Journal of Physiology (1991), **440**, *pp.* 207–224
With 9 figures
Printed in Great Britain

TIME-DEPENDENT CHANGES IN Ca^{2+} SENSITIVITY DURING PHASIC CONTRACTION OF CANINE ANTRAL SMOOTH MUSCLE

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(*Received 18 September 1990*)

SUMMARY

1. Relationships between cytosolic Ca^{2+} concentration ($[Ca^{2+}]_{cyt}$), myosin light chain (MLC) phosphorylation and muscle tension were examined in circular smooth muscle of canine gastric antrum.

2. Electrical slow waves induced a transient increase in $[Ca^{2+}]_{cyt}$ and muscle tension. $[Ca^{2+}]_{cyt}$ increased before the initiation of contraction and reached a maximum before the peak of the phasic contractions. Following the first Ca^{2+} transient, a second rise in $[Ca^{2+}]_{cyt}$ was often observed. The second Ca^{2+} transient was of similar magnitude to the first, but only in some cases was this increase in $[Ca^{2+}]_{cyt}$ associated with a second phase of contraction. Relaxation occurred more rapidly than the restoration of resting levels of $[Ca^{2+}]_{cyt}$.

3. Acetylcholine (ACh; 3×10^{-7} M) increased the amplitude of Ca^{2+} transients, caused MLC phosphorylation and increased the force of contraction. The decay of contraction and MLC dephosphorylation preceded that of $[Ca^{2+}]_{cyt}$.

4. Increasing external K^+ (to 25–40 mM) caused a sustained increase in $[Ca^{2+}]_{cyt}$, but little change in resting tension. This suggests that the Ca^{2+} sensitivity decreased as $[Ca^{2+}]_{cyt}$ increased. Increasing K^+ to 59·5 mM further increased the level of $[Ca^{2+}]_{cyt}$, induced MLC phosphorylation and caused a transient contraction. When normal levels of K^+ were restored, the rates of MLC dephosphorylation and relaxation exceeded the rate of decay in $[Ca^{2+}]_{cyt}$.

5. Removal of external Ca^{2+} in depolarized muscles decreased $[Ca^{2+}]_{cyt}$ below the resting level without affecting resting tension. Readmission of Ca^{2+} to depolarized muscles caused force to develop at $[Ca^{2+}]_{cyt}$ levels below the original resting level, suggesting that Ca^{2+} sensitivity was increased when the resting level of $[Ca^{2+}]_{cyt}$ was decreased.

6. The phosphatase inhibitor, calyculin-A (10^{-6} M), induced tonic contraction and MLC phosphorylation without an increase in $[Ca^{2+}]_{cyt}$. During these contractures, electrical activity caused transient increases in $[Ca^{2+}]_{cyt}$ and phasic contractions which were superimposed upon the Ca^{2+}-independent contracture. In the presence of calyculin-A, relaxation occurred in two phases. The initial, rapid phase of relaxation

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was not significantly affected by calyculin-A, but the slow phase was significantly decreased.

7. These results suggest that the relationship between $[Ca^{2+}]_{cyt}$, MLC phosphorylation and contraction changes as a function of $[Ca^{2+}]_{cyt}$ in canine antral muscles. This may be due to a Ca^{2+}- and time-dependent phosphatase that regulates the level of myosin phosphorylation.

INTRODUCTION

Recent studies using aequorin or Fura-2 support the concept that increased cytosolic Ca^{2+} ($[Ca^{2+}]_{cyt}$) leads to force development in smooth muscles (for review, see Karaki, 1989; Somlyo & Himpens, 1989). The relationship between $[Ca^{2+}]_{cyt}$ and force development appears to be dependent upon the method of stimulation. For a given increase in $[Ca^{2+}]_{cyt}$, agonists such as noradrenaline in ferret portal vein (Morgan & Morgan, 1984; DeFeo & Morgan, 1985), noradrenaline, prostaglandins and endothelin in rat aorta (Sato, Ozaki & Karaki, 1988; Sakata, Ozaki, Kwon & Karaki, 1989; Ozaki, Ohyama, Sato & Karaki, 1990*b*), and carbachol in canine trachea (Gerthoffer, Murphey & Gunst, 1989; Ozaki, Kwon, Tajimi & Karaki, 1990*a*) induce greater contraction than high K^+. These findings suggest that Ca^{2+} sensitivity of contractile elements may be increased by certain agonists. Agonist-dependent decreases in Ca^{2+} sensitivity due to cyclic AMP- and cyclic GMP-mediated effects have also been observed (Karaki, 1989). In studies of isolated smooth muscle cells Yagi, Becker & Fay (1988) found that Ca^{2+} sensitivity decreased spontaneously with time. Sakata *et al.* (1989), on the other hand, showed that a decrease in Ca^{2+} sensitivity may occur during the early phase of contraction in rat aorta. Himpens & Casteels (1990) also recently reported that the Ca^{2+} sensitivity decreased during the contraction–relaxation cycle in high-K^+ solution in guinea-pig ileum. These data are consistent with the notion that the regulation of smooth muscle contraction is not determined simply by $[Ca^{2+}]_{cyt}$.

In the distal portion of the stomach, peristalsis is organized into a series of rhythmic contractions. These contractions are twitch-like, reaching a maximum within 1–2 s and then decreasing to a low resting level. Stimulation by moderate levels of external K^+, ACh or pentagastrin increases the amplitude of phasic contraction, but does not usually elevate the level of tone between contractions (for review see Szurszewski, 1987). Golenhofen (1976) classified this type of muscle as 'phasic muscle', and contrasted its activity to 'tonic muscle' or 'mixed-type muscle', such as gastric fundus, guinea-pig taenia caecum or ileal longitudinal muscles which maintain force over time (i.e. generate tone). There is a close correlation between $[Ca^{2+}]_{cyt}$ and muscle tension in guinea-pig taenia caecum muscles when they are stimulated with either high K^+ or carbachol (Ozaki, Satoh, Karaki & Ishida, 1988; Mitsui & Karaki, 1990). But in guinea-pig ileal muscles, muscle tension reaches a peak and then decreases during a sustained rise in $[Ca^{2+}]_{cyt}$ (Himpens, Matthjis & Somlyo, 1989). In the present study we further studied the relationship between $[Ca^{2+}]_{cyt}$ and tension in phasic gastrointestinal smooth muscle from canine antrum, using the fluorescent Ca^{2+} indicator Fura-2 (Ozaki, Sato, Satoh & Karaki, 1987*c*; Sato *et al.* 1988). In parallel studies we measured myosin light chain (MLC)

phosphorylation and attempted to correlate this regulatory step with changes in $[Ca^{2+}]_{cyt}$ and muscle tension.

METHODS

Mongrel dogs of either sex were killed with sodium pentobarbitone (45 mg/kg). After opening the abdomen the entire stomach was removed and placed in a bath of Krebs–Ringer bicarbonate solution (KRB). A sheet of muscularis from the ventral surface, 6–9 cm proximal to the pyloric junction, was removed from the underlying submucosa. The muscle sheet was pinned out in a dish and a 1 × 10 mm strip was cut parallel to the circular muscle fibres from the intermediate sphincter region. We were interested in measuring fluorescence and contractions of pure circular muscle, so the longitudinal layer was removed from part of each strip. But since slow wave activity originates at the border between the circular and longitudinal layer (Bauer, Publicover & Sanders, 1985*a*) the longitudinal layer was left intact over about one-third to one-half of the length of the strips to facilitate generation of spontaneous activity (Fig. 1). We also removed the submucosal half of the circular layer and studied only the myenteric region of the circular layer, since electrical and mechanical characteristics differ between the myenteric and submucosal regions (Bauer & Sanders, 1985; Bauer *et al.* 1985*a*; Bauer, Reed & Sanders, 1985*b*). Electrical stimulation was performed in some experiments by the placement of a suction electrode near the region where the myenteric border was left intact. Stimuli, 500 ms in duration and 5–20 V in amplitude, were delivered at rate of 0·01–0·025 pulses/s.

