Journal of Physiology (1989), **413**, *pp.* 489–503
With 10 text-figures
Printed in Great Britain

DESENSITIZATION TO CYTOPLASMIC Ca^{2+} AND Ca^{2+} SENSITIVITIES OF GUINEA-PIG ILEUM AND RABBIT PULMONARY ARTERY SMOOTH MUSCLE

BY B. HIMPENS, G. MATTHIJS AND A. P. SOMLYO*

From the Pennsylvania Muscle Institute, University of Pennsylvania School of Medicine, B42 Anatomy–Chemistry Building, Philadelphia, PA 19104-6083, USA

(*Received 8 August 1988*)

SUMMARY

1. The free cytoplasmic Ca^{2+} concentration ($[Ca^{2+}]_i$) was measured in the tonic rabbit pulmonary artery and the phasic ileum smooth muscle.

2. Force development and $[Ca^{2+}]_i$ were determined during either cumulative or non-cumulative additions of $[Ca^{2+}]_o$ to smooth muscles depolarized with 140 mM-K^+ solutions.

3. The level to which $[Ca^{2+}]_i$ declined in Ca^{2+}-free, 140 mM-K^+ solutions was significantly lower in the ileum (40 ± 4 nM) than in pulmonary artery (77 ± 5 nM) smooth muscle.

4. The level of $[Ca^{2+}]_i$ reached during non-cumulative superfusion with 10 μM and 1 mM $[Ca^{2+}]_o$ was higher in the pulmonary artery than in the ileum.

5. The force level reached for a given $[Ca^{2+}]_i$ was also higher in the pulmonary artery than in the ileum.

6. During maintained depolarization there was a marked decrease in the sensitivity of ileum smooth muscle tension to $[Ca^{2+}]_i$.

7. We conclude that significant differences exist in the Ca^{2+} sensitivity of the regulatory/contractile apparatus among different smooth muscles; the lower sensitivity of depolarized ileum than pulmonary artery to $[Ca^{2+}]_o$ is due to both differences in Ca^{2+} metabolism and in the Ca^{2+} sensitivity of the regulatory contractile system. We suggest that these two mechanisms also contribute to the decline in force during a phasic K^+ contracture, and that desensitization to $[Ca^{2+}]_i$ contributes to the decline of the K^+ contracture in the ileum.

INTRODUCTION

The two broad classes of smooth muscle, phasic and tonic, can be distinguished on the basis of their membrane electrical properties and their responses to maintained depolarization (Somlyo, Vinall & Somlyo, 1969). The excitatory electrical response of tonic smooth muscles is a graded depolarization without action potentials, and their contractile response is maintained or may even increase during a prolonged depolarization. Phasic smooth muscles generate action potentials and respond to

* Address reprint requests to Dr A. P. Somlyo, Department of Physiology, School of Medicine, University of Virginia, Charlottesville, VA 22908, USA.

depolarization with K^+-rich solution with an initial phasic contraction that declines to a variable, maintained level.

When depolarized with K^+-rich, Ca^{2+}-free solutions, tonic smooth muscles are contracted by lower concentrations of externally added Ca^{2+} than phasic smooth muscles (Somlyo *et al.* 1969). The response of phasic smooth muscle to externally added Ca^{2+} concentrations declines during prolonged incubation in K^+-rich, Ca^{2+}-free solution. The above observations could be accounted for by a lower permeability of depolarized phasic smooth muscles to externally added Ca^{2+}, possibly due to a time-dependent inactivation of the voltage-gated Ca^{2+} influx, but also to differences between the respective Ca^{2+} sensitivities of the regulatory/contractile proteins of tonic and phasic smooth muscles. At the time of these observations, methods for measuring the free cytoplasmic Ca^{2+} concentration ($[Ca^{2+}]_i$) and directly testing these two hypotheses were not available. The availability of the reliable fluorescent Ca^{2+} indicator, Fura-2 (Grynkiewicz, Poenie & Tsien, 1985) and the feasibility of introducing it into strips of smooth muscle in which cytoplasmic Ca^{2+} and force development can be measured simultaneously (Himpens & Somlyo, 1988), have now permitted us to re-examine some of the mechanisms responsible for the differences between tonic and phasic smooth muscles. The purpose of the present study was to determine whether the transient response of a phasic smooth muscle to depolarization with K^+-rich solution and its declining sensitivity to external added Ca^{2+} concentrations during depolarization, compared with a tonic smooth muscle, are due to differences in the levels of cytoplasmic Ca^{2+} or to differences in the Ca^{2+} sensitivity of the regulatory/contractile proteins. In keeping with the often complex explanations of simple observations in biology, we found that both $[Ca^{2+}]_i$ and Ca^{2+} sensitivity were different in, respectively, the phasic smooth muscle of the guinea-pig ileum and the tonic smooth muscle of the rabbit pulmonary artery. We also report here our first observations on the Ca^{2+}- and time-dependent desensitization of smooth muscle to cytoplasmic Ca^{2+}. A preliminary report of some of these findings has been presented to the American Biophysical Society (Himpens, Matthijs & Somlyo, 1988*a*).

METHODS

Male and female New Zealand White rabbits weighing *ca* 3 kg were instantaneously killed by cervical dislocation by a humane procedure, as approved by the Institutional Animal Care and Use Committee. The heart and lungs were removed and transferred to a Krebs solution. The left and the right pulmonary arteries were excised and cleaned of their periarterial connective tissue and the endothelium was gently scraped away. Small strips of approximately 5×10 mm were cut along the direction of the circular muscle layer of the left and right branch of the pulmonary artery. Male guinea-pigs (*ca* 300 g body wt) were instantaneously killed by a blow on the head by a humane procedure, as approved by the Institutional Animal Care and Use Committee. The ileum was removed and slid onto a glass rod of 5 mm diameter. The longitudinal layer was dissected free from the circular muscle, and thin sheets of about 5 mm wide and 30 mm long were prepared (Himpens & Casteels, 1987; Himpens & Somlyo, 1988).

