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Ca^{2+} REGULATION OF THE CONTRACTILE APPARATUS IN CANINE GASTRIC SMOOTH MUSCLE

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SUMMARY

1. The relationships between cytosolic Ca^{2+} ($[Ca^{2+}]_{cyt}$; expressed as a fluorescence ratio at 400 nm and 500 nm using Indo-1) and contractile force was examined in strips of circular smooth muscles of canine gastric antrum. Rhythmic increases in $[Ca^{2+}]_{cyt}$ were observed and contractions were biphasic.

2. In most muscles (70%), the amplitude of the second phase of the Ca^{2+} transient was less than or equal to the first phase of the Ca^{2+} transient, but the second phase of the contraction was much smaller than the first phase, suggesting a decrease in Ca^{2+} sensitivity during the second contractile phase. In 30% of muscles, the amplitude of the second phase of the Ca^{2+} transient was 2- to 3-fold greater than the first phase. In these muscles, the second phase of contraction was 10-fold greater than the first phase of contraction. Thus, a non-linear relationship between $[Ca^{2+}]_{cyt}$ and force greatly amplifies force development when $[Ca^{2+}]_{cyt}$ exceeds a threshold level.

3. Acetylcholine (ACh, 0·3–1 μM) increased the amplitudes of Ca^{2+} transients and basal $[Ca^{2+}]_{cyt}$ between phasic contractions. The increase in basal $[Ca^{2+}]_{cyt}$ did not cause tone to develop. ACh increased the amplitude of Ca^{2+} transients 2- to 3-fold and this was associated with a 15 to 20-fold increase in the force of phasic contractions. Pentagastrin (0·5 nM) and cholecystokinin octapeptide (CCK, 40 nM) had similar effects on Ca^{2+} transients and phasic contractions.

4. Bay K 8644 (0·1 μM) and TEA (5 mM) also increased the amplitudes of Ca^{2+} transients by 2- to 3-fold and phasic contractions by 15- to 30-fold. There was no significant difference observed between the $[Ca^{2+}]_{cyt}$–force relationships in the presence of agonists (i.e. ACh, pentagastrin and CCK) or when $[Ca^{2+}]_{cyt}$ was increased by Bay K 8644 or TEA. These data suggest that agonist-dependent increases in Ca^{2+} sensitivity may not significantly regulate the $[Ca^{2+}]_{cyt}$–force relationship in antral muscles.

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5. D600 (5 μM), added during stimulation with ACh (0·3 M), decreased $[Ca^{2+}]_{cyt}$ and force without affecting the $[Ca^{2+}]_{cyt}$–force relationship.

6. Mechanisms exist for agonist-mediated enhancement of the Ca^{2+}–force relationship. In α-toxin-permeabilized antrum, ACh (10 μM) with GTP (100 μM) or GTPγS (100 μM) increased the Ca^{2+}-induced contraction at clamped levels of Ca^{2+}. Phorbol 12,13-dibutyrate (PDBu, 10 μM) also increased the contractile force at a given level of Ca^{2+}. These data suggest that agonists that increase phosphatidyl inositol turnover might enhance Ca^{2+} sensitivity, but this effect was not substantial at physiological levels of Ca^{2+} in these muscles.

7. Addition of PDBu (0·5 μM) to intact muscles reduced Ca^{2+} transients and contractions, suggesting that activation of C kinase may have inhibitory effects on Ca^{2+} influx.

8. These results suggest that there is a very steep relationship between $[Ca^{2+}]_{cyt}$ and force in antral muscles. Although the muscle possesses agonist-dependent mechanisms to increase Ca^{2+} sensitivity, it appears that these systems do not participate in agonist-induced contractions. This phasic muscle appears to be already primed to produce forceful contractions with modest increases in $[Ca^{2+}]_{cyt}$.

INTRODUCTION

In the distal stomach, peristalsis is organized into a series of circumferential contractions that spread toward the pyloric sphincter (Kelly & Code, 1971). These contractions are phasic, reaching a maximum within 1–2 s of their onset and then relax to a low level of tension between contractions. Stimulation with ACh or pentagastrin at concentrations less than or equal to ED_{50} levels increases the amplitude of phasic contractions, but does not elevate tone between contractions (for review, see Szurszewski, 1987). The activation of contractions in antral muscles appears to be mainly due to the influx of Ca^{2+} through voltage-dependent Ca^{2+} channels because dihydropyridines essentially abolish spontaneous contractions and responses to most excitatory agonists (Morgan, Muir & Szurszewski, 1981; Hohnsbein & Golenhofen, 1985; Vogalis, Publicover, Hume & Sanders, 1991).

The influx of Ca^{2+} causes rhythmic depolarizations in antral muscles known as electrical slow waves (see Sanders & Publicover, 1989), and the amount of Ca^{2+} influx during slow waves appears to significantly increase cytoplasmic Ca^{2+} concentration ($[Ca^{2+}]_{cyt}$; see Ozaki, Stevens, Publicover & Sanders, 1991*b*). These Ca^{2+} transients are biphasic in antral muscles (Ozaki, Gerthoffer, Publicover, Fusetani & Sanders, 1991*a*). The first phase of Ca^{2+} transients is associated with the upstroke depolarization of slow waves, and they, like the upstroke depolarizations, are relatively constant in amplitude from event to event in a given preparation. After partial repolarization of the upstroke, a long-lasting 'plateau' depolarization occurs. The plateau phase leads to a second phase in the Ca^{2+} transient because at the voltage level of the plateau, there is a sustained influx of Ca^{2+} (Ozaki *et al.* 1991*b*; Vogalis, Publicover, Hume & Sanders, 1991). This phase of the Ca^{2+} transient is highly variable and is related to the amplitude and duration of the plateau potential (Ozaki *et al.* 1991*b*; Vogalis *et al.* 1991). Ca^{2+} transients initiate contractions, and like Ca^{2+} transients, the contractile responses are biphasic.

The relationship between $[Ca^{2+}]_{cyt}$ and contractile force in smooth muscles is

complicated, however, and the amplitude of contractions is not necessarily a good indicator of $[Ca^{2+}]_{cyt}$. For example, in antral muscles we have demonstrated that muscle force and myosin light chain phosphorylation decrease more rapidly than restoration of $[Ca^{2+}]_{cyt}$ after phasic contractions (Ozaki *et al.* 1991*a*). This phenomenon may be due to a Ca^{2+}- and time-dependent decrease in the Ca^{2+} sensitivity of the contractile apparatus during phasic contractions. Similar dissociation between $[Ca^{2+}]_{cyt}$ and force has been described in other 'phasic' and 'mixed' smooth muscles (Yagi, Becker & Fay, 1988; Somlyo, Kitazawa, Himpens, Matthijs, Horiuchi, Kobayashi, Goldman & Somlyo, 1989; Himpens & Casteels, 1990). The relationship between $[Ca^{2+}]_{cyt}$ and force development also depends upon the method of stimulation in many smooth muscles (see reviews: Karaki, 1989; Somlyo & Himpens, 1989). Depolarization with elevated external K^+ elicits less forceful contractions for a given increase in $[Ca^{2+}]_{cyt}$ than stimulation by certain agonists, suggesting that these agonists can increase the Ca^{2+} sensitivity. Thus, modulation of the Ca^{2+} sensitivity of the contractile apparatus appears to be an important means of regulating contractile responses in some smooth muscles.