KRB contained (mM): Na^+, 137·5; K^+, 5·9; Ca^{2+}, 2·5; Mg^{2+}, 1·2; Cl^-, 134; HCO_3^-, 15·5; $H_2PO_4^-$, 1·2; dextrose, 11·5; at 37 °C. This solution had a pH of 7·3–7·4 when bubbled with 97 % O_2–3 % CO_2. High-K^+ solution was made by replacing NaCl with equimolar KCl in the normal solution.

$[Ca^{2+}]_{cyt}$ was measured simultaneously with muscle tension by the Fura-2 method as described previously (Ozaki *et al.* 1987*c*; Sato *et al.* 1988) with some modifications. Muscle strips were treated with 5×10^{-6} M-acetoxymethyl ester Fura-2 (Fura-2/AM) for 2 h in artificial culture medium (Medium 199, Gibco) at 37 °C. The non-cytotoxic detergent cremophor EL (0·01–0·02 %) was added to increase the solubility of the dye. After dye loading, muscle strips were rinsed with KRB for approximately 20 min before starting experiments. In preliminary experiments, we confirmed that Fura-2 loading did not change the contractile responses due to KCl, ACh or electrical stimulation. Fura-2 fluorescence was measured with a dual excitation fluorimeter (CAF102, Japan Spectroscopic). The muscle strip was held horizontally in a 5 ml volume organ bath at 37 °C. One end of the muscle was connected to a force transducer (UC2, Gould) to monitor the mechanical activity. The muscle strip was illuminated alternately (60 Hz) with two excitation wavelengths (340 and 380 nm) and the amount of 500 nm fluorescence induced by 340 nm light (F_{340}) and that induced by 380 nm light (F_{380}) was measured. The ratio of the fluorescence (F_{340}/F_{380}) was calculated by an internal computer.

We calculated $[Ca^{2+}]_{cyt}$ by the method described by Scanlon, Williams & Fay (1987). At the end of the experiment, fluorescent responses to the maximum and minimum levels of $[Ca^{2+}]_{cyt}$ were determined with 2×10^{-5} M-ionomycin and 1·5 mM-Ca^{2+}, and 2×10^{-5} M-ionomycin and 8 mM-EGTA, respectively. Addition of ionomycin caused a 1·5- to 2-fold increase in the magnitude of the F_{340} signal and a 50 % reduction in F_{380}. This procedure therefore increased F_{340}/F_{380} by a factor of 1·5–2·5. These changes were 3–5 times greater than those induced by 40·4 mM-K^+. Addition of 8 mM-EGTA in the presence of ionomycin decreased F_{340} below, and increased F_{380} above, the resting levels. However, in all the muscles strips tested, small oscillatory changes in $[Ca^{2+}]_{cyt}$ and muscle tension could still be observed after treatment with 2×10^{-5} M-ionomycin for 10–20 min in the presence of Ca^{2+}, suggesting that $[Ca^{2+}]_{cyt}$ in the presence of ionomycin did not reach a maximum value. Thus the level of $[Ca^{2+}]_{cyt}$ measured by this technique may overestimate actual intracellular levels. We also assumed the dissociation constant (K_d) of Fura-2 to be 224 nM (Grinkiewicz, Poenie & Tsien, 1985), but the K_d for Fura-2 may be increased in cytoplasm (Konishi, Olson, Hollingworth & Baylor, 1988; Mitsui & Karaki, 1989). An error in estimation of the K_d would tend to cause an underestimation of $[Ca^{2+}]_{cyt}$. We did not subtract the background fluorescence before ratios were calculated since leakage of the dye was less than 10 % throughout the experiments (10–20 min). It was previously shown that gastrointestinal smooth muscles have background fluorescence at both 340 and 380 nm excitations (pyridine nucleotides and flavine nucleotides) which change in the same direction in response to changes in $[Ca^{2+}]_{cyt}$ (Ozaki *et al.* 1988). In contrast, when tissues are well

loaded with Fura-2, the background is minimal and F_{340} and F_{380} change in opposite directions in response to $[Ca^{2+}]_{cyt}$. Therefore, we monitored F_{340} and F_{380} and used data (F_{340}/F_{380}) only from experiments in which these signals changed in opposite directions. Because of the errors in calibration described above, traces are presented without calibration. Instead we have displayed relative changes in the F_{340}/F_{380} ratio.

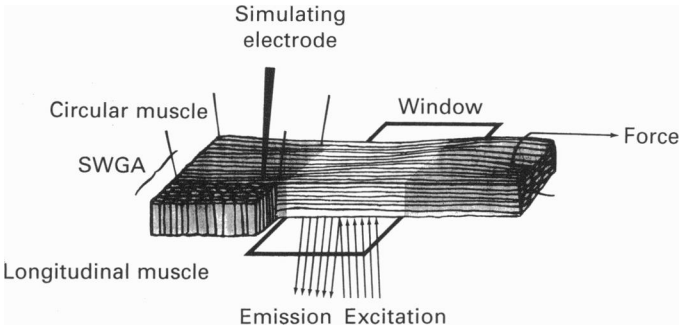

Fig. 1. Schematic representation of cross-sectional preparations used for measurement of $[Ca^{2+}]_{cyt}$ and muscle tension. Strips of muscle were cut parallel to circular fibres, and a portion of the longitudinal layer was removed. The strips were positioned in a specialized chamber such that the fluorescence of a region of pure circular muscle could be recorded while measuring force at one end of the strip. A portion of the longitudinal layer, including the slow wave generating area (SWGA), was left intact. In preparations that were not spontaneous, activity could be evoked by electrical stimulation via a suction electrode placed on the tissue near the SWGA.

Myosin phosphorylation was measured by freezing muscle strips by immersion into acetone containing 5% (w/v) trichloroacetic acid (acetone/TCA) cooled to −80 °C with crushed dry ice. All strips were thawed in acetone/TCA at room temperature for 30 min, and then transferred to acetone for 30 min. MLCs were extracted by stirring the strips vigorously in 0·25 ml/gm wet weight of 8 M-urea, 20 mM-Tris, 22 mM-glycine, 10 mM-dithiothreitol, 5 mM-EGTA and 2 mM-Na_2EDTA. Proteins in the extract were separated by glycerol polyacrylamide gel electrophoresis (Persechini, Kamm & Stull, 1986), blotted to nitrocellulose, and MLC labelled with rabbit anti-MLC antibody. Phosphorylated and dephosphorylated forms of the MLC were visualized using goat anti-rabbit immunoglobulin G (IgG) conjugated to alkaline phosphatase (Hathway & Haeberle, 1985). Fractional phosphorylation was determined by scanning densitometry.

Drugs used were Fura-2/AM (Molecular Probes), cremophor EL, acetylcholine chloride, atropine sulphate (Sigma), D600 (Knoll) and ionomycin (Calbiochem). Calyculin-A was isolated from the marine sponge *Discordemia calyx* as reported by Kato, Fusetani, Matsunaga & Hashimoto (1986). Anti-MLC antibody was prepared by immunizing rabbits with 20 kDa MLC purified from chicken gizzard according to Hathaway & Haeberle (1983). Antibody was purified from rabbit serum by affinity chromatography using 20 kDa MLC bonded to sepharose 4B. Goat anti-rabbit IgG–alkaline phosphatase was prepared by Promega Biotec (Madison, WI, USA).

