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FREE-CALCIUM AND FORCE TRANSIENTS DURING DEPOLARIZATION AND PHARMACOMECHANICAL COUPLING IN GUINEA-PIG SMOOTH MUSCLE

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SUMMARY

1. Fura2 was loaded by permeation and hydrolysis of the acetoxymethyl ester into smooth muscle cells of intact thin sheets of the longitudinal layer of the small intestine of the guinea-pig, to record Ca^{2+} transients during contraction.

2. Cytoplasmic Ca^{2+} ($[Ca^{2+}]_i$) was monitored by computing the ratio of the fluorescence signal excited at 340 and 380 nm wavelengths. The dye loading and the exposure to UV light required for the experiments had no significant effect on the contractile parameters observed.

3. Spontaneous, rhythmic increases in $[Ca^{2+}]_i$ were often observed, preceding the onset of force. Removal of extracellular Ca^{2+} caused a very transient increase in $[Ca^{2+}]_i$ accompanied by a phasic force transient; this was followed by a decline in $[Ca^{2+}]_i$ and tension below control levels. Elevated Ca^{2+} from 1·2 to 15 mM also caused a fall in $[Ca^{2+}]_i$ and a relaxation of basal tension.

4. Elevation of $[K^+]_o$ increased $[Ca^{2+}]_i$. Graded concentrations of K^+ caused graded changes in both fluorescence ratio and tension.

5. Carbachol evoked a transient increase in $[Ca^{2+}]_i$ and contraction. Thereafter, in spite of the continued presence of the drug, both signals declined, presumably as the result of cholinergic desensitization. The initial phasic force response to carbachol was usually followed by an 'after-contraction', that was only occasionally accompanied by a similar (small) secondary rise in the fluorescence signal.

6. In depolarized smooth muscle, both in the presence and in the absence of extracellular Ca^{2+}, carbachol induced a transient increase in $[Ca^{2+}]_i$, indicating that Ca^{2+} release from intracellular stores is a major mechanism of pharmacomechanical coupling.

7. In some preparations an applied stretch caused, after a few seconds, a rise in $[Ca^{2+}]_i$ and force development.

INTRODUCTION

It is generally accepted that Ca^{2+} is the primary regulator of contraction in smooth, as in striated, muscle (Filo, Bohr & Ruegg, 1965; reviewed in Johansson & Somlyo, 1980). The rise of cytoplasmic free Ca^{2+} can be induced by electromechanical

coupling, through depolarization of the surface membrane and action potentials, or by pharmacomechanical coupling, a mechanism independent of surface membrane potential (Somlyo & Somlyo, 1968*a*). The source of activator Ca^{2+} may be extracellular or intracellular, but the relative contributions of these two sources have generally not been quantified. The sarcoplasmic reticulum is the intracellular source of activator Ca^{2+}, and can store sufficient Ca^{2+} for activating maximal contractions (Bond, Kitazawa, Somlyo & Somlyo, 1984). In vertebrate smooth muscle, the initial activation of contraction by Ca^{2+} is through the formation of a Ca_4^{2+}–calmodulin complex that combines with and activates the catalytic subunit of myosin light chain kinase: phosphorylation of myosin light chains by this enzyme permits the activation of myosin ATPase by actin and contraction (Sobieszek, 1977; Hoar, Kerrick & Cassidy, 1979; for review Hartshorne, 1987). However, it has been suggested that a relatively high cytoplasmic Ca^{2+} concentration is required only for the physiological activation, but not necessarily for the maintenance of tension (Dillon, Aksoy, Driska & Murphy, 1981), and also that Ca^{2+}-regulated mechanisms independent of light chain kinase, such as caldesmon, (Sobue, Morimoto, Kanda, Fukunaga, Myamoto & Kakiuchi, 1982; for review Marston & Smith, 1985) or direct Ca^{2+} binding to myosin (Chacko, 1981), may operate in smooth muscle. Furthermore, the possibility of Ca^{2+}-independent modulation of smooth muscle (Somlyo & Somlyo, 1968*a*) remains to be considered (Morgan & Morgan, 1984*a*).