In the present study, the relationship between $[Ca^{2+}]_{cyt}$ and force in canine antral muscles was investigated using several agents to enhance the amplitude of Ca^{2+} transients. We attempted to determine whether agonists which utilize specific receptors and second messenger systems augment the $[Ca^{2+}]_{cyt}$–force relationship to a greater extent than agents which increase Ca^{2+} transients by simply increasing the influx of Ca^{2+} through voltage-dependent Ca^{2+} channels. Using α-toxin-permeabilized muscles, we also studied whether a mechanism is present in antral tissues for agonists to enhance the Ca^{2+} sensitivity.

METHODS

Mongrel dogs of both sexes were killed with sodium pentobarbitone (45 mg kg^{-1}). 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, 7–9 cm proximal to the pyloric sphincter, was removed from the underlying submucosa. Strips of muscle (1 × 15 mm) were cut parallel to the circular muscle fibres. For contractile studies, muscle strips were pinned out in cross-section (Bauer, Publicover & Sanders, 1985*a*) and the submucosal half of the circular layer was removed. This left the myenteric region of the circular muscle and the longitudinal layer. The contractile activity of the myenteric circular muscle was studied, but the longitudinal layer was left attached because spontaneous electrical activity originates at the border between the circular and longitudinal fibres (Bauer, Reed & Sanders, 1985*b*).

We measured relative changes in $[Ca^{2+}]_{cyt}$ using Indo-1 fluorescence (Grynkiewicz, Poenie & Tsien, 1985) and muscle force simultaneously using a specially designed fluorescence chamber as previously described (Ozaki *et al.* 1991*b*). In fluorescence experiments, muscles were treated with 5 μM acetoxymethyl ester of Indo-1 (Indo-1 AM) and 0·01 % cremophore EL for 2 h at 30–32 °C. After dye-loading, muscle strips were rinsed with KRB for approximately 30 min before starting experiments. During experiments the chamber was constantly perfused with warmed, oxygenated KRB, and the temperature was maintained at 37·5 ± 0·5 °C with a thermistor probe placed near the muscle.

Muscles were illuminated with 340 nm light (produced by a 75 W xenon lamp) and fluorescent emissions at 400 nm (F_{400}) and 500 nm (F_{500}) were monitored with paired photomultiplier tubes (CAF 102, Japan Spectroscopic). In this study, relative changes in $[Ca^{2+}]_{cyt}$ are expressed as relative changes in the ratio of F_{400} and F_{500} (F_{400}/F_{500}). Ratios were determined using an analog divider circuit (Ozaki *et al.* 1991*b*). Ratio, force, F_{400} and F_{500} signals were sampled at a rate of forty-five samples per second for storage and display. This was approximately an order of magnitude higher than the frequency required to avoid aliasing of the highest frequencies contained in the signals.

Although the relationship between F_{400}/F_{500} and log $[Ca^{2+}]_{cyt}$ is sigmoidal in shape over the full range, the relationship near the K_d for the binding of Ca^{2+} to Indo-1 is approximately linear. A series of experiments was performed to determine whether the Indo-1 responses measured in this study were near the mid-point of the relationship between $[Ca^{2+}]_{cyt}$ and F_{400}/F_{500}. The dynamic range of the Indo-1 signals was characterized by depolarizing muscles with elevated external K^+ solution (120 mM) to force a maximal increase in $[Ca^{2+}]_{cyt}$. High K^+ solution increased F_{400}/F_{500} by an average of $780 \pm 60\%$ ($n = 7$) of the amplitude of the first component of the Ca^{2+} transient (see description of spontaneous activity of antral muscles in Results). Addition of ionomycin (10 μM) caused further increases in F_{400}/F_{500}. The levels of F_{400}/F_{500} induced by high K^+ and ionomycin were well above the levels of F_{400}/F_{500} caused by ACh, pentagastrin, and CCK stimulation (see Results). Removal of extracellular Ca^{2+} and addition of EGTA (2 mM) decreased F_{400}/F_{500} below the basal level by a factor 10 times the amplitude of the first Ca^{2+} transient ($n = 2$) indicating that Indo-1 signals were also capable of following F_{400}/F_{500} well below basal levels. These studies indicate that the measurements of F_{400}/F_{500} in response to experimental manipulations occurred over the mid-range (approximately linear range) of the relationship between F_{400}/F_{500} and log $[Ca^{2+}]_{cyt}$. Therefore, F_{400}/F_{500} is assumed to be proportional to log $[Ca^{2+}]_{cyt}$ as has been used in numerous studies of a variety of intact smooth muscle tissues (cf. Karaki, 1989).

The fluorescence of pyridine nucleotides increases in relation to $[Ca^{2+}]_{cyt}$ in smooth muscles and this can interfere with Indo-1 measurements of F_{400}/F_{500} (Ozaki, Satoh, Karaki & Ishida, 1988). To avoid these artifacts, the F_{400} and F_{500} signals were constantly monitored, and only data from experiments in which the ratio of F_{400}/F_{500} changed in opposite directions were used.

We also considered the degree of synchrony between muscle cells within the sample window, and the synchrony between fluorescence measurements recorded from the sample window and contractions measured by the force transducer. The window in the tissue bath limited the collection of light to approximately 0·4 mm across the thickness of the tissue by 3 mm in the axis parallel to the smooth muscle fibres. The conduction velocities of slow waves have been measured in these directions to be 6·5 and 45 mm s^{-1}, respectively (Bauer *et al.* 1985*a*). The calculated time for a slow wave to propagate across the face of the sample window is 61 ms across the thickness of the tissue and 66 ms in the direction along the long axis of the muscle fibres. The distance between the sample window and the attachment point to the force transducer was similar to the dimensions of the window. Thus, the maximum total asynchrony of force and fluorescence measurements was less than 150 ms. Since maximal fluorescence and contractile responses were sustained for periods greater than 150 ms, potential problems resulting from asynchrony did not significantly distort measurements of peak responses.

Permeabilized muscle was prepared with staphylococcal α-toxin as described previously (Nishimura, Kolber & Van Breemen, 1988; Kitazawa, Kobayashi, Horiuchi, Somlyo & Somlyo, 1989). Small strips, 0·1–0·2 mm in diameter and 1·5–2·0 mm in length, were prepared from antral muscles. Permeabilization was accomplished by incubating the muscle strips with α-toxin (30 μg ml^{-1}) for 10–15 min in Ca^{2+}-free solution. The 'intracellular buffer' solution used in this study contained 130 mM potassium propionate, 4 mM $MgCl_2$, 2 mM Na_2ATP, 5 mM creatine phosphate, 10 U ml^{-1} creatine phosphokinase, 20 mM Tris-maleate (pH 6·8), 2 mM EGTA and indicated concentrations of free Ca^{2+}. The apparent binding constant of the Ca^{2+}–EGTA complex was 10^6 M^{-1}. Experiments were performed at room temperature (22–24 °C).