The numerical data were expressed as means ± standard error. Differences were evaluated by Student's t test, and a probability of less than 0·05 was considered statistically significant.

RESULTS

Phasic responses

Circular muscles of the canine antrum are electrically and mechanically active (0·9–1·6 cycles/min; Bauer & Sanders, 1985; Bauer *et al.* 1985*a*) in the absence of extrinsic stimuli. Approximately 30% of the muscle strips used in the present study

were spontaneously active. This low level of spontaneous activity may have resulted from removal of the pacemaker region from one-half to two-thirds of the length of each muscle strip (see Methods and Fig. 1). Muscles that were not spontaneously active were paced with extrinsic electrical stimuli.

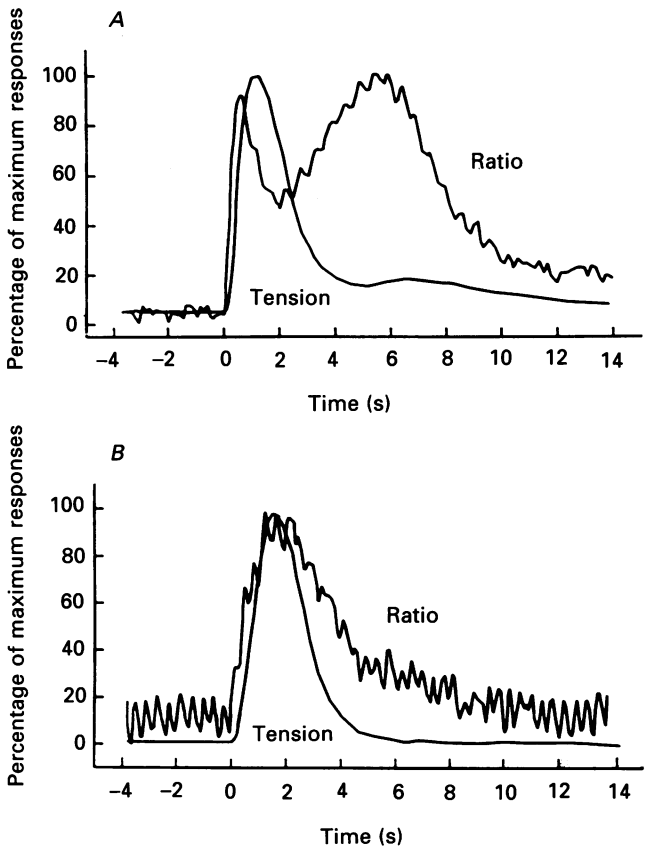

Fig. 2. Spontaneous contractions and Ca^{2+} transients in antral circular muscle. The initiation of the increase in $[Ca^{2+}]_{cyt}$ preceded the increase in force. In the population of muscles studied, two types of activity were observed. Panel *A* shows responses of a muscle in which Ca^{2+} transients and contractions consisted of two phases. Although the peak of the second phase of the Ca^{2+} transient was similar in magnitude to the first, the amplitude of the contractile response was usually far less than the first. Panel *B* shows a typical response of a group of muscles that generated single-phase Ca^{2+} transients. These muscles all displayed single-phase contractions. Note that in both groups of muscles during relaxation tension falls more rapidly than $[Ca^{2+}]_{cyt}$. Data in these traces are normalized against the maximum of the Ca^{2+} transient and the contractile maximum.

Spontaneous activity or activity induced by electrical stimulation (0·01–0·025 pulses/s) was characterized by an increase in $[Ca^{2+}]_{cyt}$ which reached a maximum in $0{\cdot}8 \pm 0{\cdot}1$ s ($n = 10$). Force development occurred when $[Ca^{2+}]_{cyt}$ reached approximately 30% of the peak, and this occurred approximately 0·3 s after the first

resolvable increase in $[Ca^{2+}]_{cyt}$. Force reached a maximum within 0·25 s after $[Ca^{2+}]_{cyt}$ had reached a maximum.

Ca^{2+} transients consisted of two phases in many muscles (seventeen of twenty-seven preparations; see Fig. 2*A*). Usually the second phase of the Ca^{2+} transient was

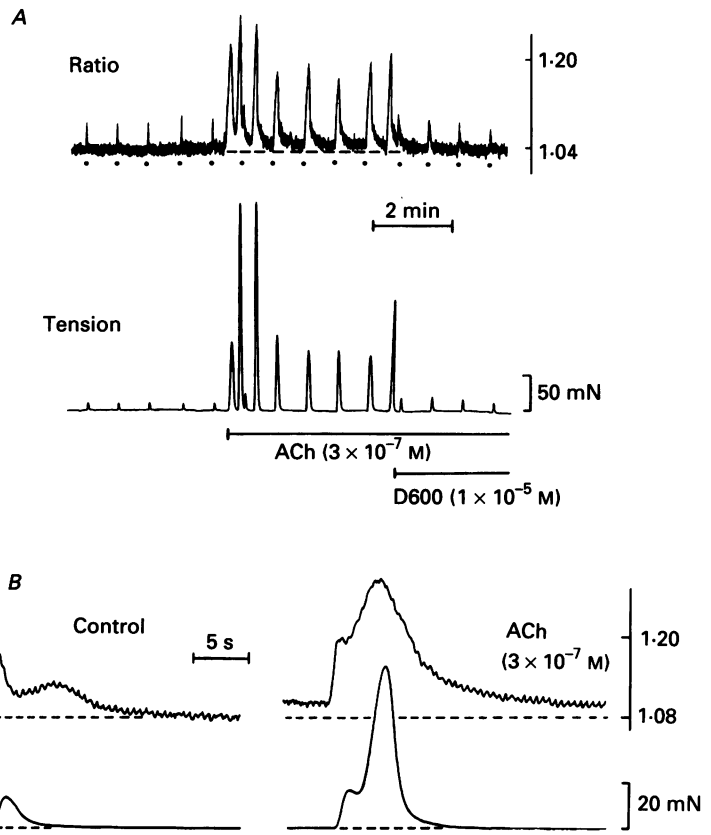

Fig. 3. Effect of ACh on $[Ca^{2+}]_{cyt}$ and muscle tension. Before adding ACh (3×10^{-7} M), small Ca^{2+} transients and contractions were observed (in this case the muscle was driven by electrical stimuli at a frequency of 0·02 pulses/s, denoted by filled dots). Addition of ACh increased the amplitude of the Ca^{2+} transients, increased resting $[Ca^{2+}]_{cyt}$ and caused some spontaneous transients to occur between applied stimuli. The increase in the $[Ca^{2+}]_{cyt}$ during Ca^{2+} transients caused an increase in muscle tension, but there was no increase in resting tension. Panel *B* shows expanded traces of Ca^{2+} transients and contractions before and during ACh applied to another muscle (which was spontaneously active). Under control conditions, the Ca^{2+} transient consisted of two phases, but the mechanical response was monophasic. The predominant effect of ACh was to increase the amplitude of the second phase of the Ca^{2+} transient and to evoke a second phase of contraction. D600 (10^{-5} M) decreased the steady-state $[Ca^{2+}]_{cyt}$ to the original level, decreased the amplitude of Ca^{2+} transients, and decreased the amplitude of phasic contractions (see end of panel *A*).