The importance of determining cytoplasmic free Ca^{2+} for understanding both Ca^{2+}-dependent and independent regulation of contraction led several laboratories to attempt its measurement. Early studies in contracting smooth muscle were based on the use of the Ca^{2+}-sensitive photoprotein aequorin (Fay, Shlevin, Granger & Taylor, 1979; Neering & Morgan, 1982; Morgan & Morgan, 1984*a*, *b*), followed by the use of the fluorescent dye, quin2 (Pritchard & Ashley, 1986; Williams & Fay, 1986, Himpens & Casteels, 1987). Ca^{2+}-selective electrodes can provide an independent measure of resting free Ca^{2+} (Yamaguchi, 1986), but are not technically suitable for measurements during contraction, due to their relatively slow time constant and dislodgement of the electrode tip. The respective advantages and disadvantages of various free Ca^{2+} indicators, including that of the first generation of fluorescent indicators, e.g. quin2 (Tsien, 1981; Tsien, Pozzan & Rink, 1982) have been reviewed in depth by Blinks (1986). A significant complicating factor in cellular free Ca^{2+} measurement is the possibility that the level of free Ca^{2+} estimated with different indicators may be influenced by the source (extracellular or intracellular) of free Ca^{2+}. This possibility is usually considered in the case of macromolecular and non-linear indicators, such as aequorin, that may diffuse and distribute anisotropically (Eisenberg, Mathias & Gilai, 1979). However, cellular non-uniformities could also influence the low molecular weight fluorophors that are sensitive to environmental microviscosity. Consequently, it is desirable to explore the reputed free Ca^{2+} transients in smooth muscle with a variety of indicators and with particular attention to the question whether dissociation between the Ca^{2+} transient and contraction is true evidence of Ca^{2+}-independent regulation.

Fura2 is a second-generation fluorescent Ca^{2+} indicator, having the advantage over quin2 of better fluorescence quantum yield, somewhat lower affinity for Ca^{2+}, lower relative sensitivity towards Mg^{2+} and nearly complete insensitivity, in the

physiological range, to pH (Grynkiewicz, Poenie & Tsien, 1985). The dye can be introduced as the acetoxymethyl ester into cells without disrupting the cell membrane, and is hydrolysed by cellular esterases to the Ca^{2+}-sensitive, free-acid form (Grynkiewicz *et al.* 1985). We describe here the relationship between $[Ca^{2+}]_i$ and tension determined with a method that we have developed for measuring fura2 fluorescence in thin sheets of contracting smooth muscle, virtually free of movement artifacts. Cytoplasmic Ca^{2+} was monitored by computing the Ca^{2+}-sensitive ratio of fluorescence signal excited at, respectively 340 and 380 nm (Tsien, Rink & Poenie, 1985). A preliminary report of some of these findings has been presented to the American Biophysical Society (Himpens & Somlyo, 1987).

METHODS

Guinea-pigs of either sex weighing approximately 300 g 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 quickly removed and, after rinsing gently, slid on a glass rod of 5 mm diameter. The longitudinal layer was dissected free from the cirular muscle, and thin sheets of about 5 mm wide and 30 mm long were prepared and transferred to an oxygenated Krebs–Ringer solution.

To load the strip in a cuvette containing 1 ml of HEPES-buffered Krebs solution containing 1·2 mM-Ca^{2+}, 2 μM-fura2AM dissolved in dimethyl sulphoxide (final concentration 0·5 %) premixed with Pluronic F127 (final concentration of 0·01 %) (Cohen, Salzberg, Davila, Ross, Landowne, Waggoner & Wang, 1974) was added. Pluronic F127 is a high molecular weight surfactant polyol (polyoxypropylene–polyoxyethylene block co-polymer) that helps to solubilize large dye molecules in physiological media (Poenie, Alderton, Steinhardt & Tsien, 1986). We have found that, in the absence of Pluronic F127 in the Krebs solution, fura2AM was not completely soluble at its final concentration and was gradually removed from the medium, as has been reported for indo-1 (Schackmann & Chock, 1986), and attached to the walls of the cuvette (data not shown). Loading was performed at room temperature for 4 h with the cuvettes rotating at 30 rev/min. The strips were removed from the loading solution and transferred to a fresh Krebs solution. The intracellular fura2 content was 30 μmol per litre of cell water as estimated by thin-layer chromatography.

