*), i.e. in relaxing solution, 1×10^{-6} M-Ca was treated for 2 min (procedure 1), the tissue was then rinsed with the relaxing solution containing 0·1 mM-EGTA for 2 min (procedure 2) and 5 mM-caffeine was applied (procedure 3). When Na-free solution was applied during procedure 1, the amplitude of the resulting caffeine-induced contraction was reduced to $0{\cdot}76 \pm 0{\cdot}09$ times the control, and with applications of the Na-free solution during procedures 2 or 3, the amplitudes of the caffeine-induced contraction were reduced to $0{\cdot}12 \pm 0{\cdot}16$ times or $1{\cdot}0 \pm 0{\cdot}04$ times the control, respectively. Furthermore, when the Na-free solution was applied throughout the experiments (procedures 1–3), the amplitude of the caffeine-induced contraction was reduced to $0{\cdot}03 \pm 0{\cdot}03$ times the control. This means that in the absence of Na_i, the Ca store site fails to accumulate the Ca due to the marked leakage of Ca.

TABLE 3. Effects of Na on the caffeine-induced contraction in skinned muscles

	Procedure 1	Procedure 2	Procedure 3	Amplitude of contraction
(1)	10 mM-Na	10 mM-Na	10 mM-Na	1·00
(2)	Na-free	10 mM-Na	10 mM-Na	$0{\cdot}76 \pm 0{\cdot}09$
(3)	10 mM-Na	Na-free	10 mM-Na	$0{\cdot}12 \pm 0{\cdot}16$
(4)	10 mM-Na	10 mM-Na	Na-free	$1{\cdot}0 \pm 0{\cdot}04$
(5)	Na-free	Na-free	Na-free	$0{\cdot}03 \pm 0{\cdot}03$

Procedure 1, 1×10^{-6} M-Ca was treated on the skinned tissue for 2 min.

Procedure 2, the tissue was rinsed with Ca-free 0·1 mM-EGTA containing solution for 2 min just after procedure 1.

Procedure 3, 5 mM-caffeine was applied just after procedure 2.

Effects of Mn on the Ca and caffeine-induced contractions in intact and skinned muscles

In Krebs solution, applications of 1 mM-$MnCl_2$ suppressed the spontaneously generated contraction. Applications of $MnCl_2$ with Ca in Ca-free solution failed to generate the Ca-induced but not the caffeine-induced contraction, whereas application of $MnCl_2$ following pre-treatment with Ca in Ca-free EGTA-containing (2 mM) solution enlarged the amplitude of the subsequently evoked caffeine-induced contraction compared with that observed in the absence of $MnCl_2$ (1–3 mM) (*d vs. f* in Fig. 5*A* and *B*). To investigate the mechanism of enlargement of the caffeine-induced contraction, the amplitudes of this contraction were compared under various durations of treatment with Ca-free $MnCl_2$-containing solution, following pre-treatment with 2·5 mM-Ca. As shown in Fig. 8, the amplitude of the caffeine-induced contraction evoked just after application of 2·5 mM-Ca in the Ca-free solution was registered as a relative tension of 1·0. When applications of Ca-free solution were prolonged, the amplitude of the caffeine-induced contraction was reduced, time dependently. However, the amplitude of the caffeine-induced contraction was consistently larger in the presence rather than in the absence of $MnCl_2$, up to 5 min.

To confirm the above results obtained with the intact muscles, the effects of $MnCl_2$ on the mechanical activity in skinned muscles of the antrum were observed using two

different experimental procedures. Fig. 9*A* shows the effects of Mn on the skinned muscles and the pMn–tension relationship in the presence of 2 mM-EGTA, and Fig. 9*B* the effects of Mn on the Ca-induced contraction and the effects of Mn on the pCa–tension relationship, in the presence of 4 mM-EGTA. In both experimental conditions, the amplitude of 1×10^{-5} M-Ca-induced contraction was registered as the relative tension of 1·0. The minimum concentration of Mn required to evoke the mechanical response in skinned muscle was just above 1·0 mM ($2{\cdot}5 \times 10^{-8}$ M free Mn) and application of 3 mM-Mn (1 mM free Mn) produced a distinct tension development.