The protocols for loading with Fura-2AM and for making the fluorescence measurements have been described by Himpens & Somlyo (1988). Briefly, the procedure consists of loading the strip in a cuvette containing 1 ml of HEPES-buffered Krebs solution containing 1·2 mM-Ca^{2+} and 2 μM-Fura-2AM dissolved in dimethyl sulphoxide (final concentration, 0·5%) pre-mixed with the high molecular weight surfactant polyol, Pluronic F127 (final concentration of 0·01%) as a dispersing agent. Strips were at room temperature with the cuvettes rotating at 30 rev/min, overnight for the pulmonary artery and for 4 h for the longitudinal ileum. We have previously measured the

concentration of cytoplasmic free Fura-2 achieved by our loading procedures in the ileum, and established that this had no effect on the rate or the amplitude of force development (Himpens & Somlyo, 1988). The strips, transferred from the loading solution to a fresh Krebs solution, were then washed for $1\frac{1}{2}$ h. The method of mounting and a description of the apparatus have been published (Himpens & Somlyo, 1988). The instrumentation consisted essentially of an illuminating light source with a rotating wheel containing the interference filters of 340 and 380 nm connected to it. The UV light of the two wavelengths was then passed through one end of a bifurcated light pipe to the muscle sheet, which was mounted in the tissue chamber and was attached to a force transducer. The fluorescence emitted by the Fura-2 in the cell was transferred to a photomultiplier through the other end of the light pipe and an interference filter of 510 nm. The two signals were then separated and digitized for further data analysis.

Cytosolic Ca^{2+} concentrations were calculated using an internal calibration and using the formula described by Grynkiewicz *et al.* (1985). The minimum fluorescence was obtained by superfusing the muscle with a 140 mM-K^+, Ca^{2+}-free solution containing 2 mM-EGTA at pH 8·6 to optimize the ionomycin effect. Five minutes after superfusion with this solution, 50 μM-ionomycin was added. After determining the R_{min}, the tissue was superfused with an excess of calcium (10 mM-Ca^{2+} solution at pH 8·6) which gave the maximal signal ratio, R_{max}. After reaching a maximum, the fluorescence (both the 340 and 380 signal) frequently declined, due to leakage of Fura-2 and its subsequent removal from the superfused strip, with the ratio remaining unchanged, but becoming noisier. The autofluorescence was determined after each experiment at the two excitation wavelengths, in order to subtract the values from the total fluorescence and obtain the net Ca^{2+}-sensitive Fura-2 fluorescence. This was done by superfusing the strip with a 20 mM-Mn^{2+} Krebs solution after the R_{min} and R_{max} ratios were determined. The remaining fluorescence represents the proportion which had to be substracted from each signal before making the ratio signal. The above calibration was used in each of the experiments in which absolute values of Ca^{2+} are indicated in the figures. Due to some uncertainties in the value of the K_d (Himpens, Matthijs, Somlyo, Butler & Somlyo, 1988*b*) the absolute ratio is also shown in the figures besides the computed $[Ca^{2+}]_i$. To determine the contribution to the 'autofluorescence' of Fura-2AM and its metabolites bound to organelles, we measured the autofluorescence prior to and following overnight loading with Fura-2AM (2 μM) at room temperature, in pulmonary artery (12 h, three strips) and ileum (4 h, four strips). The difference between the 'autofluorescence' signal contributed by Fura-2 loading and the pre-loading autofluorescence (expressed as a percentage of autofluorescence contribution to the resting signal) was 6–8 % at 340 nm and 12–13 % at 380 nm. The somewhat greater increase of the 380 nm signal due to loading with Fura-2AM is consistent with the binding of the Ca^{2+}-insensitive unhydrolysed or partially hydrolysed ester (Cobbold & Rink, 1987; Scanlon, Williams & Fay, 1987), and would have no significant effect on the Ca^{2+}-sensitive ratio signal. The contribution of bound dye was not significantly different between ileum and pulmonary artery smooth muscle and, furthermore, the 'autofluorescence' measured after loading is subtracted before obtaining the ratio signal. Therefore, differences in the amounts of organelle-bound, unhydrolysed or partially hydrolysed ester in the two smooth muscles could not account for the differences in resting $[Ca^{2+}]_i$ (see Results).

All values are means ± S.E.M., and n is the number of observations. Comparisons were made using Student's t test.

The standard physiological solution was a HEPES-buffered modified Krebs solution at pH 7·3 containing (mM): Na^+, 135·5; K^+, 5·9; Ca^{2+}, 1·2; Mg^{2+}, 1·2; Cl^-, 143·8; HEPES, 11·6 and glucose, 11·6. Solutions with increased $[K^+]_o$ were obtained by replacing Na^+ by an equivalent amount of K^+. The different intermediate Ca^{2+} concentrations were obtained by using Ca-EGTA buffered solutions of which the final $[Ca^{2+}]$ was determined with the Ca^{2+} electrode (Somlyo, Bond, Somlyo & Scarpa, 1985). Fura-2AM was obtained from Molecular Probes, Pluronic F127 from BASF Wyandotte Corporation and ryanodine from Calbiochem. Okadaic acid used in this study was a generous gift from Dr Y. Tsukitani, Fujisawa Pharmaceutical Company, Tokyo.

RESULTS

Tonic smooth muscle

The resting fluorescence level remained constant during superfusion with a Krebs solution containing 5·9 mM-K^+ and 1 mM-Ca^{2+}: the mean $[Ca^{2+}]_i$ was 114 ± 8 nM

($n = 19$). The $[Ca^{2+}]_i$ increased during stimulation with the 140 mM-K^+, 1 mM-Ca^{2+} solution to a peak of 325 ± 40 nM, followed by a plateau of 266 ± 24 nM over 20 min ($n = 18$). The $[Ca^{2+}]_i$ declined to 77 ± 5 nM ($n = 18$) during the subsequent superfusion with the 140 mM-K^+, Ca^{2+}-free solution containing 2 mM-EGTA. The resting tension in normal Krebs was $14 \pm 3\%$ ($n = 18$), as normalized to the level obtained in Ca^{2+}-free solution as 0% and the value obtained during the maximum of contraction