The autofluorescence, measured in six preparations prior to the addition of fura2, was 20 ± 4 % of the resting signal as measured in a Gilford Fluoro IV fluorimeter. In preparations not loaded with fura2, there was no change in autofluorescence (510 nm) during contractions evoked by high K^+ or carbachol (see Results). We did not attempt to determine absolute values of $[Ca^{2+}]_i$, since we did not substract the autofluorescence from the signal, the possible amount of fura2AM remaining in the specimen (Luckhoff, 1986) was unknown, the 380 nm signal included a variable component due to reflection and we did not have an accurate method of relating cuvette calibration to intracellular conditions. Absolute calibrations (Grynckiewicz *et al.* 1985) were obtained in four experiments. Maximum fluorescence (F_{max}) and minimum fluorescence (F_{min}) ratios were determined in the presence of 50 μM-ionomycin at pH 8·6 with 1·2 mM-Ca^{2+} and with 20 mM-Mn^{2+}. Assuming a dissociation constant (K_D) of 224 nM (Tsien *et al.* 1985) the $[Ca^{2+}]_i$ was 135 ± 25 nM in resting conditions. The use of different time-sharing demodulators and several changes in filter sets during the course of this study precluded the use of these values for comparing absolute values of the ratio signal obtained in different experiments. However, none of the conclusions drawn required such comparison.

Experimental set-up

Longitudinal muscle strips (3 mm long, 100 μm thick, 3 mm width in relaxed state) were stretched in a thermostatted tissue chamber of 1 ml. One end of the strip was attached to an isometric force transducer (Workshop of Department of Physiology, KU Leuven, Belgium, frequency response 200 Hz), while the three remaining sides were pinned by 0·2 mm stainless-steel minuten pins into the silicone (Sylgard, Dow Corning Corporation, Midland, MI, U.S.A.) covering the bottom of the chamber along the periphery of the light pipe. Continuous perfusion at 3 ml/min

with solutions at room temperature (23–25 °C) with a LKB 2115 multiperspex peristaltic pump, removed any fura2 leaking out of the cells, and air bubbles were minimized by using an air-trap. Perfusion of the medium through the chamber also allowed us to monitor the recovery of the muscle strips after activation with agonists.

It was necessary to measure continuously the fluorescence at 510 nm, during repeated alternating excitation at wavelengths of 340 and 380 nm (Grynkiewicz *et al.* 1985), while ensuring that fluorescent light was collected from the same region of the tissue exposed to each of the two wavelengths (340 and 380 nm). To ensure optimal collection of the fluorescent light from the 6·6 mm^2 of a 13 mm^2 tissue segment, we used a bifurcated, randomized light pipe (Welch Allyn, Skancateles Falls, NY, U.S.A.) with an individual fibre size of 100 μm. The common end formed the bottom of the chamber under the specimen. One of the bifurcated ends was connected to the light source: UV light of alternating 340 and 380 nm was obtained by placing a rotating wheel containing the two different interference filters (340 and 380 nm filters, bandpass 4 nm, transmittance 40%) and driven by air at a frequency of 150 Hz in front of the collector lens of a 200 W mercury source. The other end of the light pipe was connected to the photomultiplier (EMI 9924B), through an interference filter of 510 nm wavelength (bandpass 10 nm, transmittance 80%) to eliminate scattered light. The two signals were separated by a 4-channel demodulator (Chance, Legallais, Sorge & Graham, 1975) (Biomedical Instrumentation Group, University of Pennsylvania Medical School).

The two fluorescence signals and the tension signal were digitized, at 30 Hz for measuring the spontaneous activity and at 10 Hz for the other experiments, with the Dash 16 A/D converter (Metrabyte Corporation). The data were acquired on an IBM AT computer with the Laboratory Technologies' Labtech Notebook software and filtered on a 3 point moving average using the Hanning weights and least-squares smoothing algorithm of Savitzky & Golay (1964). Lotus 1-2-3 (Lotus Development Corporation) was used for the graphical analysis of the data.