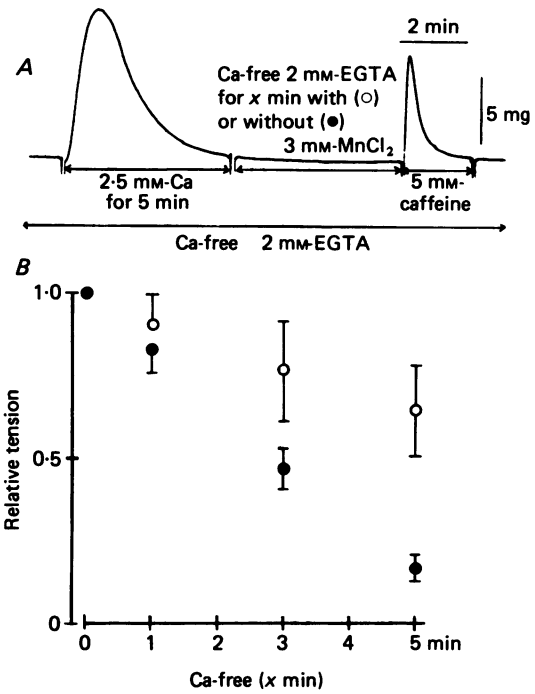

Fig. 8, Effects of $MnCl_2$ (3 mM) on the caffeine-induced contraction (5 mM) after pre-loading of Ca in the intact muscle cell. In Ca-free 2 mM-EGTA-containing solution, 2·5 mM-Ca was added for 5 min and the tissue was again rinsed with Ca-free 2 mM-EGTA and 3 mM-$MnCl_2$-containing solution for x min after which 5 mM-caffeine was applied to evoke the contraction. The amplitude of the caffeine-induced contraction evoked just after application of Ca in $MnCl_2$-free solution was registered as relative tension of 1·0. Vertical bars indicate 2 × S.D. (n = 4–5).

Application of 10 mM-Mn (8 mM free Mn) produced a further increase in the amplitude of the contraction; however, this amplitude was much smaller than the contraction evoked by 1×10^{-6} M-Ca. As described in Methods, application of more than 2 mM-Mn in 2 mM-EGTA-containing solution eliminated free EGTA in the solution. In the present experiments, 10 mM-Mn produced a larger contraction than that produced by 3 mM-Mn in the presence of 2 mM-EGTA. Thus, the possibility of a direct action of Mn on the contractile protein cannot be ruled out.

On the other hand, as shown in Fig. 9*Ba*, when 0·1 mM-Mn ($6{\cdot}4 \times 10^{-10}$ M free Mn) was applied in the presence of 3×10^{-7} M-Ca, the amplitude was further increased. In the presence of 4 mM-EGTA, Mn enhanced the amplitude of the Ca-induced

contraction at any applied concentration of Ca. To prepare $3{\cdot}3 \times 10^{-7}$ M-Ca in 4 mM-EGTA, 1 mM-$[Ca]_t$ is required. When 3×10^{-4} M-Mn is added to the solution, $[EGTA]_t$ would effectively decrease to 3·7 mM instead of 4 mM. This procedure increases the free Ca from $3{\cdot}3 \times 10^{-7}$ M in 4 mM-EGTA to $3{\cdot}7 \times 10^{-7}$ M in