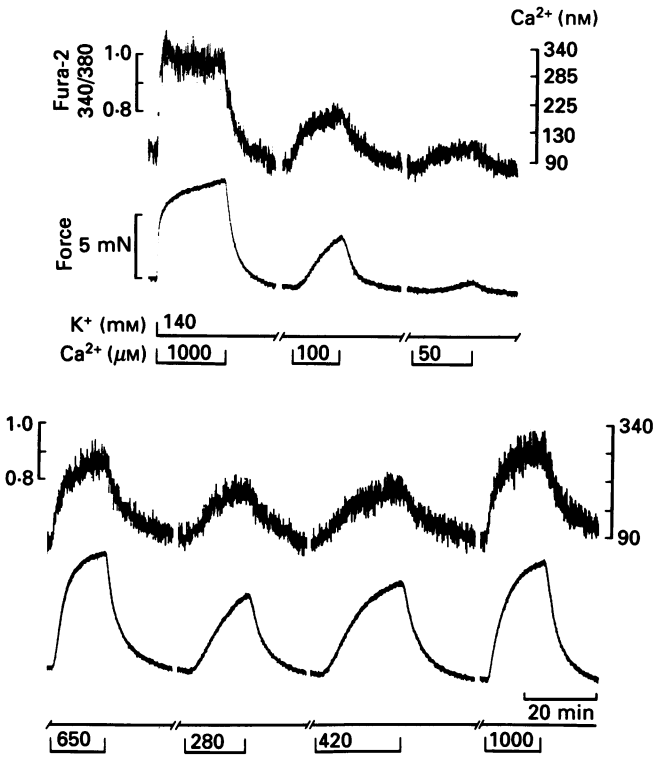

Fig. 1. Representative $[Ca^{2+}]_i$ (upper) and force traces (lower) of a strip of pulmonary artery. After a reference control stimulation with 140 mM-K^+ solution containing 1 mM-Ca^{2+}, the perfusion is changed to a Ca^{2+}-free, high-K^+ solution containing 2 mM-EGTA. After 30 min in the Ca^{2+}-free, depolarizing solution $[Ca^{2+}]_o$ is added at various concentrations. The lower panel is a continuation of the traces in the upper panel.

induced by 140 mM-K^+, 1 mM-Ca^{2+} solution as 100%. Maximum force in pulmonary artery was reached only after prolonged (20–30 min) exposure to 140 mM-K^+, 1 mM-Ca^{2+} and it was only $68 \pm 3\%$ of its maximum when the $[Ca^{2+}]_i$ peaked. In the following experiments, $[Ca^{2+}]_o$ was re-added to the perfusate either cumulatively or non-cumulatively during the continued presence of the 140 mM-K^+ solution.

Response to non-cumulative addition of $[Ca^{2+}]_i$

After obtaining the steady-state $[Ca^{2+}]_i$ value in the Ca^{2+}-free, K^+-rich solution, the depolarized strip was superfused for a defined period of time (20 min) in a random way with different $[Ca^{2+}]_o$ levels varying between 10 μM and 10 mM $[Ca^{2+}]_o$. The fixed duration of the superfusion with the different external solutions was necessary,

because force increased slowly and continuously in the pulmonary artery. Figure 1 represents an example of these measurements. After 20 min incubation with the respective $[Ca^{2+}]_o$, the maximum fluorescence and peak force were measured ($n = 5$). The $[Ca^{2+}]_i$ rose faster than force did following addition of low $[Ca^{2+}]_o$, while with higher increments of $[Ca^{2+}]_o$ the two parameters increased simultaneously. The relationship between the peak force, defined as the percentage of the maximum force

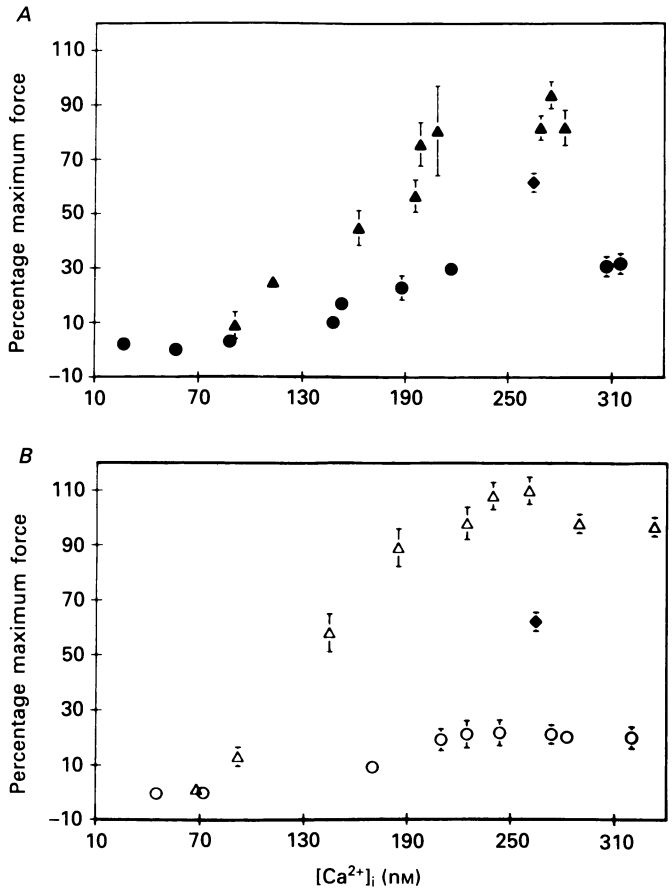

Fig. 2. Relationship between the peak force and the intracellular calcium concentration $[Ca^{2+}]_i$, reached during non-cumulative (filled symbols) and cumulative (open symbols) additions of varying concentrations $[Ca^{2+}]_o$ (shown in Figs 3 and 4) in the pulmonary artery (triangles) and the ileum (circles). The filled diamond represents the force plateau of the ileum reached at 15 min during a control K^+ contraction response to 140 mM-K^+ and 1 mM-Ca^{2+}.

output during the preceding control stimulation with the 140 mM-K^+, 1 mM-Ca^{2+} solution and the $[Ca^{2+}]_i$ followed a sigmoidal curve with an ED_{50} of about 160 nM (Fig. 2*A*). The relationship between $[Ca^{2+}]_o$ and $[Ca^{2+}]_i$ was such that a $[Ca^{2+}]_i$ of 160 nM was reached at a $[Ca^{2+}]_o$ of approximately 120 μM, and about 75% of the increase in $[Ca^{2+}]_i$ occurred at approximately 350 μM $[Ca^{2+}]_o$ (Fig. 3*A*). Maximum tension was restored upon increasing the $[Ca^{2+}]_o$ (Fig. 4*A*).