similar in amplitude to the first phase. But in the majority of these muscles ($n = 14$) the second phase of contraction was undetectable. These data demonstrate a dissociation between $[Ca^{2+}]_{cyt}$ and muscle tension. In three of the seventeen muscles

in which a second-phase Ca^{2+} transient was observed, the second contractile peak was more prominent, but the dissociation between $[Ca^{2+}]_{cyt}$ and tension was still observed. Ito, Kuriyama & Parker (1988) demonstrated that electrical stimulation induces an initial rise in $[Ca^{2+}]_{cyt}$ followed by a second rise with long latency in guinea-pig ileum. The latter phase was abolished by atropine or tetrodotoxin suggesting that this component is due to an activation of nerves within the muscle. In canine antrum, however, biphasic Ca^{2+} transients and contractions were observed in the presence of atropine (10^{-6} M) and after storage of muscles at 4 °C for 1–2 days, demonstrating that release of ACh from intrinsic nerves is not essential for the second phase of the Ca^{2+} transient. However, as described below, it is likely that this phase is regulated by neural transmitters. In ten of twenty-seven preparations, Ca^{2+} transients were monophasic (Fig. 2*B*), and these were accompanied by monophasic contractions. Whether Ca^{2+} transients were monophasic or biphasic, muscle tension decreased more rapidly than the return of $[Ca^{2+}]_{cyt}$ to resting levels (see Fig. 2*A* and *B*).

We calculated $[Ca^{2+}]_{cyt}$ using a K_d of 224 nM and considering the maximum and minimum values by the ionomycin method described by Scanlon *et al.* (1988) (see Methods for details). Resting $[Ca^{2+}]_{cyt}$ averaged 41 ± 10 nM ($n = 12$) and the peak of spontaneous $[Ca^{2+}]_{cyt}$ transients (first peak) averaged 70 ± 19 nM ($n = 11$).

Effects of acetylcholine

Addition of ACh (3×10^{-7} M) increased the amplitude of Ca^{2+} transients and phasic contractions (Fig. 3*A*). ACh also raised the baseline $[Ca^{2+}]_{cyt}$, but the latter was not associated with an increase in resting tension. The maximal increase in baseline $[Ca^{2+}]_{cyt}$ caused by 3×10^{-7} M-ACh was $83 \pm 22\%$ ($n = 7$) of the peak $[Ca^{2+}]_{cyt}$ reached during spontaneous Ca^{2+} transients. As described above, spontaneous Ca^{2+} transients resulted in contractions, yet similar increases in baseline $[Ca^{2+}]_{cyt}$ did not cause development of tone. Figure 3*B* shows expanded traces and demonstrates that ACh primarily increased the amplitude of the second phase of the Ca^{2+} transient and the phasic contraction. These data are consistent with previous findings that the primary effect of cholinergic stimulation on antral muscles is to increase the amplitude and duration of the plateau phase of the slow wave and the peak of the second phase of contraction (Szurszewski, 1987). Figure 3*B* again demonstrates the lack of change in resting tensions, despite an increase in baseline $[Ca^{2+}]_{cyt}$. Addition of D600 (10^{-5} M) in the continued presence of ACh decreased steady-state $[Ca^{2+}]_{cyt}$ back to the resting level and greatly reduced the amplitude of the Ca^{2+} transients and phasic contractions (Fig. 3*A*, end of trace). The effects of D600 on phasic contractions are consistent with effects of this agent on electrical slow wave activity (El-Skarkawy, Morgan & Szurszewski, 1978).

During the relaxation phase of the phasic contraction induced by ACh we noted a dissociation of force and $[Ca^{2+}]_{cyt}$ similar to that observed during spontaneous phasic contractions. To determine whether MLC dephosphorylation correlated better with tension or $[Ca^{2+}]_{cyt}$ we measured MLC phosphorylation under conditions identical to those in Fig. 3. Basal phosphorylation was measured in seven unstimulated muscles and averaged $0{\cdot}13 \pm 0{\cdot}02$ moles inorganic phosphate (P_i)/moles light chain. Other strips were then stimulated with ACh (3×10^{-7} M) and frozen at

various times after peak tension was observed. Myosin phosphorylation increased to $0{\cdot}29 \pm 0{\cdot}05$ moles P_i/moles light chain ($n = 6$) within 3 s of the onset of contraction and returned to basal levels within 3 s of the peak tension. The half-times ($t_{\frac{1}{2}}$) for dephosphorylation and relaxation were calculated after normalizing the mean data

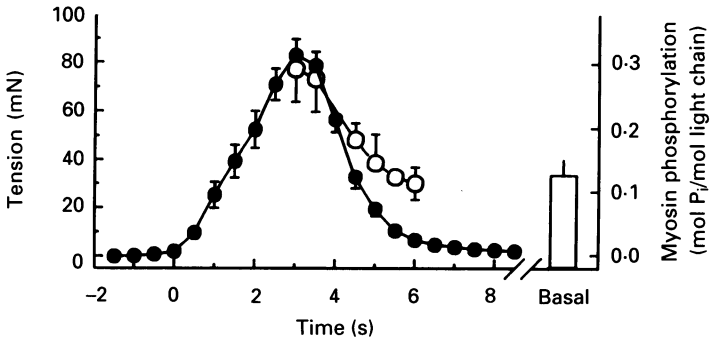

Fig. 4. Changes in muscle tension (●) and MLC phosphorylation (○) during a phasic contraction in response to ACh (3×10^{-7} M). In these experiments all longitudinal muscle was removed from a set of five muscle strips from the same animal. Muscle strips were stimulated with ACh for 5 min and strips were frozen at the peak of a phasic contraction and at various times during the relaxation phase. A paired muscle in each experiment was frozen in KRB solution without ACh to estimate basal phosphorylation (average basal phosphorylation shown in bar graph inset in figure). Representative tension data were obtained by digitizing the phasic contraction occurring just before freezing each muscle strip. $n = 3$–7 for each phosphorylation data point and $n = 7$ for each tension data point.

from Fig. 4 and subtracting basal phosphorylation and basal tension. The $t_{\frac{1}{2}}$ for dephosphorylation (1·7 s) was similar to the $t_{\frac{1}{2}}$ for relaxation (1·1 s).

Tonic responses

The lack of correlation between baseline $[Ca^{2+}]_{cyt}$ and force in response to ACh suggests dissociation between Ca^{2+} and force. Therefore we investigated this relationship further in muscles that were depolarized with elevated external K^+. Increasing K^+ to 25·9 mM increased resting $[Ca^{2+}]_{cyt}$ to 72 ± 27 nM ($n = 4$). This increase was equivalent to the peak increase in $[Ca^{2+}]_{cyt}$ that occurred during spontaneous and electrical stimulated contractions (see above), yet the sustained rise in $[Ca^{2+}]_{cyt}$ did not yield a sustained increase in force (Fig. 5*A*). When phasic contractions were initiated during exposure to 25·9 mM-external K^+, there was an average $1{\cdot}5 \pm 0{\cdot}2$-fold ($n = 4$) increase in amplitude of Ca^{2+} transients, and this was associated with a $3{\cdot}2 \pm 1{\cdot}2$-fold ($n = 4$) fold increase in the amplitude of phasic contractions (Fig. 5*A*). A further increase in external K^+ (to 40·4 mM) increased $[Ca^{2+}]_{cyt}$ to an average of 129 ± 42 nM ($n = 6$) and slightly elevated the resting tension to 86 ± 20 % ($n = 6$) of the amplitude of the spontaneous phasic contractions. Figure 5*B* shows the effects of increasing external K^+ to 59·5 mM. $[Ca^{2+}]_{cyt}$ increased rapidly, reached a maximum within 1 min, and was well maintained for at least 5 min. Muscle tension also increased, reached a maximum at about 2 min, and then fell to a level averaging 57 % of the peak contraction. Figure 6 shows the averaged responses to

59·5 mM-external K^+. Restoration of normal external K^+ resulted in a rapid decline in tension and a significantly slower decrease in $[Ca^{2+}]_{cyt}$.