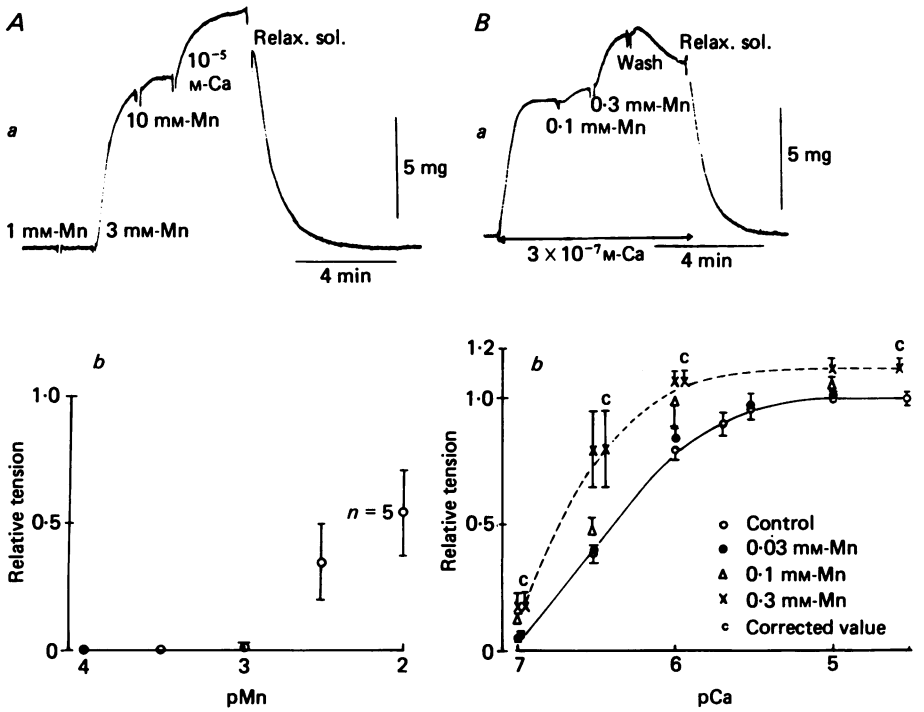

Fig. 9. Effects of $MnCl_2$ on the mechanical responses evoked from skinned muscle tissues of the antrum. The experimental procedures were the same as described in Fig 7. *Aa*, effects of $MnCl_2$ (1–10 mM) and also 1×10^{-5} M-Ca on skinned muscles. *Ab*, pMn–tension relationship observed in skinned muscles. *Ba*, effects of 0·1 and 0·3 mM-Mn on the contraction evoked by 3×10^{-7} M-Ca. *Bb*, effects of three different concentrations of $MnCl_2$ on the pCa–tension relationship. Relax. sol. in *Aa* and *Ba* is tissue that was rinsed with the relaxing solution (see Method); wash in *Ba*, 0·3 mM-$MnCl_2$ was washed with 3×10^{-7} M-Ca-containing solution. Vertical bars in *Ab* and *Bb* indicate 2 × S.D. in $n = 5$. In *A*, 2 mM-EGTA and in *B*, 4 mM-EGTA were added; the resulting concentrations of free Mn and EGTA are shown in Table 1. *c* and dotted line in *Bb*, amplitudes of the corrected Ca-induced contraction in the presence of 0·3 mM-$MnCl_2$. Corrected values shifted to the right compared with uncorrected ones. Continuous line pCa–tension relationship observed in the control solution.

3·7 mM-EGTA. The recalculated pCa–tension relationship observed in the presence of 3×10^{-4} M-$MnCl_2$ (dotted line) was inserted in Fig. 9*Bb*. Application of 1×10^{-4} M-$MnCl_2$ ($6{\cdot}4 \times 10^{-10}$ M free Mn) slightly enlarged the Ca-induced contraction, at any given concentration of Ca, and in the presence of 3×10^{-4} M-$MnCl_2$ ($2{\cdot}0 \times 10^{-9}$ M free Mn), the amplitude of the Ca-induced contraction was enlarged to an even greater extent.

The effects of procaine and Mn on Ca accumulation in the store sites and on the caffeine-induced Ca release in skinned muscles were then observed. After the tissue

was skinned by saponin, 1×10^{-6} M-Ca was applied for 2 min (procedure 1), after which the tissue was rinsed with the relaxing solution for 2 min (procedure 2) and 5 mM-caffeine was applied (procedure 3; see Methods). Application of 5 mM-procaine with 1×10^{-6} M-Ca produced the same contraction that evoked by Ca alone, and when 5 mM-caffeine was applied after pre-treatment with procaine with Ca, the amplitude of the caffeine-induced contraction was enlarged ($1{\cdot}12 \pm 0{\cdot}06$ times the control, $n = 5$), whereas when 10 mM-procaine was applied with 1×10^{-6} M-Ca (procedure 1), the Ca-induced contraction was slightly inhibited ($0{\cdot}92 \pm 0{\cdot}08$ times the control), but the resulting caffeine-induced contraction was enhanced ($1{\cdot}23 \pm 0{\cdot}09$ times the control, $n = 4$). On the other hand, when 10 mM-procaine was applied after treatment with 1×10^{-6} M-Ca (procedures 2 and 3), 10 mM-caffeine produced no contraction ($0{\cdot}02 \pm 0{\cdot}01$ times the control, $n = 5$).