Response to cumulative increases in $[Ca^{2+}]_o$

When Ca^{2+} was added cumulatively to the depolarized smooth muscle strip of the pulmonary artery, each increase in $[Ca^{2+}]_o$ resulted in a stepwise increase in force and $[Ca^{2+}]_i$ as illustrated in Fig. 5. After superfusing for 30 min with the Ca^{2+}-free, K^+-

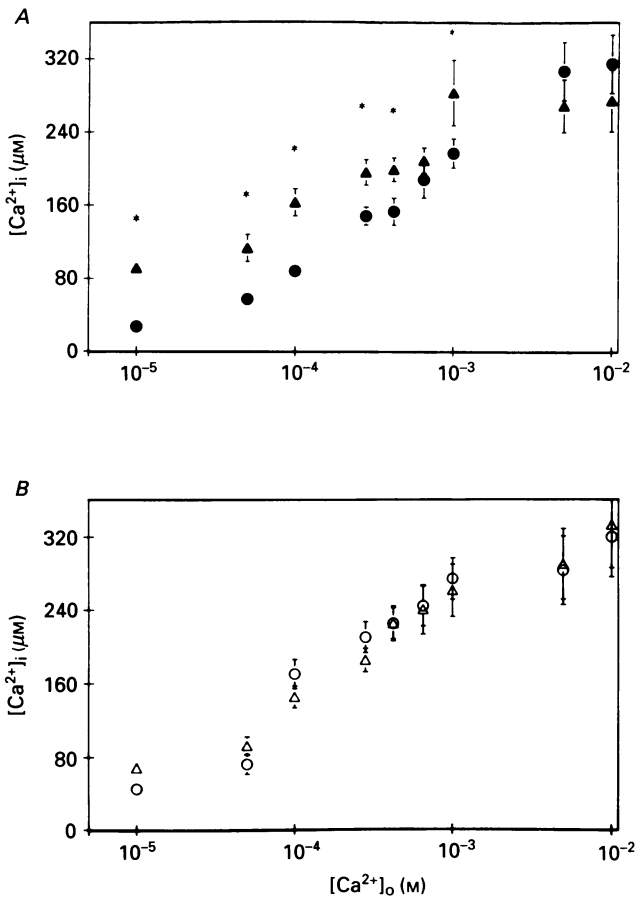

Fig 3. Relationship between the intracellular and extracellular calcium concentration during non-cumulative (filled symbols) and cumulative (open symbols) additions of $[Ca^{2+}]_o$ in the pulmonary artery (triangles) and the ileum (circles). The asterisk during non-cumulative additions (*A*) indicates significant ($P < 0{\cdot}05$) differences between the two muscles. The scale of the abscissa is logarithmic.

rich solution, $[Ca^{2+}]_i$ was gradually increased each 15 min. The ED_{50} for force, 140 nM $[Ca^{2+}]_i$ (Fig. 2*B*), was reached at an $[Ca^{2+}]_o$ of about 100 μM (Fig. 3*B*). The relationship between the $[Ca^{2+}]_o$ and the peak force (Fig. 4*B*) demonstrates the recovery of the tension upon increasing the $[Ca^{2+}]_o$.

Thus the $[Ca^{2+}]_i$ *vs.* force, the $[Ca^{2+}]_o$ *vs.* $[Ca^{2+}]_i$ and the force *vs.* $[Ca^{2+}]_o$ relationships are rather similar during cumulative and non-cumulative additions of $[Ca^{2+}]_o$ to depolarized pulmonary artery.

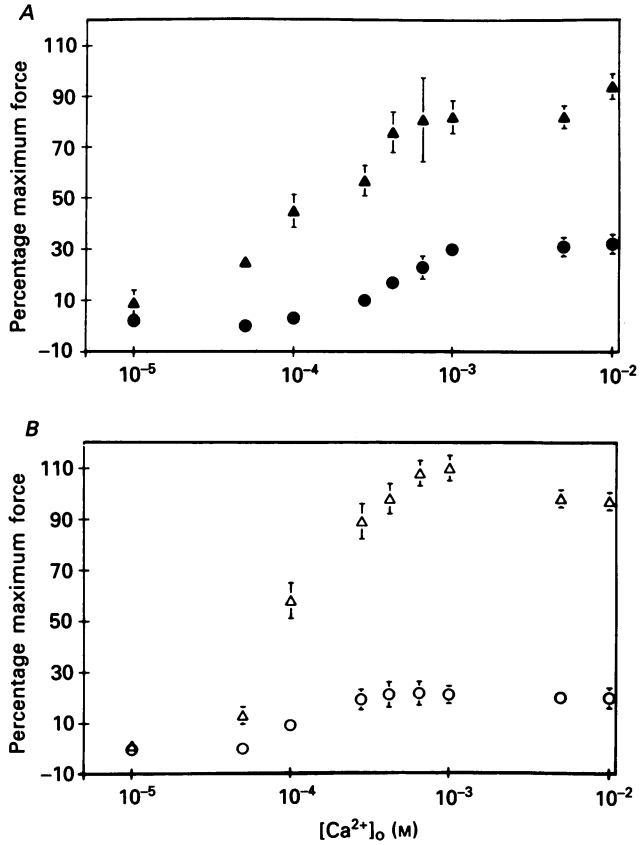

Fig. 4 Relationship between the extracellular calcium concentration and the peak force during non-cumulative (filled symbols) and cumulative (open symbols) additions of $[Ca^{2+}]_o$ in the pulmonary artery (triangles) and the ileum (circles). The scale of the abscissa is logarithmic.

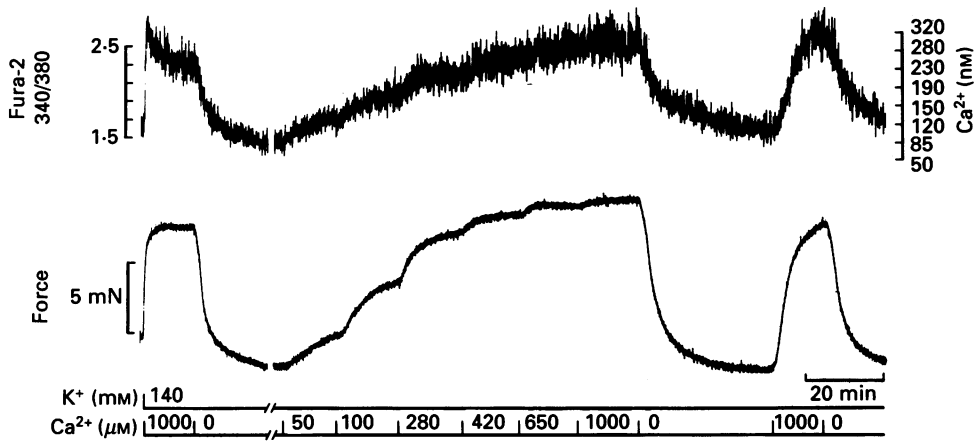

Fig. 5. $[Ca^{2+}]_i$ (upper trace) and force (lower trace) over 15 min intervals during the cumulative addition of $[Ca^{2+}]_o$ to the pulmonary artery.

Phasic smooth muscle

The mean $[Ca^{2+}]_i$ in smooth muscle of the ileum was 82 ± 5 nM ($n = 17$) at rest. During the contraction induced by the 140 mM-K^+, 1 mM-Ca^{2+} solution, the $[Ca^{2+}]_i$ values for the peak and the plateau were respectively 555 ± 45 nM and 265 ± 21 nM ($n = 31$). Resting tension, normalized to the level obtained by incubation with a Ca^{2+}-free, 140 mM-K^+ solution as 0% and the value obtained during the peak of a high K^+ contraction as 100%, was $22 \pm 2{\cdot}5$% ($n = 32$). During superfusion with the 140 mM-K^+, Ca^{2+}-free solution containing 2 mM-EGTA, the muscle relaxed and the $[Ca^{2+}]_i$ declined to 40 ± 4 nM ($n = 29$).