The KRB used in this study 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. When aerated with a 97% O_2–3% CO_2 gas mixture, the pH was $7·4 \pm 0·1$. Indo-1 AM (Molecular Probes), acetylcholine (ACh), pentagastrin, phorbol 12,13-dibutyrate (PDBu), nicardipine, atropine disulphate, tetrodotoxin (Sigma), cholecystokinin-(23,26)-octapeptide (CCK, Boehringer Mannheim) and D600 (Knol AG) were used. Staphylococcal α-toxin, purified from culture media of *Staphylococcus aureus* Wood 46 strain, was a generous gift from Dr Iwao Kato (Chiba University, Japan).

Numerical data are expressed as means ± standard error of the mean. Differences were evaluated by Student's t test, and P values less than 0·05 were taken as a statistically significant difference.

RESULTS

Spontaneous activity of antral muscles

Ca^{2+} transients in canine gastric antrum are biphasic and induce biphasic contractile responses (Ozaki *et al.* 1991*a*, *b*). The first phase of the Ca^{2+} transient

occurs in response to the upstroke depolarization of each electrical slow wave. Force development occurred when F_{400}/F_{500} reached $20 \pm 2{\cdot}9\%$ ($n = 15$) of the peak, and this occurred approximately 0·3 s after the first resolvable increase in F_{400}/F_{500}. This phase is quite constant in amplitude from event to event in a given preparation.

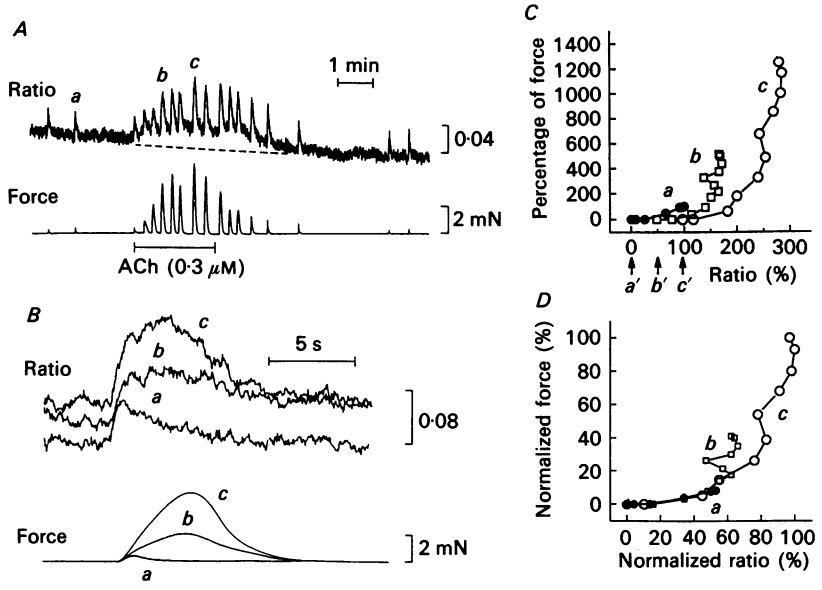

Fig. 1. Effect of ACh (0·3 μM) on F_{400}/F_{500} and muscle force. Before adding ACh, small Ca^{2+} transients (top trace) and contractions (bottom trace) were observed. Addition of 0·3 μM ACh increased the amplitude and the frequency of the Ca^{2+} transients and elevated basal F_{400}/F_{500}. The increase in F_{400}/F_{500} during Ca^{2+} transients increased the force of phasic contractions, but there was no increase in basal force. Panel *B* shows expanded traces of Ca^{2+} transients and contractions before (denoted by *a*) and during ACh (denoted by *b* and *c*). 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. In this muscle it was not possible to clearly distinguish the two contractile phases (see text for details). Panel *C* shows temporal $[Ca^{2+}]_{cyt}$–force relationship during the rising phase of the transient responses. The relationship between F_{400}/F_{500} and force shifts to the right after the addition of ACh since ACh increased basal F_{400}/F_{500} with no basal increase in force (basal F_{400}/F_{500} are denoted by *a′*, *b′* and *c′*). Note that, at higher F_{400}/F_{500}, the slope of the $[Ca^{2+}]_{cyt}$–force relationship increases. Panel *D* is a replot of the data in panel C after subtracting the increase in basal F_{400}/F_{500} (values are normalized to maximum ACh response). This figure shows that $[Ca^{2+}]_{cyt}$–force curves become steeper above the range of $[Ca^{2+}]_{cyt}$ achieved during spontaneous activity.

Therefore, in the present study, we considered Ca^{2+} transients and contractions during this phase as reference responses (i.e. 100%). The second phase of the Ca^{2+} transient is far more variable; it depends upon the level of depolarization and the duration of the plateau phase of slow waves (Ozaki *et al.* 1991*b*; Vogalis *et al.* 1991). After the peak of the plateau potential and during repolarization, F_{400}/F_{500} and force decrease. In most of the muscles (70%; $n = 50$), the amplitude of the second phase of the Ca^{2+} transient was less than or equal to the amplitude of the first phase of the Ca^{2+} transient, and the second phase of the contractile response was much smaller than the first phase of contraction. In 30% of the muscles ($n = 24$), the second phase

of Ca^{2+} transients was greater in amplitude than the first phase and the second phase of contraction was much greater than the first phase. Addition of atropine (0·2 μM) or TTX (0·1 μM) did not reduce Ca^{2+} transients and contractions in these muscles.

Effects of ACh, pentagastrin and cholecystokinin

Addition of ACh (0·3 μM) increased the amplitude of Ca^{2+} transients and phasic contractions (Fig. 1*A*). ACh also increased basal F_{400}/F_{500}. Although this increase

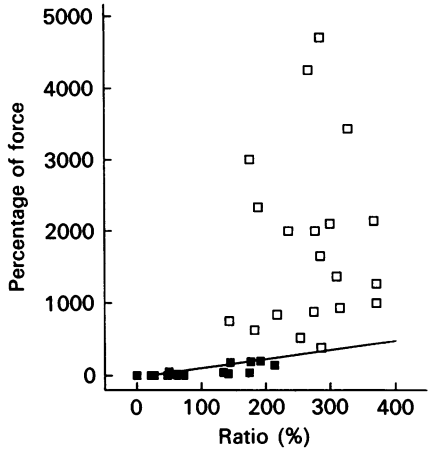

Fig. 2. $[Ca^{2+}]_{cyt}$–force relationship in the presence of ACh. Contractile force was plotted against F_{400}/F_{500} (at second phase) in the absence (■) and presence (□) of 0·3 μM ACh in all muscles tested ($n = 20$ tissues). Continuous line indicates 1:1 relationship between F_{400}/F_{500} and force, i.e. F_{400}/F_{500} and force at resting (0, 0%) and spontaneous activities during the first phase (100, 100%).

averaged 65 ± 11% ($n = 20$) of the increase in F_{400}/F_{500} during the first phase of Ca^{2+} transients, it did not elicit an elevation in tone between phasic contractions. ACh increased the average amplitude of Ca^{2+} transients by a factor of 3·2 and increased the amplitude of the second phase of contractions by 32-fold ($n = 20$). Figure 1*B* illustrates this point with records displayed at expanded time scales. ACh primarily increased the amplitude of the second phase of Ca^{2+} transients and the second phase of contractile responses. These data are consistent with previous reports in which the primary effects of cholinergic stimulation on antral muscles were an increase in the amplitude and duration of the plateau phase of electrical slow waves, and an increase in the peak of the second phase of contractions (Szurszewski, 1975; El-Sharkawy, Morgan & Szurszewski, 1978).