When 1×10^{-6} M-Mn ($2{\cdot}5 \times 10^{-10}$ M free Mn) with 1×10^{-6} M-Ca was applied to the skinned muscle during procedure 1, the amplitude of the Ca-induced contraction was increased ($1{\cdot}21 \pm 0{\cdot}08$ times the control, $n = 5$), while the amplitude of the caffeine-induced contraction was not affected ($0{\cdot}98 \pm 0{\cdot}06$ times the control). When Mn was applied during procedure 2, the amplitude of the caffeine-induced contraction was enhanced ($1{\cdot}18 \pm 0{\cdot}05$ times the control, $n = 12$). In these experiments, 1×10^{-4} M-EGTA was treated throughout the procedures 1–3, and to prepare 1×10^{-6} M free Ca, $5{\cdot}1 \times 10^{-5}$ M total Ca was added. Therefore, during procedure 1, enhancement of the Ca-induced contraction by addition of 1×10^{-6} M-Mn may have contributed to the resulting increase in free Ca in the cell, and during procedure 2, if even a trace of Ca (over 10^{-7} M free Ca) remains in the solution, the resulting caffeine-induced contraction may be the effect of this Ca as well as the contamination of a trace of Mn. This conclusion is derived from the findings that increases in the Ca and caffeine-induced contractions were 1·21 times and 1·18 times the control. Therefore, the effects of Mn observed on the skinned muscle may indicate that Mn, in the very low concentrations, mainly accelerates the Ca–calmodulin interaction and enhances the mechanical response, while the other actions of Mn on the Ca store sites remain obscure.

DISCUSSION

From the effects of various agents (diltiazem, $MnCl_2$, procaine and caffeine) on the Ca and caffeine-induced contractions in the Ca-free solution, and contractions evoked by 143·6 mM-K_o and Na-free solutions (Table 2), the results suggest that influxes of Ca required for the generation of contraction after depletion of the stored Ca can be classified tentatively into three different types, namely, voltage-dependent Ca influx (this influx was suppressed by diltiazem and Mn), diltiazem-insensitive but Mn-sensitive Ca influx, and Mn-insensitive Ca influx. These conclusions are derived from the observations that diltiazem blocked the spontaneously generated contraction, K-induced contraction and tonic response of Ca-induced contraction in the depolarized muscle, under pre-treatment with Ca-free solution, but had no effect on the Ca-induced contraction in the polarized muscle. $MnCl_2$ not only possessed the same action as diltiazem but also blocked the Ca-induced contraction, in both polarized and depolarized muscles. The subsequently applied caffeine produced a small contraction under pre-treatment with Ca-free solution. If $MnCl_2$ had completely suppressed the

Ca influx, caffeine would not produce a contraction even if Mn did accelerate the interaction of Ca and calmodulin. Furthermore, in these muscle tissues, influxes of Ca can be classified into two components: (i) that the influx of Ca directly increases the amount of free Ca which activates the contractile protein, and (ii) that the Ca is directly sequestered into the storage site while that activating the Ca-induced Ca release mechansim indirectly increases the amount of free Ca in the cell. The latter was suppressed by application of procaine. In skinned muscles of vascular tissues (mesenteric and coronary arteries), procaine completely suppressed the caffeine-induced contraction, and when procaine was applied with Ca in the Ca-free solution in intact tissues, the resulting caffeine-induced contraction was enhanced. Therefore, procaine probably inhibits the activation of the Ca-induced Ca-release mechanism from the Ca storage sites (Itoh *et al.* 1981*a*, 1982). The amplitude of the Ca-induced contraction produced by simultaneous applications of procaine with Ca in the polarized muscle under pre-treatment with Ca-free solution was small, and this contraction may be induced by directly increased levels of free Ca following the influx of Ca. When caffeine was applied with Ca in Ca-free 128 mM-K_o containing solution, the amplitude of the Ca-induced contraction was enlarged. Presumably, influxes and releases of Ca from the store site may contribute to this enhancement.