In the following experiments $[Ca^{2+}]_o$ was re-added either cumulatively or non-cumulatively to depolarized preparations.

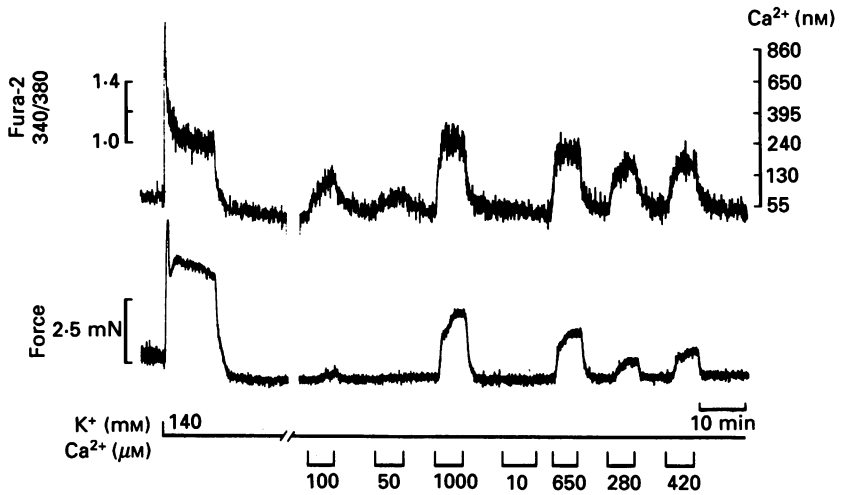

Fig. 6. Force (lower trace) and $[Ca^{2+}]_i$ (upper trace) in depolarized guinea-pig ileum during non-cumulative additions of different $[Ca^{2+}]_o$.

Response to non-cumulative increases $[Ca^{2+}]_o$

Figure 6 demonstrates the effect of different $[Ca^{2+}]_o$ levels on a fully depolarized strip of the guinea-pig longitudinal layer of the ileum ($n = 9$). Addition of low $[Ca^{2+}]_o$ (below 100 μM-Ca^{2+}), failed to cause force development, in spite of a detectable increase in the $[Ca^{2+}]_i$. Force was depressed and the maximum tension was only 33% of the peak force. The maximum increase of $[Ca^{2+}]_i$, about 320 nM (Fig. 2*A*), was reached by superfusion with 10 mM $[Ca^{2+}]_o$ (Fig. 4*A*). The relationship between the $[Ca^{2+}]_i$ and the $[Ca^{2+}]_o$ (Fig. 3*A*) shows that at lower concentrations of external Ca^{2+}, the $[Ca^{2+}]_i$ is higher in the pulmonary artery than in the ileum. Pre-incubation with ryanodine (10^{-5} M) increased the tension (46 ± 9%) and $[Ca^{2+}]_i$ (259 ± 39 nM) ($n = 5$), but these values were not significantly different from the data obtained without ryanodine pre-incubation.

Cumulative increases of $[Ca^{2+}]_o$

When $[Ca^{2+}]_o$ was added cumulatively (Fig. 7), the depression of the force/$[Ca^{2+}]_i$ ratio was even more pronounced than during non-cumulative additions. The maximum force with 10 mM $[Ca^{2+}]_o$ was only 20%, while the $[Ca^{2+}]_i$ was about 300 nM (Fig. 2*B*). This desensitization could not be inhibited by blocking the neuronal

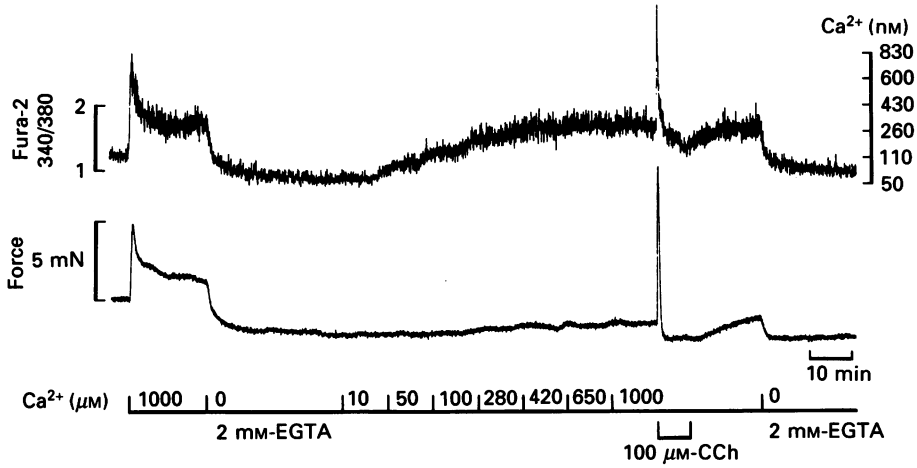

Fig. 7. $[Ca^{2+}]_i$ (upper trace) and force (lower trace) during the cumulative addition of $[Ca^{2+}]_o$ to the depolarized ileum. During the plateau response to 1 mM $[Ca^{2+}]_o$, 10 μM-carbachol (CCh) was added evoking rapid $[Ca^{2+}]_i$ and tension spikes. The large force response to the rapid pulse of increased $[Ca^{2+}]_i$ indicates that the preceding low level of force represents desensitization rather than damage of the contractile apparatus.

release by atropine (10^{-5} M) or TTX (3×10^{-6} M). The use of the inhibitor of the myosin light-chain phosphatase, okadaic acid (10^{-5} M) (Bialojan, Rüegg & Takai, 1988), did not increase the force, but instead a rather small decline could be observed. In order to exclude that the increased fluorescence was due to fluctuations of the Mg^{2+} concentration due to chelation by the EGTA, the Ca^{2+}-free solution containing 2 mM-EGTA and 2 mM-Mg^{2+} was also replaced by one containing 2 mM-EGTA and 5 mM-Mg^{2+} for 20 min. No change in the tension or the fluorescence could be observed ($n = 3$). We excluded the possibility that the depression of the force/$[Ca^{2+}]_i$ ratio was the result of an inhibition of the contractile apparatus due to swelling caused by the low-Ca^{2+}, low-Na^+, K^+-rich solutions used: the addition of 50 mM-sucrose to reverse any such swelling (Jones, Somlyo & Somlyo, 1973) to the 1 mM $[Ca^{2+}]_o$ solution after prolonged incubation (1 h) in the Ca^{2+}-free, K^+-rich solution (Fig. 8) resulted in a slight depression of the tension ($n = 5$). Furthermore, the original observations of the depressant effect of incubation in Ca^{2+}-free depolarizing solutions on phasic smooth muscle were made in the presence of depolarizing solutions containing K_2SO_4, rather than KCl, which would not have caused swelling (Somlyo & Somlyo, 1969).