$[Ca^{2+}]_{cyt}$–force relationships were plotted during the rising phase of spontaneous contractions and during the rising phase of contractions in the presence of ACh (Fig. 1*C*). This graph demonstrates two important points: (i) the steepness of the $[Ca^{2+}]_{cyt}$–force relationship increased during ACh stimulation suggesting a non-linear relationship between $[Ca^{2+}]_{cyt}$ and force; and (ii) the Ca^{2+}–force relationship shifted to the right in the presence of ACh suggesting a decrease in Ca^{2+} sensitivity that may have been due to the increase in basal $[Ca^{2+}]_{cyt}$ (see Ozaki *et al.* 1991*a*; Ozaki,

Gerthoffer, Publicover & Sanders, 1991*c*). We also plotted the $[Ca^{2+}]_{cyt}$–force relationships after the subtraction of the increase in basal $[Ca^{2+}]_{cyt}$ with F_{400}/F_{500} and force normalized to the maximum responses (Fig. 1*D*). These plots illustrate that the $[Ca^{2+}]_{cyt}$–force relationships have similar slopes during the early phase of contraction.

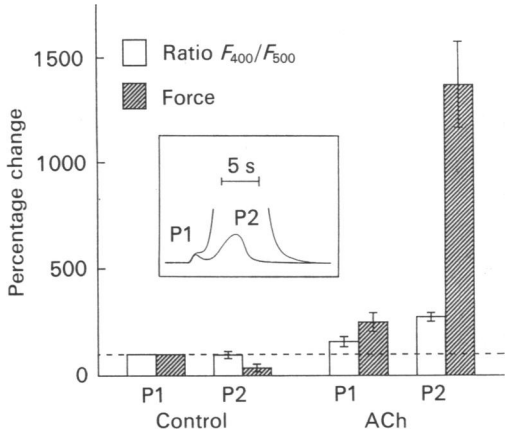

Fig. 3. $[Ca^{2+}]_{cyt}$–force relationship in the presence of ACh. In 50 % of muscle strips (10 out of 20 tissues), after the treatment with 0·3 μM ACh, force increased in two phases (P1 and P2; see inset). Ca^{2+} transients were also biphasic. During spontaneous activity, the amplitude of the second phase of Ca^{2+} transients was similar to the first phase. However, this Ca^{2+} transient induced smaller contractions than during P1. In the presence of ACh, the second phase of Ca^{2+} transient increased by 2·8-fold (values calculated from basal level to peak), and contractile amplitude increased by 37-fold. During the first phase of Ca^{2+} transients and contraction, $[Ca^{2+}]_{cyt}$ did not rise high enough to reach the non-linear portion of the $[Ca^{2+}]_{cyt}$–force curve; during the first phase of Ca^{2+} transient $[Ca^{2+}]_{cyt}$ increased by 1·6-fold (over control P1) and force increased by 2·5-fold. Results are expressed as means ±S.E.M. ($n = 10$).

Figure 2 is a scatter plot of the $[Ca^{2+}]_{cyt}$–force relationship during the second phase of contraction of twenty muscles in response to ACh (0·3 μM). Average F_{400}/F_{500} and force of control muscles (during second phase of Ca^{2+} transients) were 87 ± 17 and 50 ± 17 % (compared to the first phase), respectively. Average F_{400}/F_{500} and force during the second phase of Ca^{2+} transients in the presence of ACh were 270 ± 15 and 1810 ± 200 %, respectively.

Although contractions of antral muscle are usually biphasic (see Szurszewski, 1987), after addition of ACh it was not always possible to distinguish the peaks of the two phases in many muscles. However, in half of the muscles studied it was possible to characterize the effects of ACh on the first and second contractile peaks independently because the peaks were clearly separated in time (see inset in Fig. 3). In ten muscles, the amplitude of the first phase of the Ca^{2+} transient increased by a factor of 1·6 and the first phase of contraction increased by a factor of 2·5 in response to ACh. During the second phase of the Ca^{2+} transient F_{400}/F_{500} increased by a factor of 2·8, but force increased by a factor of 37 (compared to amplitude of second phase contractions in muscles before ACh). These data demonstrate the increase in the slope of the $[Ca^{2+}]_{cyt}$–force relationship at higher $[Ca^{2+}]_{cyt}$.

Responses to pentagastrin were also characterized by a steep $[Ca^{2+}]_{cyt}$–force relationship during phasic contractions. Figure 4*A* shows the effect of pentagastrin (0·5 nM) on Ca^{2+} and force transients. Pentagastrin (0·5 nM) raised baseline F_{400}/F_{500} (average 35±4% of the level reached during the first phase of Ca^{2+} transients, n =

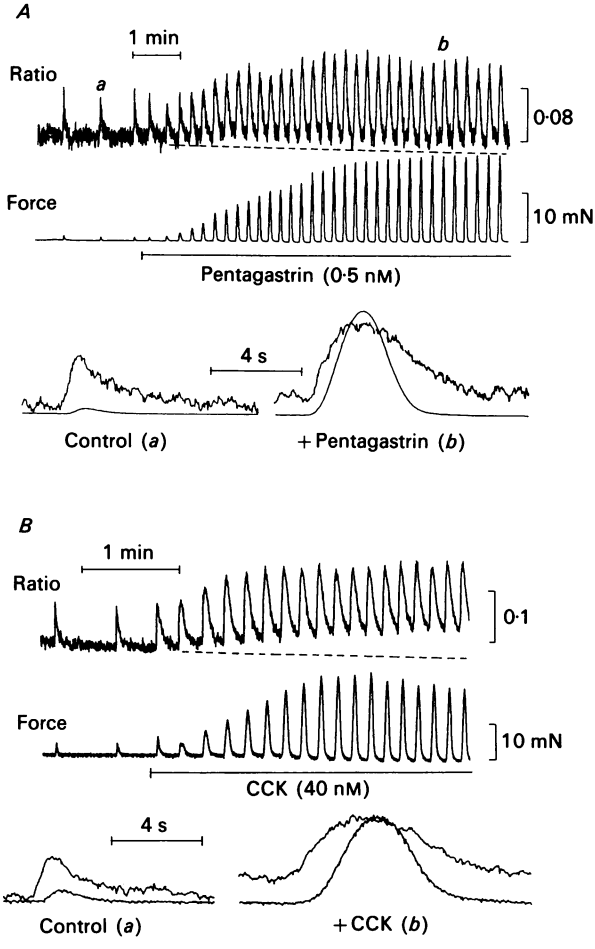

Fig. 4. Effects of pentagastrin and CCK on F_{400}/F_{500} and force. In panel *A*, before adding pentagastrin, small Ca^{2+} transients (top trace) and contractions (bottom trace) were observed. Addition of pentagastrin (0·5 nM) increased the amplitude and frequency of Ca^{2+} transients, and increased basal F_{400}/F_{500}. The increased Ca^{2+} transients increased amplitude of phasic contractions. Lower panel shows Ca^{2+} transients and contractions at expanded time scale before and in presence of pentagastrin. Panel *B* shows the effects of CCK (40 nM). Similar results were obtained with both agonists.