The subsequently generated caffeine-induced contraction in the Ca-free solution following pre-loading of Ca was used as an indicator of the amount of Ca stored in the cell. The amplitude of the ACh-induced contraction was, however, larger than the caffeine-induced one recorded under the same conditions, yet caffeine continued to produce a small contraction even after application of ACh. The reversed sequence was not observed after application of caffeine. Presumably, ACh to a greater extent than caffeine releases the Ca distributed within the cell surface membrane and/or just beneath the surface membrane, and caffeine more than ACh may extrude the Ca into the extracellular spaces, because relaxation of the tissue was much faster in the case of the caffeine-induced contraction. In the skinned muscle, 5 mM-caffeine completely released the stored Ca after Ca was pre-loaded but 1×10^{-5} M-ACh did not release the Ca (authors' unpublished observations). Thus, caffein probably releases the Ca mainly stored in the sarcoplasmic reticulum, while ACh releases the intracellular Ca through activations of muscarinic receptors distributed on the surface membrane. When ACh was concomitantly applied with Ca, the resulting contraction was larger than that in caffeine plus Ca. Presumably, ACh also increases the Ca influx.

The Ca extrusion from smooth muscle cells may require the influx of Na as the Na–Ca exchange process (Reuter, Blaustein & Haeusler, 1973; Blaustein, 1977; Brading, 1978) or the activation of Ca ATPase (Aaronson & Van Breemen, 1981), under physiological conditions. Influxes of Ca in Na-containing solution may also in part be modified by Na ions distributed in the intra- and extra-cellular fluids. In the skinned muscles of the antrum, removal of Na abolished stores of Ca in the cell, and in intact muscles, reduction of $[Na]_o$ to 15·5 mM, markedly reduced the caffeine-induced contraction and prolonged the duration of the Ca-induced contraction. In the guinea-pig taenia coli, the intracellular Na ion may be diminished after 30 min incubation in the Na-free solution (Brading, 1981; Casteels, 1981). Aaronson & Van Breemen (1981) reported that in 5 mM-Na_o, the $[Na]_o/[Na]_i$ ratio was reduced from 6·3 to > 1, in this same tissue. In different visceral muscles, the $[Na]_i$ is estimated

to range between 15 and 30 mM (Brading, 1981; Jones, 1980). Reduction of $[Na]_o$ to 15 mM (NaCl-free solution) in the stomach muscle may reduce $[Na]_i$, thus accelerating the leakage of Ca from the store sites, and effluxes of Ca into the extracellular spaces may also be inhibited.

In the guinea-pig mesenteric artery, Itoh *et al.* (1981*b*) observed that in case of 15 mM-Na_o the contraction developed to 0·8 times the 128 mM-K_o-induced contraction and that this contraction was insensitive to diltiazem. Furthermore, the contraction evoked by 128 mM-K_o was composed of diltiazem-sensitive and insensitive Ca influxes. Under conditions of Na-deficient solution the noradrenaline and caffeine-induced contractions were enhanced. Thus, they postulated that the influx of Ca under conditions of Na deficiency may occur via the Na channel and the Ca influx would thus be increased.

In the skinned muscles of the antrum, removal of Na completely released the Ca stored in the cell. Brading, Burnett & Sneddon (1980) and Brading & Sneddon (1980) reported that in the case of guinea-pig taenia coli in Na-free solution, carbachol released the stored Ca to a lesser extent, as compared to findings in the control solution. Our present observations in the guinea-pig stomach support this view, i.e. there is no Ca due to leakage from the store sites.

In Ca-free solution, applications of Ca produced the contraction after depletion of the stored Ca in the stomach, whereas no contraction occurred in the mesenteric and coronary arteries (Itoh *et al.* 1981*b*; Hirata, Itoh & Kuriyama, 1980). In the stomach muscle tissue, the influx of Ca may directly activate the contraction. These observations also suggest that properties of the Ca store sites in the alimentary canal and vascular tissue differ.

The experimental conditions used herein for the generation of the Ca-induced contraction were not physiological and therefore the properties of these contractions evoked in Ca-free or Na-free solution may not be valid under normal circumstances. Under physiological conditions, the contraction is evoked mainly by voltage-dependent Ca influx and these influxes of Ca may be partly sequestered into the store site, as has been postulated in the case of guinea-pig mesenteric artery (Itoh *et al.* 1981*a*). Thus, the Ca-induced Ca-release mechanism in the Ca store site may exist but not play a major role to regulate the free Ca in the cell. In this stomach muscle tissue the membrane was spontaneously active, while in the vascular tissues membranes were not (exept for the portal vein), these differences of properties of the myoplasmic membrane may also have a causal relation with differences in the property of Ca store sites in both tissues.