Since the 140 mM-K^+ solutions were prepared by substituting K^+ for Na^+, we also wished to determine whether our observations could be influenced by the removal of Na_o^+ through its effect on, for example, the Na^+–Ca^{2+} exchanger or pH_i (Aickin,

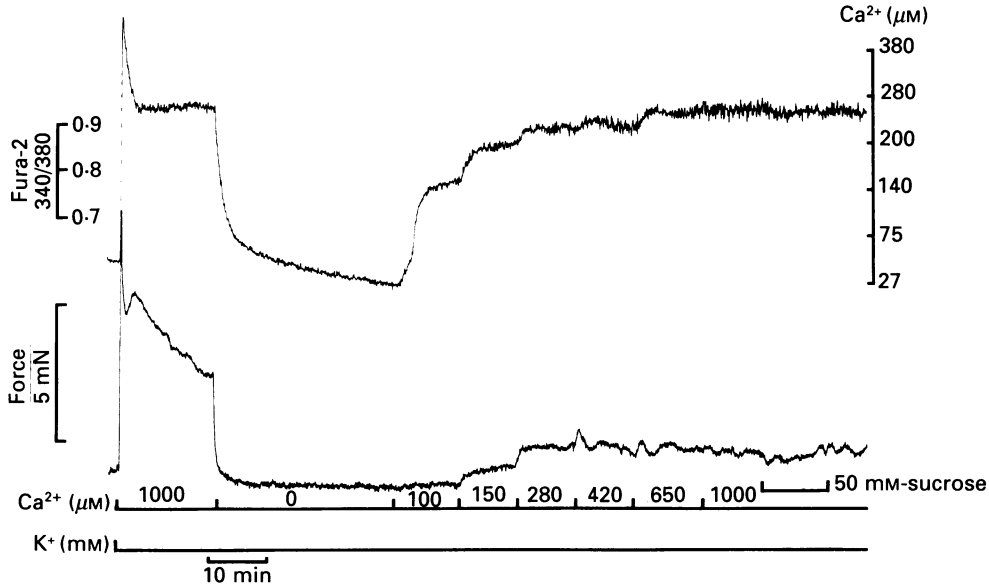

Fig. 8. The lack of effect of increasing the osmolarity, with 50 mM-sucrose, on $[Ca^{2+}]_i$ (upper trace) and force (lower trace) during the cumulative addition of $[Ca^{2+}]_o$ in a depolarized muscle strip of ileum.

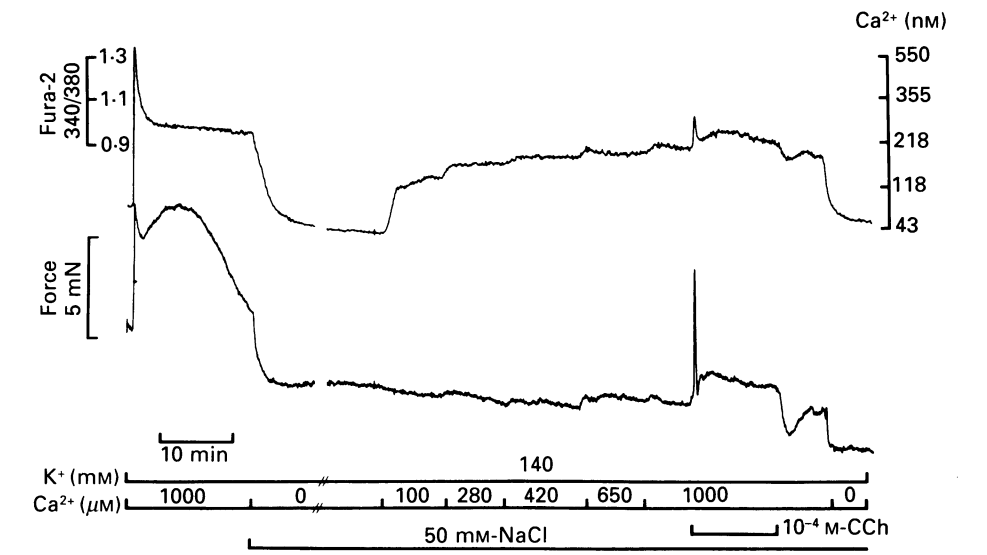

Fig. 9. The lack of effect of added Na^+ on desensitization to Ca^{2+} during cumulative additions of $[Ca^{2+}]_o$. Note that in spite of the presence of $[Na^+]_o$ and the return of $[Ca^{2+}]_i$ to the 'tonic' level upon addition of $[Ca^{2+}]_o$, the contractile response is markedly depressed.

1986). However, the presence of 50 mM-Na_o had no effect on the markedly reduced force response to additions of Ca_o^{2+} (Fig. 9). Carbachol (10^{-4} M) added to the depolarized preparation at the height of the maximal $[Ca^{2+}]_o$ induced contraction evoked a large Ca^{2+} 'spike' and additional force development greater than the peak of the control K^+ contracture (Fig. 7), indicating that the contractile apparatus was