7), increased the amplitude of Ca^{2+} transients, and increased the force of phasic contractions. The increase in basal F_{400}/F_{500} in response to pentagastrin was smaller than the increase caused by ACh, and the increase in basal F_{400}/F_{500} did not induce tonic contraction. Average F_{400}/F_{500} and force of control muscles (during second phase of Ca^{2+} transients) were 80±3·6 and 23±12% (compared to the first phase),

respectively. Average F_{400}/F_{500} and force during the second phase of Ca^{2+} transients in the presence of pentagastrin were 200 ± 22 and $2010 \pm 590\%$, respectively ($n = 7$; see Fig. 6).

Cholecystokinin octapeptide (CCK, 40 nM) also produced a large increase in force with a relatively small increase in F_{400}/F_{500} (Fig. 4*B*). Average F_{400}/F_{500} and force of

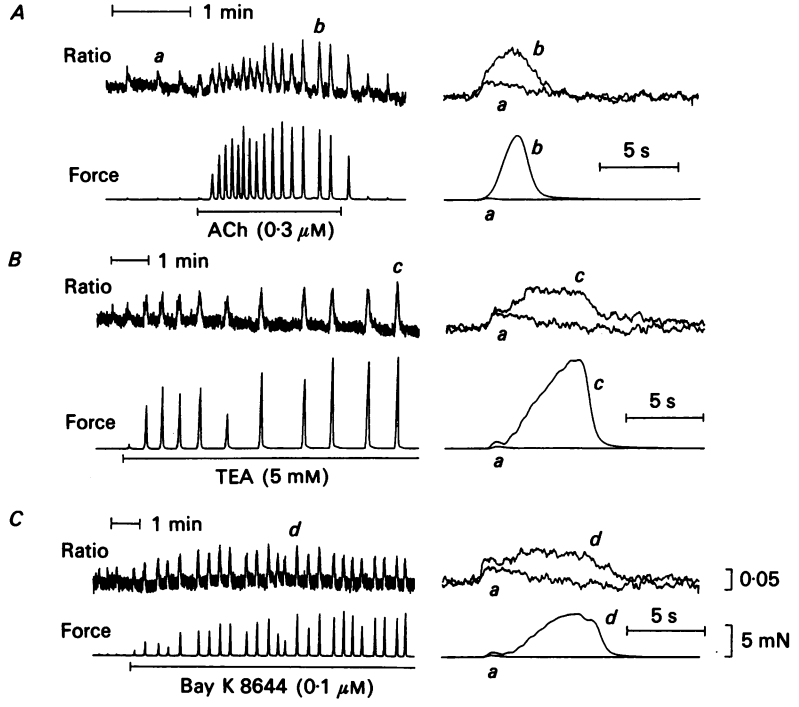

Fig. 5. Effects of ACh, TEA and Bay K 8644 on Ca^{2+} and mechanical transients recorded from a single muscle strip. ACh (0·3 μM, panel *A*) increased resting $[Ca^{2+}]_{cyt}$. TEA (5 mM, panel *B*) and Bay K 8644 (0·1 μM, panel *C*) slightly increased basal $[Ca^{2+}]_{cyt}$, but this effect was less than the increase observed with ACh. TEA and Bay K 8644 increased the amplitudes of Ca^{2+} transient by 2- to 3-fold and increased force by a factor of 30–60.

control muscles (during second phase of Ca^{2+} transients) were 34 ± 12 and $0 \pm 0\%$ (compared to the first phase), respectively. Average F_{400}/F_{500} and force of CCK-treated muscles (during second phase of Ca^{2+} transients and contractions) were 230 ± 38 and $2070 \pm 750\%$, respectively ($n = 3$; see Fig. 6).

Effects of Bay K 8644 and TEA

The results in the previous section show that the $[Ca^{2+}]_{cyt}$–force relationship becomes very steep when $[Ca^{2+}]_{cyt}$ approximately doubles over levels reached during spontaneous Ca^{2+} transients. Others have suggested that agonists increase the Ca^{2+} sensitivity of the contractile apparatus in other smooth muscles (Morgan & Morgan, 1984; DeFeo & Morgan, 1985; Sato, Ozaki & Karaki, 1988; Gerthoffer, Murphey &

Gunst, 1989; Himpens, Kitazawa & Somlyo, 1990). We tested this hypothesis by comparing the $[Ca^{2+}]_{cyt}$–force relationships in the presence of agonists with the relationship when $[Ca^{2+}]_{cyt}$ was elevated by non-receptor-dependent mechanisms. We used Bay K 8644, a Ca^{2+} channel agonist, and TEA, a K^+ channel blocker

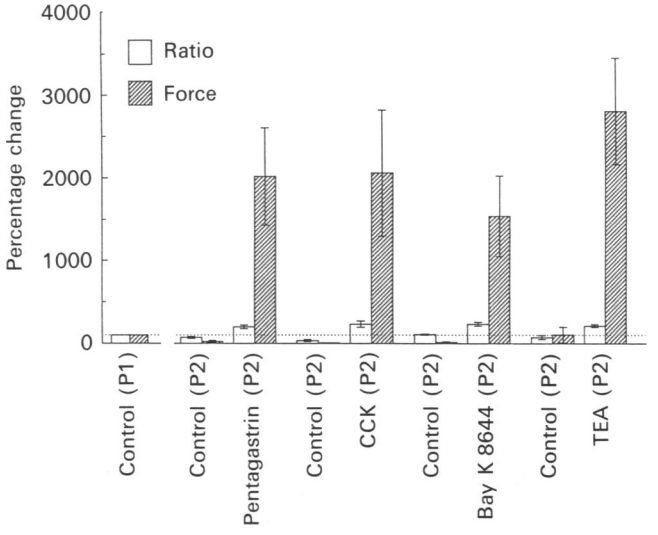

Fig. 6. Summary of peak changes in F_{400}/F_{500} and force in response to pentagastrin (0·5 nM; $n = 7$), CCK (40 nM; $n = 3$), Bay K 8644 (0·1 μM; $n = 4$), and TEA (5 mM, $n = 4$). Peak Ca^{2+} transient and force during the second phases are plotted.

which indirectly increases the open probability of Ca^{2+} channels by increasing the amplitude and duration of show waves (Szurszewski, 1978). Both of these agents increased the amplitude of Ca^{2+} transients and phasic contractions. Figure 5*A–C* compare the effects of ACh (0·3 μM), TEA (5 μM) and Bay K 8644 (0·1 μM) on F_{400}/F_{500} and force in the same muscle strip using the same amplitude scales. In this example, ACh (0·3 μM) increased Ca^{2+} transient by about 3-fold and force of phasic contractions by a factor of 50. TEA increased Ca^{2+} transient by 2·5-fold, and increased contractile force by a factor of 60. Bay K 8644 (0·1 μM) increased Ca^{2+} transients by 1·6-fold and increased contractions by a factor of 30. The average changes in F_{400}/F_{500} and force are shown in Fig. 6. These data demonstrate that when $[Ca^{2+}]_{cyt}$ increases above some threshold level, muscle force increases sharply. This appears to be the result of a non-linear relationship between $[Ca^{2+}]_{cyt}$ and force. The data do not support a specific, agonist-dependent enhancement in Ca^{2+} sensitivity of the contractile apparatus.