$MnCl_2$ suppressed the Ca spike in visceral smooth muscles (Brading *et al.* 1969; Kuriyama, 1981; Osa, 1974; Itoh *et al.* 1981*a*). $MnCl_2$ also possessed multiple actions on the mechanical response of smooth muscle cells of the antrum, namely, this agent suppressed the spontaneous contraction, suppressed the Ca-induced contraction evoked by application of Ca in the Ca-free solution in polarized and depolarized muscles, and prevented the leakage of Ca from the store site, under conditions of Ca-free solution. It was also reported that $MnCl_2$ modifies the Ca influx and penetrates the cell, thus causing either a suppression (Keene, Seidel & Bohr, 1972; Katase & Tomita, 1972; Osa, 1974; Yoshida *et al.* 1977) or an acceleration of the mechanical responses (Shibata, 1969; Ogasawara *et al.* 1980) in various visceral muscles.

In cells of the antrum, Mn in an extremely low concentration (2×10^{-9} M) may accelerate the interaction of Ca–Ca receptor for contractile protein. It is also feasible that Mn itself binds with the Ca receptor for contractile proteins and generates the contraction. As shown in Fig. 8*A*, the amplitude of the 10 mM-Mn (8 mM free Mn)-induced contraction was much smaller than the contraction evoked by 1×10^{-6} M-Ca and such may in part be due to a reduction in the Mg–ATP concentration with production of the Mn–ATP complex. In the guinea-pig taenia coli, reduction in the Mg–ATP produced a smaller contraction (Saida & Nonomura, 1978; Iino, 1981). Furthermore, Mn may suppress the leakage of Ca from the store sites. However, to elucidate the latter two actions more detailed experiments are required. Calmodulin is known to possess four Ca binding sites (two of them show the low K_d value (high affinity) and other two the high K_d value), and the former two sites are thought to bind with Ca for the generation of contraction (Teo & Wang, 1973; Dedman, Potter, Jackson, Jonson & Means, 1977; Seamon, 1989; Kilhoffer, Demaille & Gerard, 1980). Wolf, Poirier, Brostrom & Brostrom (1977) reported that Mn also binds with calmodulin in a similar manner to Ca (high and low K_d values). Whether or not these properties directly reflect the Mn action in skinned muscle remains to be clarified, but the results do suggest that Mn is capable of binding with calmodulin. The minimum concentration of Mn required to produce the contraction was about 10^3 times higher than that of Ca. The Ca receptor for contractile protein is considered to be calmodulin; however, the possible role of leiotonin C has not been ruled out (Mikawa, Nonomura, Hirata, Ebashi & Kakiuchi, 1978). If Mn does indeed act on these Ca binding sites, the sensitivity of the Ca receptor of contractile protein might be modified.

As Mn possessed a higher apparent dissociation constant than Ca for EGTA, there are many unsolved problems in the analysis of the Mn action on contractile proteins, as is the case of the skeletal muscle (Saida & Suzuki, 1981). $MnCl_2$ blocked the spontaneous contraction with no generation of the tonic contraction, but enhanced the caffeine-induced contraction in the Ca-free solution. The response of tissues to $MnCl_2$, either enhancement or blockade of the contraction, presumably depends on the permeability of membranes in various tissues.

In conclusion, antral smooth muscles cells of the guinea-pig stomach possess multiple Ca channels for induction of the Ca influx. In physiological solution, Mn and diltiazem inhibited the spontaneously generated contraction presumably due to suppression of the voltage-dependent Ca influx. In the Ca-free polarized and depolarized muscles after depletion of stored Ca, the influx of Ca by addition of Ca may be related to at least three different channels. Furthermore, some of the Ca influx directly increases the amount of free Ca, while a part of Ca is sequestered into the store site and may activate the Ca-induced Ca-release mechanism, thus increasing the amount of free Ca. These results indicate that the mode of Ca in the muscle cell of the antrum is not the same as that in the mesenteric artery (Itoh *et al.* 1981*a*, *b*). Reduction in $[Na]_o$ enhanced the amplitude and prolonged the duration of the Ca-induced contraction. Presumably these effects might be related to the accelerated leakages of Ca from the store sites, as estimated from skinned muscles and inhibition of the Ca extrusion from the cell. $MnCl_2$ acts on the surface membrane and suppresses most of the Ca influxes except for some fractions directly sequestered into the store

site. If $MnCl_2$ penetrates the cell membrane, such as occurs in Ca-free solution, the interaction of Ca and Ca receptors of contractile proteins is accelerated and contractile protein is activated.

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