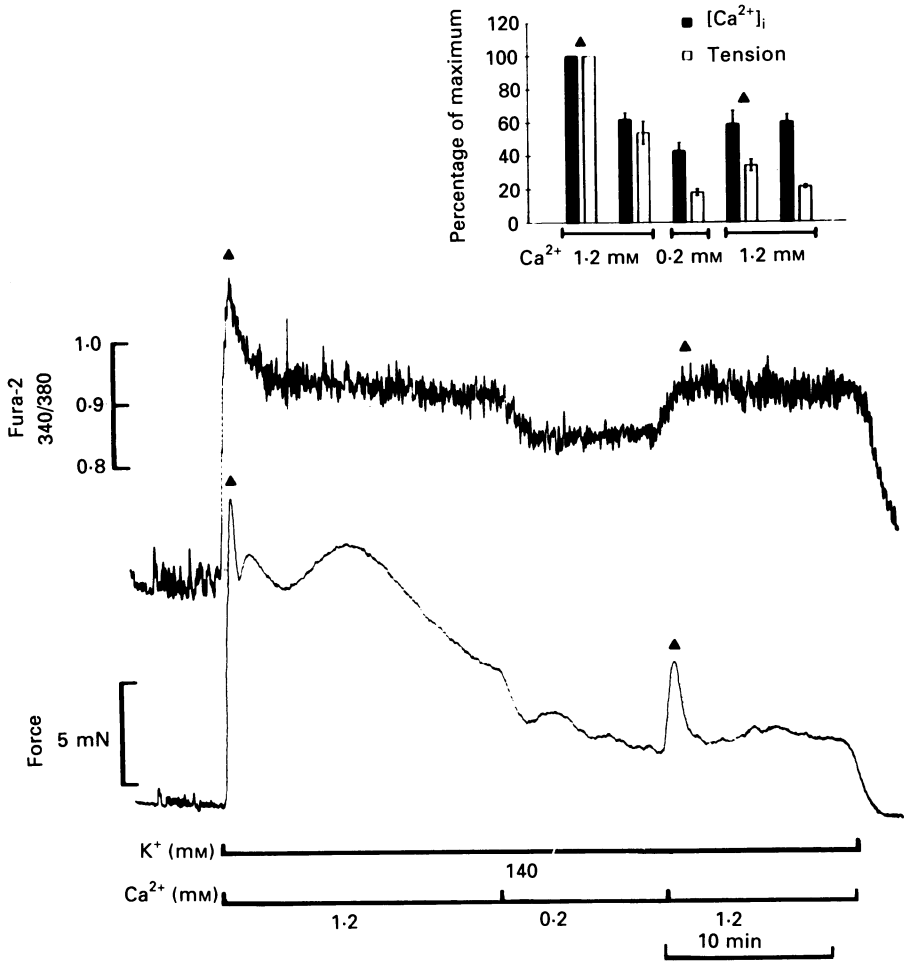

Fig. 10. The effect of a reduction of $[Ca^{2+}]_o$ from 1·2 to 0·2 mM, during exposure to a solution containing 140 mM-K^+, on $[Ca^{2+}]_i$ (upper record) and force development (lower record) in the ileum. After superfusing the tissue with a solution containing 140 mM-K^+ and 1·2 mM-Ca^{2+} for 15 min, the $[Ca^{2+}]_o$ was reduced to 0·2 mM-Ca^{2+} for another 15 min, and then increased again to 1·2 mM. The bar graph (inset) summarizes the results of six similar experiments. The Fura-2 ratio and the tension are normalized against the values obtained during the initial peak. The triangle symbolizes the initial peak values. Note that after an initial force transient accompanying the increase in $[Ca^{2+}]_o$ and $[Ca^{2+}]_i$, tension returns to the 'desensitized' level.

not damaged. The $t_{\frac{1}{2}}$ of the contraction was 6 ± 1 s and the $t_{\frac{1}{2}}$ of relaxation was 21 ± 2 s ($P < 0{\cdot}01$). Interestingly, after the initial 'spike' response, fluorescence and force were depressed during the continued presence of carbachol. Both signals rose after washing out the agonist.

We also investigated the effect of decreasing the $[Ca^{2+}]_o$ from 1·2 to 0·2 mM-Ca^{2+} during a prolonged K^+ contracture (Fig. 10). During the initial stimulation with the 1·2 mM-Ca^{2+}, 140 mM-K^+-containing solution, the $[Ca^{2+}]_i$ declined from 521 ± 46 to 286 ± 25 nM over 15 min, while tension declined to 54 ± 7 % of its peak value ($n = 6$).

At this time, $[Ca^{2+}]_o$ was lowered from 1·2 to 0·2 mM. The $[Ca^{2+}]_i$ and tension declined respectively to 201 ± 18 nM and 18 ± 2 % of the peak, and were significantly different from the values in 1·2 mM $[Ca^{2+}]_o$ ($P < 0.05$) and 15 min superfusion in the depolarizing solution. Finally the superfusion was changed again to the 1·2 mM $[Ca^{2+}]_o$. Initially there was always a transient peak in force (34 ± 3.5 %; $P < 0.05$) which then declined to a level, not statistically different from the maintained level obtained with 0·2 mM $[Ca^{2+}]_o$ (21 ± 1 %). The $[Ca^{2+}]_i$ rose, however, to reach a level of 278 ± 36 mM during the transient peak and had a value of 274 ± 21 nM after 15 min superfusion with 1·2 mM $[Ca^{2+}]_o$. This value was significantly different from the 0·2 mM $[Ca^{2+}]_o$ value, but not from the data obtained during the initial plateau value during superfusion with 1·2 mM $[Ca^{2+}]_o$.

DISCUSSION

The time course of the cytoplasmic Ca^{2+} transient and the Ca^{2+} sensitivity of the contraction in depolarized ileum and pulmonary artery (present study; Himpens *et al.* 1988*b*) shed some light on the mechanisms underlying the phasic and tonic characteristics of smooth muscles. The major differences between the tonic pulmonary artery and the phasic ileum, observed in this study, are summarized in Table 1.

The markedly lower $[Ca^{2+}]_i$ in the ileum than in the main pulmonary artery during depolarization in Ca^{2+}-free solutions, is the first direct indication of such differences in cytoplasmic Ca^{2+} control among different smooth muscles, and may contribute to the more rapid loss of the contractile response of phasic smooth muscle in Ca^{2+}-free solutions. In earlier electron microscopic studies this difference between phasic and tonic smooth muscle was tentatively ascribed to the larger volume of sarcoplasmic reticulum in the tonic muscle studied (Devine, Somlyo & Somlyo, 1972). The possibility that the sarcoplasmic reticulum in phasic smooth muscles was more readily depleted of its Ca^{2+} content or that the excitation–contraction coupling mechanism is more readily uncoupled in Ca^{2+}-free solution has also been considered. The lower $[Ca^{2+}]_i$ in phasic ileum (present study) supports the notion of a contribution by the latter two mechanisms, as low $[Ca^{2+}]_i$ could accelerate the loss of Ca^{2+} from the sarcoplasmic reticulum and also inhibit Ca^{2+}-dependent phospholipase C activity (Sasaguri, Hirata & Kuriyama, 1985; Rapoport, 1987; Roth, 1987; Mallows & Bolton, 1987) which mediates pharmacomechanical Ca^{2+} release in smooth muscle (reviewed by Somlyo, Walker, Goldman, Trentham, Kobayashi, Kitazawa & Somlyo, 1988).