The effects of tetrodotoxin (0·2 μM) and atropine (0·1 μM) on the Bay K 8644-induced responses were tested ($n = 2$). These compounds had no effect on $[Ca^{2+}]_{cyt}$ and force suggesting that endogenous release of ACh did not increase the steepness of the $[Ca^{2+}]_{cyt}$–force relationship. Of course it is not possible to rule out all endogenous substances (i.e. those not blocked by TTX and atropine) that might have affected the $[Ca^{2+}]_{cyt}$–force relationship.

Effects of D600 and nicardipine

Ca^{2+} channel blockers, such as D600 and dihydropyridines, have been shown to inhibit gastric slow waves (El-Sharkawy *et al.* 1978; Fujii, Inoue, Yamanaka & Yoshitomi, 1985; Ozaki *et al.* 1991*b*), Ca^{2+} transients and contractions (Ozaki *et al.*

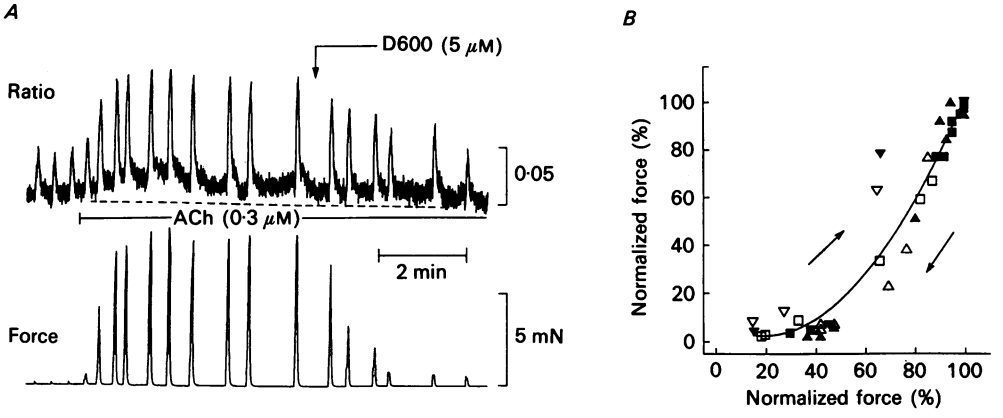

Fig. 7. Effects of D600 on ACh-stimulated responses. In panel *A*, D600 (5 μM) was added after recording a response to ACh (0·3 μM). D600 inhibited Ca^{2+} transients and contractions. Panel *B* shows the $[Ca^{2+}]_{cyt}$–force relationship during ACh treatment (closed symbols) and ACh + D600 treatment (open symbols) in three muscles (Ca^{2+} and mechanical transients plotted from peak to peak). D600 inhibited F_{400}/F_{500} and force in a parallel manner, so that rising and falling portions lie along the same curve. Values are normalized to the maximum response to ACh.

1991*a*, *b*). It has been reported that Ca^{2+} sensitivity is not affected by Ca^{2+} channel blocking agents (Karaki, Sato & Ozaki, 1991; Kobayashi, Gong, Somlyo & Somlyo, 1991). D600 (5 μM), added in the presence of ACh, reduced the amplitude of Ca^{2+} transients and contractions (Fig. 7*A*). Figure 7*B* shows the relationship between peak F_{400}/F_{500} and peak force during the rising phase of the ACh response and during the decrease in these parameters caused by D600. Under both conditions the data appeared to lie along a single Ca^{2+} sensitivity curve. Similar results were obtained with the dihydropyridine Ca^{2+} channel blocker nicardipine (1 μM). These data demonstrate the non-linear relationship between $[Ca^{2+}]_{cyt}$ and force. They also suggest that the increase in force due to cholinergic stimulation is dependent upon changes in $[Ca^{2+}]_{cyt}$ and not on a change in Ca^{2+} sensitivity.

G protein and C kinase mediated effects

Others have suggested that agonists can increase Ca^{2+} sensitivity by a G protein-dependent mechanism (Nishimura *et al.* 1988; Kitazawa *et al.* 1989). We tested whether a mechanism of this type exists in phasic gastric muscles using tissues permeabilized with α-toxin. Addition of Ca^{2+} (pCa 5·5) induced contraction which reached a peak within 10 min and then fell to a level 20–30% below the peak. Restoring low Ca^{2+} conditions caused muscles to relax to stable baseline conditions. Repeated exposures to the same concentration of Ca^{2+} elicited reproducible

contractions. The amplitude of contractions in response to elevated Ca^{2+} was slightly increased when GTP (100 μM) was added (Fig. 8*A*). Application of GTP together with ACh (10 μM) further enhanced the force of contractions in response to elevated Ca^{2+} (Fig. 8*A*). The effects of GTP and ACh on the Ca^{2+} concentration–response

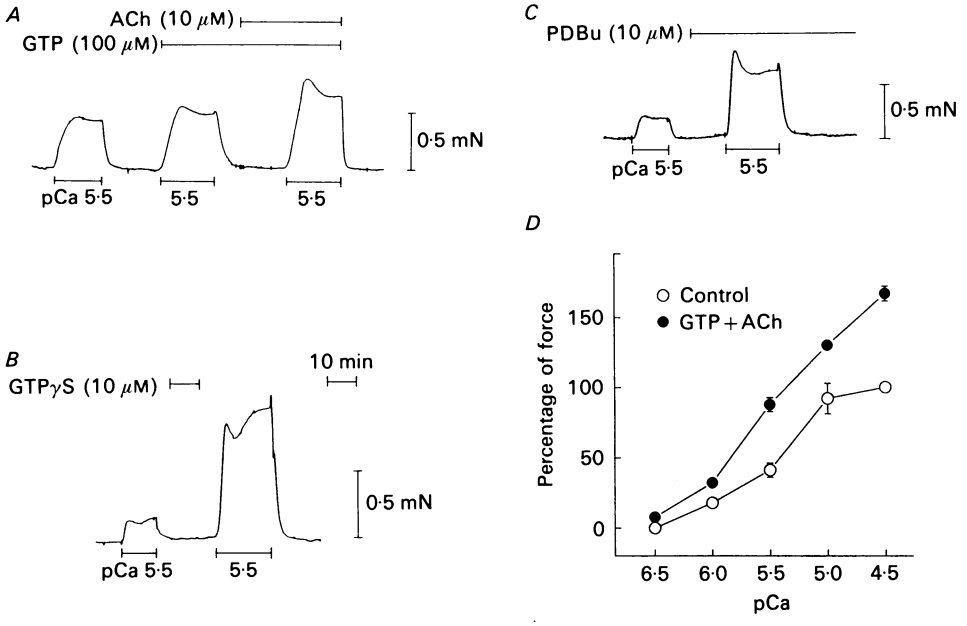

Fig. 8. Ca^{2+} sensitization in muscle permeabilized with α-toxin. Panel *A*, after observing a contraction induced by Ca^{2+} at pCa 5·5 as control, GTP (100 μM) was added and exposure to pCa 5·5 was repeated. With GTP, the Ca^{2+}-induced contraction was enhanced. ACh (10 μM) with GTP further increased the amplitude of the Ca^{2+}-induced contraction. Panel *B*, after a response to pCa 5·5, this muscle was pretreated with GTPγS for 10 min. GTPγS treatment greatly enhanced the Ca^{2+}-induced contraction. Panel *C*, contractions to pCa 5·5 shown before and in presence of PDBu (10 μM). The Ca^{2+}-induced contraction was enhanced by PDBu. Panel *D*, pCa–force curves were constructed in the absence (○) and presence of 100 μM GTP and 10 μM ACh (●). Results are expressed as means ± S.E.M. of four to nine experiments. The difference between curves is small when $[Ca^{2+}]$ was less than 1 μM.

relationship are shown in Fig. 8*D*. Note that while GTP and ACh clearly increased the amplitude of contraction at higher pCa, in the physiological range of $[Ca^{2+}]$ (i.e. less than 1 μM) there was very little enhancement in force by ACh and GTP.