Myosin phosphorylation was measured during the onset of K^+-induced contraction and after restoration of normal K^+ concentration (Fig. 7). Our aim was to test for

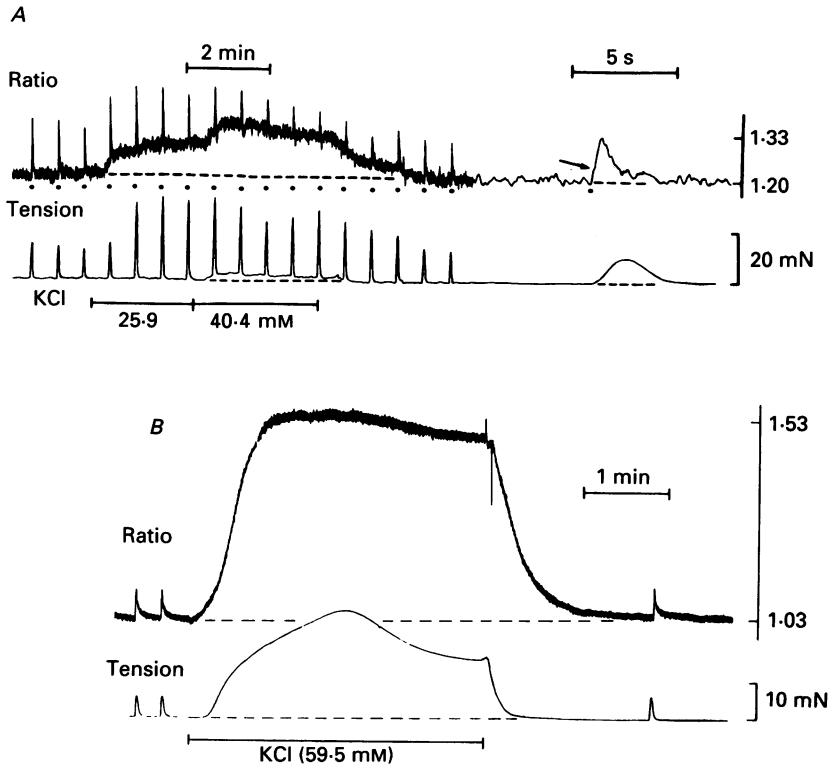

Fig. 5. Effect of elevated external K^+ on $[Ca^{2+}]_{cyt}$ and muscle tension. In panel *A* external concentration of K^+ was sequentially increased from 5·9 to 25·9 mM and 40·4 mM. After washing the muscle with normal KRB, twitch responses were monitored on an expanded time scale. Arrow indicates the level of $[Ca^{2+}]_{cyt}$ that had been reached when muscle tension began to increase. The actual threshold level of $[Ca^{2+}]_{cyt}$ necessary for contraction may be below this level considering the elasticity of the muscle strip. Although high K^+ (25·9 mM) increased the steady-state level of $[Ca^{2+}]_{cyt}$ above 'threshold level', it did not increase muscle tension. Muscle was electrically stimulated at 0·025 pulses/s, denoted by filled dots. In panel *B*, external solution was replaced with solution containing 59·5 mM-K^+ for period indicated. This caused a large increase in $[Ca^{2+}]_{cyt}$ and muscle tension. $[Ca^{2+}]_{cyt}$ reached a relatively stable plateau, but muscle tension reached a peak and then relaxed.

dissociation of phosphorylation and changes in $[Ca^{2+}]_{cyt}$ similar to those observed during the relaxation phase of ACh-induced phasic contractions (Figs 3 and 4). Myosin phosphorylation in K^+-stimulated muscles increased from basal levels of $0{\cdot}15 \pm 0{\cdot}02$ moles P_i/moles light chain ($n = 5$) to $0{\cdot}33 \pm 0{\cdot}03$ ($n = 5$) at 1 min, and was maintained at about 0·3 moles P_i/moles light chain until the bath solution was

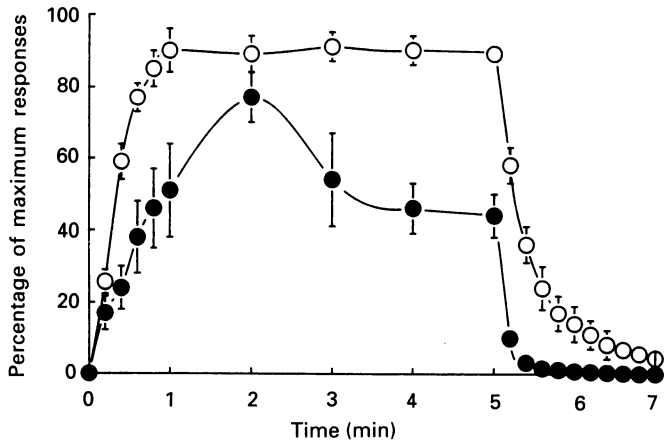

Fig. 6. Changes in $[Ca^{2+}]_{cyt}$ (○) and muscle tension (●) in the presence of elevated external K^+ (59·5 mM). This figure shows the correlation between $[Ca^{2+}]_{cyt}$ and muscle tension from five experiments like that described in Fig. 5*B*. In these experiments depolarization causes a rise in $[Ca^{2+}]_{cyt}$ that reached a plateau level within 1 min. Tension reached a peak, but then relaxes to approximately 57 % of the maximum. Replacement with 5·9 mM-K^+ solution caused rapid relaxation, and much slower restoration of resting $[Ca^{2+}]_{cyt}$.

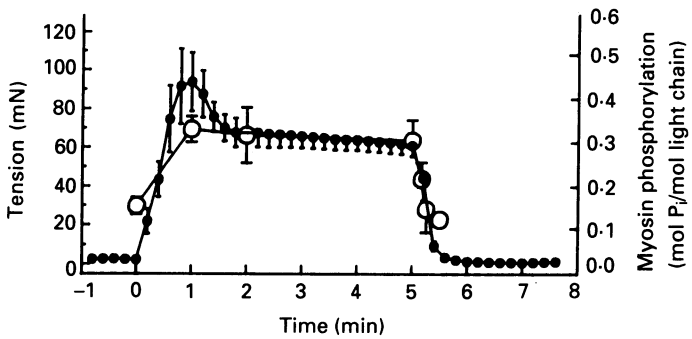

Fig. 7. Changes in MLC phosphorylation (○) and muscle tension (●) in the presence of elevated external K^+ (59·5 mM). This figure shows that MLC phosphorylation closely paralleled changes in tension. Experimental protocol for these experiments was the same as for those in Fig. 6. Tension data was obtained by digitizing a control response to K^+ obtained prior to freezing each muscle strip. $n = 5$ for each phosphorylation and tension data point.

exchanged for normal KRB solution. The muscles relaxed at a rate of $t_{\frac{1}{2}} = 16{\cdot}6$ s, and myosin was dephosphorylated at a similar rate ($t_{\frac{1}{2}} = 15{\cdot}6$ s), returning to basal levels within 30 s. The time course of dephosphorylation was quite similar to relaxation, but preceded the return of $[Ca^{2+}]_{cyt}$ to resting levels.