A marked desensitization of the regulatory/contractile proteins to $[Ca^{2+}]_i$ occurred in ileum depolarized in Ca^{2+}-free solutions, during increases of $[Ca^{2+}]_o$. Tension rose to only 35 % of the peak force, even in the presence of 320 nM $[Ca^{2+}]_i$ obtained at a $[Ca^{2+}]_o$ of 10 mM. In contrast, during conventional depolarization with K^+-rich (1 mM-Ca^{2+}) solution, 62 % of the peak force is reached at a $[Ca^{2+}]_i$ of 265 nM at the plateau phase of the K^+ contracture. During desensitization to Ca^{2+}, carbachol could evoke a normal maximal contraction (Fig. 7). Furthermore, the reversal of cell swelling by increased osmolarity (with sucrose) did not enhance, but rather depressed force below the 'desensitized' level, indicating a true desensitization rather than a

reversible damage of the contractile apparatus due to osmotic swelling in the K^+-rich solution (Jones *et al.* 1973). The best-known, biochemically identified, Ca^{2+}-independent mechanism of contractile regulation is through phosphorylation of myosin light-chain kinase by the cyclic AMP-dependent protein kinase (kinase A), with phosphorylation reducing the activity of the myosin light-chain kinase when

TABLE 1. Differences between tonic pulmonary artery and phasic ileum

	Pulmonary artery	Ileum
Resting $[Ca^{2+}]_i$ (nM)	114±8 (19)	82±5 (31) ($P < 0.01$)
Resting tension (% of peak tension)	14±3 (18)	22±2·5 (32) ($P < 0.01$)
$[Ca^{2+}]_i$ (nM) in 0 mM $[Ca^{2+}]_o$	77±5 (18)	40±4 (29) ($P < 0.01$)
Peak $[Ca^{2+}]_i$ (nM)	325±40 (18)	555±45 (31) ($P < 0.01$)
Tension at peak $[Ca^{2+}]_i$ (%)	68±3 (13)	100
'30 min' $[Ca^{2+}]_i$ (nM)*	266±24 (17)	265±21 (31)
'30 min' tension (%)	100	62±3·5 (32)
$[Ca^{2+}]_i$ ED_{50}		
Non-cumulative†	160 nM	> 310 nM
Cumulative†	140 nM	> 310 nM
$[Ca^{2+}]_o$ ED_{50}		
Non-cumulative†	120 μM	> 10 mM
Cumulative†	100 μM	> 10 mM

* $[Ca^{2+}]_i$ at 30 min after depolarization with 140 mM-K^+, 1 mM-Ca^{2+}.
† Cumulative and non-cumulative additions of $[Ca^{2+}]_o$.
Number of experiments is given in parentheses.

the latter is not saturated by calmodulin (Conti & Adelstein, 1981). This mechanism can inhibit smooth muscle contraction, if kinase A activity is increased while the muscle is relaxed or during a submaximal contraction (Pfitzer, Rüegg, Zimmer & Hofmann, 1985). Hence, it is possible that stimulation of adenylate cyclase by the small increases in $[Ca^{2+}]_i$ during cumulative additions of $[Ca^{2+}]_o$, my account for the desensitization to $[Ca^{2+}]_i$.

It is equally tempting to assign the mechanism of desensitization to activation of myosin light-chain phosphatase in view of the very low (near baseline) level of myosin light-chain phosphorylation found during the tonic phase of K^+ contractures of ileum smooth muscle (Himpens *et al.* 1988*b*). The decline in tension accounting for the phasic character of K^+ contractions in the ileum may also be, at least in part, due to desensitization, as the levels of $[Ca^{2+}]_i$ during maintained depolarization were not lower in the ileum than in the pulmonary artery, in which a tonic maintenance, or even rise, of tension occurs during the latter phase of the K^+ contracture. The presence of a myosin light-chain phosphatase which is activated during incubation in high-K^+, Ca^{2+} solutions could prevent the initial phosphorylation of myosin light chains that is required for activation of contraction in all types of smooth muscle (for review, see Hartshorne, 1987), and so cause desensitization. The very rapid dephosphorylation of myosin light chains in ileum smooth muscle during K^+ contracture (Himpens *et al.* 1988*b*) is consistent with high myosin light-chain phosphatase activity playing a role in desensitization of ileum smooth muscle. The lower sensitivity of the regulatory/contractile apparatus to $[Ca^{2+}]_i$ in the ileum than in the tonic pulmonary artery (Fig. 2) could also be the consequence of

desensitization, and specific differences in muscle Ca^{2+} sensitivity have also been observed in saponin-permeabilized smooth muscles (Endo, Kitazawa, Yagi, Iino & Kakuta, 1977).

The level of $[Ca^{2+}]_i$ reached at a given $[Ca^{2+}]_o$ was higher in the tonic than in the phasic smooth muscle during non-cumulative additions of $[Ca^{2+}]_o$. This result is compatible with the earlier suggestion that the permeability of the plasma membrane to Ca^{2+} declines in phasic smooth muscles during prolonged depolarization with K^+-rich solutions (Somlyo & Somlyo, 1969), possibly due to inactivation of voltage-gated Ca^{2+} channels. We have not formally ruled out the possibility that the lower $[Ca^{2+}]_i$, at a given $[Ca^{2+}]_o$, in the ileum, compared to the pulmonary artery, is due to its faster removal by Ca^{2+} pumps, but consider this possibility unlikely, because the sarcoplasmic reticulum is less developed in phasic than in tonic smooth muscles (Devine *et al.* 1972). During cumulative additions of $[Ca^{2+}]_o$ the rise in $[Ca^{2+}]_i$ was similar in the ileum and in the pulmonary artery. This could be due to saturation of the small volume sarcoplasmic reticulum in the ileum, but we have no direct information to explain this observation.

In conclusion, we found significant differences in both cellular Ca^{2+} metabolism and in the sensitivity of regulatory/contractile proteins to cytoplasmic Ca^{2+} in, respectively, a phasic and a tonic smooth muscle. The desensitization of the ileum, but not the pulmonary artery muscle, indicates the muscle specific variability of regulatory systems. The generalization of these observations to other tonic and phasic smooth muscles will have to await additional experiments in several species and types of smooth muscle.

B. H. and G. M. are research assistants of the N.F.W.O. (Belgium). B. H. is a recipient of Fogarty International Fellowship 1 F05 TW04004. This work has been supported by grant HL15835 to the Pennsylvania Muscle Institute from the National Heart, Lung and Blood Institute. We thank Dr E. L. Buhle for his help with the computer programs. The outstanding technical contributions of Mrs M. Tokito and M. A. Spina are also gratefully acknowledged. We are grateful to Professor T. Yoshioka and Dr Y. Tsukitani for the generous gift of okadaic acid.

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