If the enhancement in Ca^{2+} sensitivity is mediated by GTP, then this effect should be enhanced by the non-hydrolysable form, GTPγS. Muscles were treated with GTPγS (10 μM) for 10 min between exposures to elevated Ca^{2+} (pCa 5·5). GTPγS had no effect on its own, but it enhanced the contractile response to elevated Ca^{2+} (Fig. 8*B*). Similar increases in contraction were produced by phorbol 12,13-dibutylate (PDBu, 10 μM), an activator of protein kinase C (Fig. 8*C*).

In vascular and tracheal smooth muscles, Ca^{2+} sensitization can be mimicked by C kinase activation (Ozaki *et al.* 1990*a*, *b*). Therefore we tested whether phorbol ester could induce Ca^{2+} sensitization in intact antral muscles. In contrast to its effects in

other smooth muscles, PDBu (0·5 μM) decreased the amplitudes of Ca^{2+} transients by $78 \pm 10\%$, and contractions by $76 \pm 19\%$ in four muscles. PDBu selectively inhibited the second phase of Ca^{2+} transient and contraction. Examples of the effects of PDBu are shown in Fig. 9*A* and *B*.

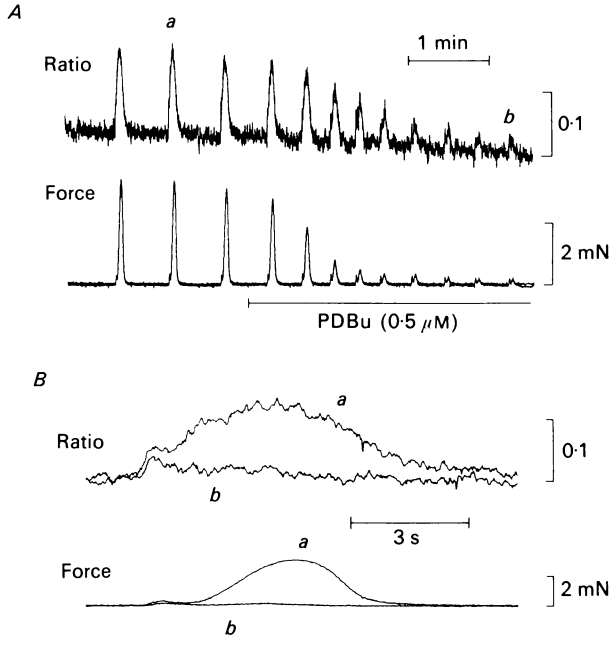

Fig. 9. Effect of PDBu on F_{400}/F_{500} and force. Panel *A*, PDBu (0·5 μM) reduced the amplitudes of Ca^{2+} transients and phasic contractions. Panel *B* shows these effects at an expanded time scale (events denoted by *a* (control) and *b* (during PDBu) are expanded). PDBu, like dihydropyridine Ca^{2+} channel blockers (Ozaki *et al.* 1991*b*), selectively reduced the second phases of Ca^{2+} transients and contractions.

DISCUSSION

Contraction of smooth muscles is thought to be initiated by myosin light chain (MLC) phosphorylation via the activation of Ca^{2+}–calmodulin-dependent MLC kinase (for reviews see, Kamm & Stull, 1985; Hartshorne, 1987). According to this model, contraction is thought to be initiated when $[Ca^{2+}]_{cyt}$ increases. When $[Ca^{2+}]_{cyt}$ decreases, MLC kinase activity decreases and myosin is dephosphorylated by phosphatases. Several investigators have shown that the relationship between $[Ca^{2+}]_{cyt}$ and force is complex and several factors regulate the *sensitivity* of the contractile apparatus to Ca^{2+} (cf. Morgan & Morgan, 1984; DeFeo & Morgan, 1985; Sato *et al.* 1988; Gerthoffer *et al.* 1989; Somlyo *et al.* 1989; Himpens *et al.* 1990; Ozaki *et al.* 1991*a*).

The method of stimulation appears to affect the mechanical output of many smooth muscles. For example, agonists such as noradrenaline in ferret portal vein (Morgan & Morgan, 1984; DeFeo & Morgan, 1985), noradrenaline, prostaglandins and endothelin in rat aorta (Sato *et al.* 1988; Sakata, Ozaki, Kwon & Karaki, 1989;

Ozaki, Ohyama, Sato & Karaki, 1990*a*), thromboxane analogues and phenylephrine in rabbit pulmonary artery (Himpens *et al.* 1990), and carbachol in canine trachea (Gerthoffer *et al.* 1989; Ozaki, Kwon, Tajimi & Karaki, 1990*b*) induce greater contraction than high K^+ for a given increase in $[Ca^{2+}]_{cyt}$. These findings have suggested that the Ca^{2+} sensitivity of the contractile apparatus can be regulated by certain agonists, and this appears to be an important mechanism in regulating the force of contractions. Experiments with muscles permeabilized with α-toxin have suggested that G proteins may mediate agonist modulation of the Ca^{2+} sensitivity (Nishimura *et al.* 1988; Kitazawa *et al.* 1989). In the present study we found that such a mechanism exists in antral circular muscles, but the approximately 20% increase in force in the presence of ACh and GTP we observed at $[Ca^{2+}]_{cyt}$ of less than 1 μM seems unlikely to explain the steep increase in the $[Ca^{2+}]_{cyt}$–force relationship above certain levels of $[Ca^{2+}]_{cyt}$. It is possible that activation of C kinase may be additive with the G protein mechanism and further enhance Ca^{2+} sensitivity in antral muscles, but experiments with phorbol esters on intact antral muscles suggest that activation of C kinase may actually be a negative feedback signal in antral muscles by decreasing the amplitude of Ca^{2+} transients, possibly through an ion channel-dependent mechanism.

Comparison of the $[Ca^{2+}]_{cyt}$–force relationships in muscles stimulated with ACh, pentagastrin or CCK with muscles stimulated with Bay K 8644 or TEA, showed steep relationships regardless of the method of stimulation. The latter techniques would be unlikely to stimulate phosphatidyl inositol turn over or to stimulate a G-protein-dependent mechanism to enhance Ca^{2+} sensitivity. Therefore, our data suggest that an agonist-dependent increase in Ca^{2+} sensitivity may not provide a significant level of regulation, at least for the three physiologically relevant agonists tested. The non-linear $[Ca^{2+}]_{cyt}$–force relationship appears to be a constitutive property of antral muscles and may facilitate the rapid development of force during phasic contractions.