Removal of extracellular Ca^{2+} and chelation of remaining Ca^{2+} with EGTA (1·2 mM) caused $[Ca^{2+}]_{cyt}$ to fall below the resting level but did not affect the resting

tension (Fig. 8). Depolarization with high K^+ (66·3 mM) in the absence of Ca^{2+} had no effect on $[Ca^{2+}]_{cyt}$ or muscle tension, but stepwise readmission of Ca^{2+} (0·5 mM) resulted in an increase in $[Ca^{2+}]_{cyt}$ and development of force. The contractile response was initiated at a level of $[Ca^{2+}]_{cyt}$ below the resting level. Cumulative addition of 1·0

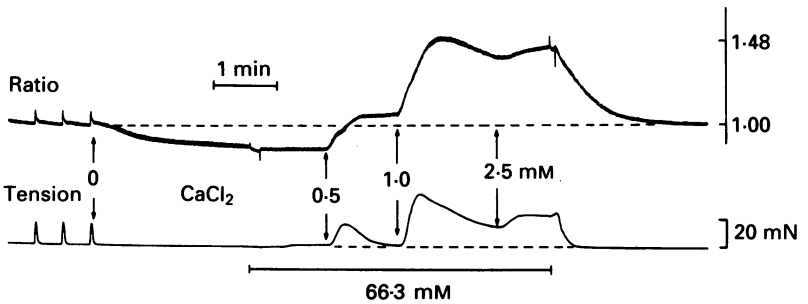

Fig. 8. Changes in $[Ca^{2+}]_{cyt}$ and muscle tension in response to Ca^{2+} depletion and readdition. After the observation of spontaneous Ca^{2+} transients and contractions, external solution was changed to Ca^{2+}-free solution containing 1·2 mM-EGTA. This caused a reduction in the resting $[Ca^{2+}]_{cyt}$ and abolished Ca^{2+} transients and phasic contractions. After 2 min in Ca^{2+}-free solution, the external medium was changed to a Ca^{2+}-free solution with elevated external K^+ (66·3 mM) (no EGTA). Then Ca^{2+} (0·5–2·5 mM) was cumulatively readmitted. This caused a progressive increase in $[Ca^{2+}]_{cyt}$ and elicited contraction. Note that initiation of contraction occurred at a $[Ca^{2+}]_{cyt}$ below that of the initial resting $[Ca^{2+}]_{cyt}$ level. Before the addition of 1·0 mM-Ca^{2+} muscle tension had fallen to the resting level, but $[Ca^{2+}]_{cyt}$ remained above the resting level.

and 2·5 mM-Ca^{2+} further increased $[Ca^{2+}]_{cyt}$ and muscle tension, but the increase in tension was not sustained despite well-maintained Ca^{2+} levels.

Effect of phosphatase inhibitor

Calyculin-A (10^{-7}–10^{-6} M), a phosphate inhibitor (Ishihara, Ozaki, Sato, Hori, Karaki, Watabe, Kato, Fusetani, Hashimoto, Uemara & Hartshorne, 1989*b*; Ishihara, Martin, Brautigan, Karaki, Ozaki, Kato, Fusetani, Watabe, Hashimoto, Uemura & Hartshorne, 1989*a*), slowly increased muscle tension (maximum reached in about 10 min), increased phosphorylated MLC content (from $0·14 \pm 0·03$ ($n = 4$) to $0·31 \pm 0·05$ ($n = 5$) moles P_i/moles light chain) without increasing resting $[Ca^{2+}]_{cyt}$ (Fig. 9*A*). In the absence of external Ca^{2+} and in the presence of 1·2 mM-EGTA (following depletion of Ca^{2+} from internal stores by 2×10^{-5} M-ionomycin), calyculin-A still induced a sustained increase in muscle tension (data not shown). This increase in muscle tension without a change in $[Ca^{2+}]_{cyt}$ suggests the presence of a mechanism for Ca^{2+}-independent MLC phosphorylation. As shown in Fig. 9, calyculin-A abolished the second phase of the Ca^{2+} transient and phasic contraction, but had no effect on the first phase. The phasic increases in $[Ca^{2+}]_{cyt}$ caused small phasic contractions to be superimposed on the large Ca^{2+}-independent increase in tone. The phasic contractions relaxed in two phases: (i) initially there was a rapid decrease to approximately half-amplitude, and (ii) this was followed by a very slow decrease.

These data suggest that calyculin-A inhibits the slow phase of relaxation but does not substantially affect the first, more rapid phase of relaxation.

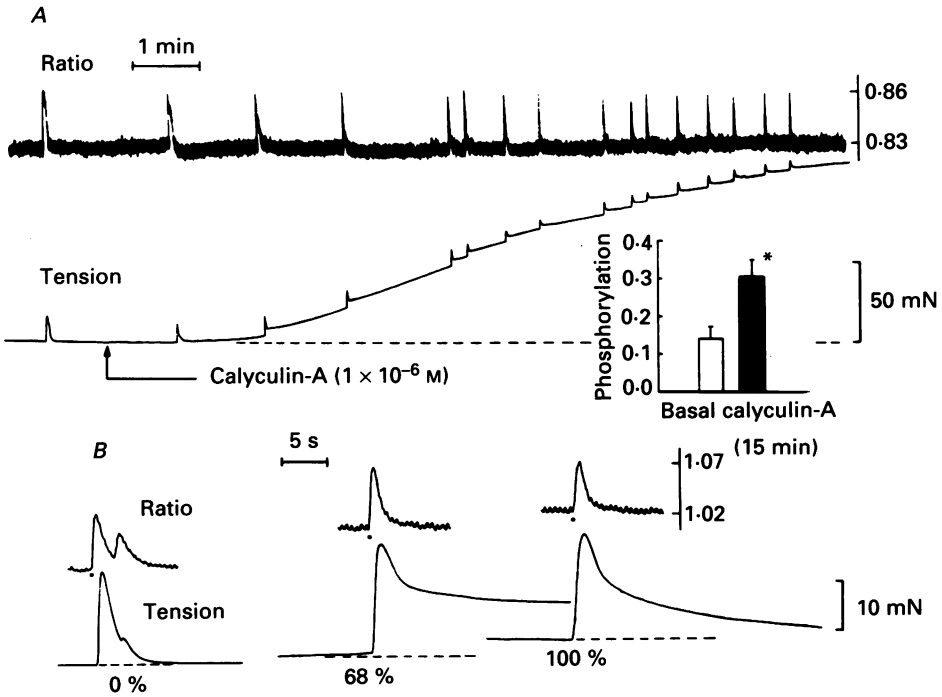

Fig. 9. Effect of calyculin-A on $[Ca^{2+}]_{cyt}$, muscle tension and MLC phosphorylation. In panel *A*, calyculin-A (10^{-6} M) was added to a spontaneously active muscle. Calyculin-A increased resting muscle tension without changing resting $[Ca^{2+}]_{cyt}$. Spontaneous Ca^{2+} transients and phasic contractions were superimposed upon the non-Ca^{2+}-dependent increase in tension. Inset shows that the average level of MLC phosphorylation increased significantly in response to 10^{-6} M-calyculin-A (bar graph compares phosphorylation levels at times 0 ($n = 4$) and 15 min ($n = 5$) after addition of calyculin-A; $P < 0{\cdot}01$). Panel *B* shows the effects of calyculin-A on $[Ca^{2+}]_{cyt}$ and muscle tension at an expanded time scale in another experiment. Ca^{2+} transients and phasic contractions (induced by electrical stimuli in this experiment) are displayed during control conditions (0 %), during the development of tone in response to calyculin-A (at 68 % of peak response) and at the maximal response to calyculin-A (100 %). The initial phase of relaxation of the phasic contractions was not affected by calyculin-A, but the later, slow phase was markedly reduced (see text for details).

DISCUSSION

Contractions are initiated by electrical slow waves in the antral region of the stomach (see Szurszewski, 1987). Slow waves consist of a rapid upstroke depolarization and a sustained plateau depolarization that persists for several seconds (Bozler, 1945; Papasova, Nagai & Prosser, 1968; Kuriyama, Osa & Tasaki, 1970; El-Sharkawy *et al.* 1978). Both phases of the slow wave are thought to be due to an inward Ca^{2+} current since they are blocked by Ca^{2+}-free solution or by adding

inorganic Ca^{2+} channel blockers (for review, see Sanders & Publicover, 1989). It is likely that Ca^{2+} entry through dihydropyridine-sensitive Ca^{2+} channels is the primary source of Ca^{2+} for phasic contractions in the stomach. In this study we have measured changes in $[Ca^{2+}]_{cyt}$ that occur during spontaneous phasic contractions, and our data support the general concept that contraction of gastric smooth muscle is primarily regulated by $[Ca^{2+}]_{cyt}$. However, our data also suggest that $[Ca^{2+}]_{cyt}$ is not the only determinant of contractile force, and other regulatory mechanisms may be involved in the maintenance of tension and relaxation.

Contraction of smooth muscles is thought to be initiated by MLC phosphorylation via the activation of Ca^{2+}–calmodulin-dependent MLC kinase (for reviews see Kamm & Stull, 1985; Hartshorne, 1987). According to this model, relaxation is thought to be initiated by the reduction of $[Ca^{2+}]_{cyt}$ and resultant decrease in MLC kinase activity. When MLC kinase activity decreases, myosin is dephosphorylated by MLC phosphatase. At present the regulation of dephosphorylation has not been clarified, and most investigators have assumed that dephosphorylation occurs by an unregulated process (for review, see Hartshorne, 1987). Somlyo and co-workers have suggested that in some smooth muscles, in which the fall in tension is greater than would be predicted by the decline in Ca^{2+}, dephosphorylation of myosin may be enhanced by activation of phosphatases (Somlyo, Kitazawa, Himpens, Matthijs, Horiuchi, Kobayashi, Goldman & Somlyo, 1989; Somlyo & Somlyo, 1990). Our data generally support this idea, but since direct assays of phosphatase activity have not yet been performed, this concept must still be viewed as a hypothesis.

The most significant finding of the present study was the observation that Ca^{2+} sensitivity decreases during the time course of spontaneous, phasic contractions in antral muscles. Yagi *et al.* (1988) found that tension induced by electrical stimulation in toad gastric muscle cells decreased before relaxation of Ca^{2+} transients, suggesting a dissociation between $[Ca^{2+}]_{cyt}$ and tension. Others have demonstrated that Ca^{2+} desensitization occurs in guinea-pig ileal muscle depolarized with elevated external K^+ solutions at room temperature (Himpens *et al.* 1989). The decrease in Ca^{2+} sensitivity in canine gastric muscles occurred independently of agonist stimulation, depolarization by elevated external K^+, or other exogenous stimuli (see Fig. 2*A*). This finding increases the probability that this mechanism plays a physiological role in 'phasic smooth muscles'. We also found that basal $[Ca^{2+}]_{cyt}$ may cause continuous Ca^{2+} desensitization during resting conditions, because we found that the level of the Ca^{2+} threshold for contraction decreased when resting $[Ca^{2+}]_{cyt}$ was decreased (see Fig. 8). The Ca^{2+} desensitization we observed was time dependent. For example, in the relatively rapid increases of $[Ca^{2+}]_{cyt}$ that occur during spontaneous activity, there was a steep relationship between $[Ca^{2+}]_{cyt}$ and contractile responses, but slow increases in $[Ca^{2+}]_{cyt}$ during K^+ depolarization greatly diminish the contractile response for a given increase in $[Ca^{2+}]_{cyt}$. These data suggest that when $[Ca^{2+}]_{cyt}$ increases rapidly, contractile responses are initiated, but when a slow increase in $[Ca^{2+}]_{cyt}$ occurs there is time for a Ca^{2+} desensitization mechanism to be activated. It is possible that Ca^{2+} desensitization is due to activation of a protein phosphatase as suggested previously (Somlyo *et al.* 1989). Our data support this hypothesis and suggest that a calyculin-A-insensitive phosphatase may be involved in the desensitization mechanism.

Several observations suggest that contractile force is not regulated simply by steady-state $[Ca^{2+}]_{cyt}$ and that the Ca^{2+} sensitivity of the processes that regulate contraction decrease during continuous elevation of $[Ca^{2+}]_{cyt}$. (i) Elevation of external K^+ to 25·9 mM increased resting $[Ca^{2+}]_{cyt}$ above the level achieved during spontaneous contractions, but this concentration of K^+ did not induce contraction. (ii) Increasing external K^+ to 40·4 mM further increased $[Ca^{2+}]_{cyt}$ to a level that was approximately equal to the maximal increase during spontaneous contractions, but contractile responses were less than 50% of the magnitude of spontaneous contractions. (iii) At 59·5 mM K^+ induced a large and sustained increase in $[Ca^{2+}]_{cyt}$, but the contractile response was biphasic; an initial force transient was followed by partial relaxation to a tonic steady-state contraction. Readdition of Ca^{2+} (0·5 and 1·0 mM) to the Ca^{2+}-depleted and K^+-depolarized antrum also induced a sustained increase in $[Ca^{2+}]_{cyt}$ although it induced only a transient contraction. (iv) Although $[Ca^{2+}]_{cyt}$ always began to decrease prior to the onset of relaxation, muscle tension fell more rapidly to the resting level than $[Ca^{2+}]_{cyt}$. (v) During spontaneous contractions, the second phasic increase in $[Ca^{2+}]_{cyt}$ induced contraction, but the magnitude of the contraction was significantly smaller at any $[Ca^{2+}]_{cyt}$ than the initial contraction. Similar dissociation between $[Ca^{2+}]_{cyt}$ and tension was demonstrated during contractile responses to ACh. (vi) Removal of external Ca^{2+} decreased $[Ca^{2+}]_{cyt}$ below the resting level, but did not alter resting tone. Readdition of 0·5 mM-Ca^{2+} increased $[Ca^{2+}]_{cyt}$ and caused contraction at a level of $[Ca^{2+}]_{cyt}$ below the resting level. Thus it appears that Ca^{2+} sensitivity is reduced by resting $[Ca^{2+}]_{cyt}$ and that the threshold level of $[Ca^{2+}]_{cyt}$ for contraction shifts in relation to $[Ca^{2+}]_{cyt}$. One explanation for these observations is that an inactivation mechanism decreases the sensitivity of the contractile element to Ca^{2+}.

To further study the mechanism of the Ca^{2+} desensitization we measured MLC phosphorylation during sustained contractions in response to elevated external K^+ and during phasic contraction induced by ACh. In either type of contraction the change in MLC phosphorylation during the relaxation phase closely correlated with the change in muscle tension but not with $[Ca^{2+}]_{cyt}$. The rates of relaxation and myosin dephosphorylation were significantly faster than the decay of $[Ca^{2+}]_{cyt}$.