Although the relationship between $[Ca^{2+}]_{cyt}$ and force is very steep, we have not quantified the absolute steepness of the function in this study. This is because antral muscles also possess a time- and Ca^{2+}-dependent mechanism that decreases the Ca^{2+} sensitivity of the contractile apparatus, and this mechanism appears to activate within the time course of phasic contractions (Ozaki *et al.* 1991*a*, *c*). As a result of the biphasic Ca^{2+} transients in antral muscles, $[Ca^{2+}]_{cyt}$ does not rise rapidly to the steep portion of the $[Ca^{2+}]_{cyt}$–force relationship. The initial phase of the Ca^{2+} transient is a modest increase in $[Ca^{2+}]_{cyt}$, and during its time course (about 1 s) the Ca^{2+} sensitivity begins to decrease (Ozaki *et al.* 1991*a*). This is evident from the fact that the second phase of Ca^{2+} transients is often similar in amplitude to the first phase, but the amplitude of the second phase of contraction is usually much less (in 70% of the muscles in the present study) than the amplitude of the first phase (Ozaki *et al.* 1991*a*, *c*). Thus, there is a drop in the Ca^{2+} sensitivity between the first and second phases of the contractile response. These observations suggest that in order to observe the basal $[Ca^{2+}]_{cyt}$–force relationship it will be necessary to (i) inhibit the mechanism responsible for Ca^{2+} desensitization, or (ii) rapidly increase $[Ca^{2+}]_{cyt}$ from basal to test levels. The latter is very difficult to accomplish in intact muscles, but might be possible in isolated cells.

At present we do not know the mechanism for the decrease in Ca^{2+} sensitivity in antral muscles. Several investigators have suggested that regulation of MLC dephosphorylation may be responsible for this effect. Yagi *et al.* (1988) first demonstrated that force induced by electrical stimulation in toad gastric muscle cells decreased before relaxation of Ca^{2+} transients, suggesting a dissociation between $[Ca^{2+}]_{cyt}$ and force. Others have demonstrated that Ca^{2+} desensitization occurs in guinea-pig ileal muscles depolarized with elevated external K^+ solutions (Himpens, Matthjis & Somlyo, 1989; Himpens & Casteels, 1990). Somlyo and co-workers have suggested that in guinea-pig ileal smooth muscle, in which the fall in force is greater than would be predicted by the decline in Ca^{2+}, dephosphorylation of MLC may be enhanced by activation of phosphatases (Somlyo *et al.* 1989). An understanding of Ca^{2+}-dependent desensitization may allow development of selective pharmacological tools to inhibit this mechanism. This appears to be necessary to fully understand the relationship between $[Ca^{2+}]_{cyt}$ and force in intact muscles.

The mechanisms responsible for the very steep $[Ca^{2+}]_{cyt}$–force relationship are of great interest. Hyperbolic $[Ca^{2+}]_{cyt}$–force relationships and MLC phosphorylation–force relationships (Kamm & Stull, 1986; Rembold & Murphy, 1988; Gerthoffer *et al.* 1989; Ozaki *et al.* 1990*b*) have been demonstrated previously in tonic smooth muscles (i.e. vascular and tracheal muscles). In the present study we also find that the $[Ca^{2+}]_{cyt}$–force relationship is very steep. It is possible that there are important differences between phasic and tonic smooth muscles in the $[Ca^{2+}]_{cyt}$–force relationship. Previously we have shown that the level of MLC phosphorylation increased from 0·1 to 0·3 (moles P_i per mole myosin light chain) during stimulation of antral contractions with ACh (Ozaki *et al.* 1991*a*). It is unclear at present whether this level of MLC phosphorylation can explain the steep $[Ca^{2+}]_{cyt}$–force relationship in antral muscles.

It is possible that thin filament regulation by actin binding proteins such as caldesmon (Sutherland & Walsh, 1986) and calponin (Takahashi, Hiwada & Kokubu, 1986; Takahashi & Nadal-Ginard, 1991) may also be involved in regulating cross-bridge cycling rates in antral muscles. Unphosphorylated forms of caldesmon or calponin inhibit actin–myosin interactions (Clark, Ngai, Sutherland, Groschel-Stewart & Walsh, 1986; Abe, Takahashi & Hiwada, 1990), and the inhibition is relieved by phosphorylation by some Ca^{2+}-dependent protein kinases, such as calmodulin-dependent protein kinase II and C kinase (Adam, Haeberle & Hathaway, 1989; Sutherland & Walsh, 1989; Winder & Walsh, 1990). This may increase cross-bridge cycling in parallel with MLC phosphorylation, thus producing 'co-operativity' (Somlyo, Goldman, Fujimori, Bond, Trentham & Somlyo, 1988), such that modest levels of MLC phosphorylation can lead to high rates of cross-bridge cycling in phasic muscles. Haeberle and co-workers (Haeberle, Hathaway & Smith, 1991) have shown that caldesmon content in 'phasic' muscles (such as ileum, taenia coli and uterus) are higher than levels of this protein in 'tonic' muscles (such as aorta, carotid artery and trachea). This is consistent with an important role for thin filament regulation in phasic smooth muscles (Gerthoffer, Murphey, Mangini, Boman & Lattanzio, 1991).

Other differences between tonic and phasic muscles also appear to exist. When vascular and tracheal smooth muscles are stimulated by agonists such as noradrenaline and carbachol, Ca^{2+} channel blockers reduce $[Ca^{2+}]_{cyt}$ to basal levels

but force remains well above the resting level (Sato *et al.* 1988; Ozaki *et al.* 1990*a*, *b*; Karaki *et al.* 1991). Thus, in these muscles agonists can maintain contraction without maintaining the stimulated level of $[Ca^{2+}]_{cyt}$. This suggests that an increase in Ca^{2+} sensitivity can be achieved at low levels of $[Ca^{2+}]_{cyt}$. In antral muscles, the Ca^{2+} channel blockers, D600 and nicardipine, inhibited Ca^{2+} transients and force in a parallel manner in the presence of ACh. In vascular and tracheal muscles, stimulation with phorbol esters induces sustained contraction at higher concentrations and greatly potentiates high-K^+-induced contraction with no change in $[Ca^{2+}]_{cyt}$ at lower concentrations (Ozaki *et al.* 1990*a*, *b*). The effect of stimulation by phorbol esters in intact antral muscles was not an increase in Ca^{2+} sensitivity but a decrease in slow wave amplitude, Ca^{2+} transients and phasic contractions. These observations suggest that the predominant effect of C kinase activation in this muscle may be to regulate ion channels, the net effect being to decrease Ca^{2+} influx.

In summary, there is a steep $[Ca^{2+}]_{cyt}$–force relationship in antral muscles regardless of the method of stimulation. Although a biochemical mechanism is present that sensitizes the contractile apparatus to Ca^{2+}, it does not appear to play a significant role in agonist-induced contractions. The steep $[Ca^{2+}]_{cyt}$–force relationship facilitates the rapid development of force during phasic contractions.

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