Our results could be explained by a mechanism that includes rapid activation of MLC phosphorylation followed by activation of a MLC phosphatase which results in Ca^{2+} desensitization. The phosphorylation and dephosphorylation mechanisms may both be regulated by $[Ca^{2+}]_{cyt}$. It is possible that phosphorylation precedes dephosphorylation because of a higher sensitivity for Ca^{2+} and/or the presence of some lag period for activation of the phosphatase. Therefore the 'activation–inactivation balance' may favour activation during the early phase of Ca^{2+} transients and the increase in $[Ca^{2+}]_{cyt}$ causes contraction. If $[Ca^{2+}]_{cyt}$ reaches a plateau there may be shift in the 'activation–inactivation balance' with time. Inactivation may become predominant, and contraction cannot be maintained. When $[Ca^{2+}]_{cyt}$ begins to decrease, the predominance of the inactivation mechanism may explain why muscle tension decreases more rapidly than $[Ca^{2+}]_{cyt}$.

A time-dependent change in the 'activation–inactivation balance' might also explain why the slow increase in baseline $[Ca^{2+}]_{cyt}$ in response to ACh or 25–40 mM-K^+ did not induce tonic contraction. Since the reduction in Ca^{2+} sensitivity occurs

within a few seconds (i.e. within the time course of phasic contractions), a slow increase in $[Ca^{2+}]_{cyt}$ may not initiate the activation process (MLC phosphorylation) before the inactivation process is enhanced (MLC dephosphorylation). Although the increase in $[Ca^{2+}]_{cyt}$ may increase the rate of MLC phosphorylation, an increase in the rate of dephosphorylation may create a condition where there is no net increase in MLC phosphorylation. Thus a slow increase in $[Ca^{2+}]_{cyt}$ might result in a shift in the threshold level of Ca^{2+} necessary to elicit the development of force. This hypothesis is supported by experiments on guinea-pig ileum (Himpens *et al.* 1989), swine carotid artery (Rembold, 1989) and rat aorta (N. Kato & H. Karaki, personal communication) in which readdition of Ca^{2+} to depolarized muscles slowly increased the level of $[Ca^{2+}]_{cyt}$ without substantial mechanical responses. In these experiments the slow rise in $[Ca^{2+}]_{cyt}$ may have activated the inactivation process we have described and shifted the Ca^{2+} threshold for contraction to higher levels and therefore inhibited the development of force.

Dephosphorylation of MLC, and therefore relaxation, may be mediated by multiple phosphatases. The phosphatase inhibitor calyculin-A (Ishihara *et al.* 1989*a*, *b*) induced a slow contraction and a significant increase in MLC phosphorylation without changing resting $[Ca^{2+}]_{cyt}$. The contraction due to calyculin-A occurred in the absence of external Ca^{2+} and in the presence of EGTA and ionomycin, suggesting that canine antrum has Ca^{2+}-independent MLC kinase activity as in other smooth muscles (Ozaki, Ishihara, Kohama, Nonomura & Karaki, 1987*a*; Ozaki, Kohama, Nonomura, Shibata & Karaki, 1987*b*; Ishihara *et al.* 1989*b*). Superimposed upon the sustained contractions elicited by calyculin-A, electrical activity induced transient increases in $[Ca^{2+}]_{cyt}$ and phasic contractions. However, in the presence of calyculin-A relaxation was reduced and occurred in two phases. The initial, rapid phase of relaxation was not affected by calyculin-A, but the rate of the second phase was greatly reduced (see Fig. 9). The two phases of relaxation may be a result of the action of two phosphatases, only one of which is sensitive to calyculin-A. In gizzard smooth muscle a type-1 phosphatase contributes to the dephosphorylation of MLC (Ishihara *et al.* 1989*a*). This phosphatase is very sensitive to calyculin-A (IC_{50} for phosphorylated MLC, $1{\cdot}6 \times 10^{-9}$ M). By comparison, calyculin-A is far less effective as an inhibitor of type-2B phosphatase (IC_{50} for phosphorylated MLC, $7{\cdot}2 \times 10^{-7}$ M; H. Ishihara & D. J. Hartshorne, personal communication). The type-2B phosphatase is Ca^{2+}–calmodulin dependent and dephosphorylates skeletal muscle MLC *in vitro* (Ingebritsen & Cohen, 1983; Stewart, Ingebritsen & Cohen, 1983). At the dose of calyculin-A used in the present study, it is possible that the initial dephosphorylation (rapid phase of relaxation) is due to type-2B phosphatase, and the slower relaxation may be due to dephosphorylation by residual phosphatase or an unknown calyculin-A-sensitive phosphatase.

We also tested the effects of another phosphatase inhibitor, okadaic acid (at 10^{-5} M), on the contractile activity of canine antrum, but okadaic acid failed to cause contraction (data not shown). The order of potencies of this compound for type-1 phosphatase and type-2B phosphatase is similar to calyculin-A (IC_{50} for MLC of type-1, $3{\cdot}4 \times 10^{-7}$ M; IC_{50} for MLC of type-2B, $3{\cdot}3 \times 10^{-6}$ M) (Ishihara *et al.* 1989*b*; H. Ishihara & D. J. Hartshorne, personal communication). Others have reported that okadaic acid induces contraction in rabbit and rat aortae, human umbilical artery,

guinea-pig taenia caecum, and sheep and canine trachea (Shibata, Ishida, Kitano, Ohizumi, Habon, Tsukitani & Kiuchi, 1982; Ozaki *et al.* 1987*b*; Ishihara *et al.* 1989*b*; Obara, Takai, Rüegg & De Lanerolle, 1989; W. T. Gerthoffer, unpublished observation). It may be necessary to permeabilize antral muscles to demonstrate the effect of okadaic acid, since Ishihara *et al.* (1989*b*) reported that okadaic acid is less permeable than calyculin-A in guinea-pig taenia caecum. Alternatively, it is possible that the spontaneously active MLC phosphatase in canine antrum may have a different sensitivity to okadaic acid than phosphatases of other smooth muscles. In guinea-pig ileum treated with α-toxin, it was demonstrated that Ca^{2+} (at pCa 6·3) induces transient contraction (Somlyo *et al.* 1989; Kitazawa & Somlyo, 1990). When these muscles were treated with okadaic acid, the amplitude of the contractile response was increased and contractions were sustained. From these results the authors suggested the involvement of a type-2B phosphatase in Ca^{2+} desensitization. Their experiment, however, did not exclude the role of a type-1 phosphatase in Ca^{2+} desensitization, because okadaic acid is also an inhibitor of this species of phosphatase (Bialojan & Takai, 1988; Ishihara *et al.* 1989*a*). Our results tend to exclude the participation of a type-1 phosphatase because (i) calyculin-A is a very potent inhibitor of type-1 phosphatase (Ishihara *et al.* 1989*a*); (ii) at the concentration used calyculin-A would only be a weak inhibitor of type-2B phosphatases (H. Ishihara & D. J. Hartshorne, personal communication). Therefore our data are consistent with the involvement of a type-2B phosphatase in Ca^{2+} desensitization, since phasic contractions were still observed in the presence of calyculin-A.

In summary, in canine antral muscle the Ca^{2+} sensitivity of the contractile element changes in a Ca^{2+}- and time-dependent manner during the time course of phasic contractions. A calyculin-A-insensitive (and perhaps Ca^{2+}-dependent) phosphatase may mediate this mechanism. Ca^{2+} desensitization may facilitate the rhythmic, peristaltic contractions of the distal stomach and protect this region of the stomach from tonic contraction.

This work was supported by NIH grants DK 32176 to N.G.P. and K.M.S. and DK 41315 to W.T.G. and K.M.S. The authors would like to thank Randel Stevens and Janine Mangini for technical assistance.

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