Figure 2 shows an example of the data acquisition. The upper part of the panel shows the original raw fluorescence signals. Upon each spontaneous contraction, there is an increase in the (510 nm) fluorescence signal during excitation with light of 340 nm and a decrease during excitation with light of 380 nm. In the middle part the computed ratio of the fluorescence signals is displayed, while in the lower part the force is shown.

Statistical analysis

Statistical error figures are expressed as standard errors of the mean.

Solutions

The standard physiological solution was a HEPES-buffered modified Krebs solution 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. The solution was bubbled with O_2 and the pH was 7·3. Solutions with increased $[K^+]_o$ were obtained by replacing Na^+ by an equivalent amount of K^+. The Ca^{2+}-free solutions always contained 2 mM-EGTA. In some experiments the spontaneous activity was sychronized by modifying the Krebs solution to contain 10 mM-K^+ and 5 mM-Ca^{2+}. Hypertonic solutions were made by adding 200 mM-NaCl to the original Krebs solution, while hypotonicity was achieved by diluting the original Krebs solution by the same amount of distilled water.

Chemicals

Fura2AM was obtained from Molecular Probes, Pluronic F127 from BASF Wyandotte Corporation, carbachol and verapamil were from Sigma.

RESULTS

Control experiments

Controls were performed in unloaded specimens to ensure that the ratio signal was unaffected by movement artifacts or changes in autofluorescence induced by the agonist. Figure 1 shows that during superfusion with high K^+- or carbachol (10^{-4} M)-containing solutions, the ratio signal is unchanged in strips not loaded with fura2 ($n = 6$).

Smooth muscle can be relaxed by exposure to UV light in the range of 310–440 nm (Furchgott, Ehrreich & Greenblatt, 1961), possibly due to activation of guanylate cyclase (Karlsson, Axelsson, Elwing & Andersson, 1986). However, UV light, at the intensity used in our experiments, had no effect on either the stimulated contractions (Fig. 1) or the spontaneous activity (data not shown). No changes in the amplitude or shape of the contraction evoked by carbachol or high K^+ ($n = 5$) could be detected even after prolonged (3 h) UV exposure.

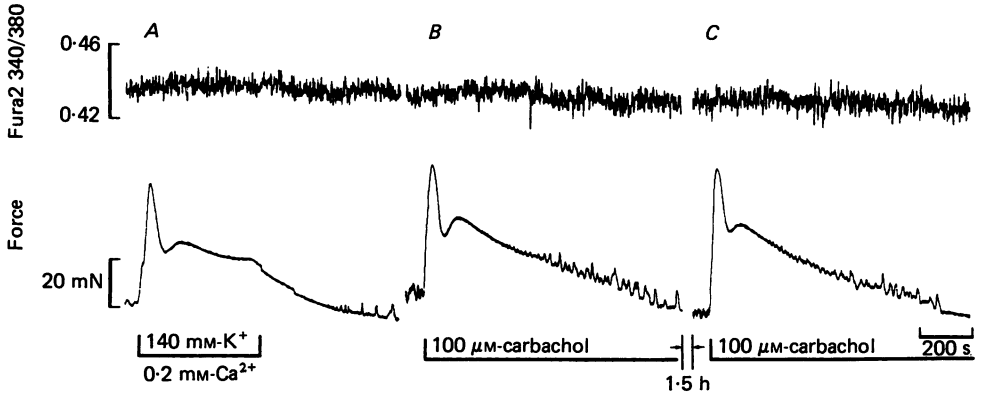

Fig. 1. Lack of effect of agonist stimulation on the light emission of unloaded strips of smooth muscle. In the upper half of the Figure the ratio of the fluorescence at 510 nm wavelength during alternating excitation at wavelengths of 340 and 380 nm is shown. In the lower part the accompanying force development is displayed. In *A* the specimen was superfused for 7 min with high (140 mM) K^+. In *B* the strip was stimulated for 10 min with carbachol (10^{-4} M). In *C* carbachol (10^{-4} M) was added again after 90 min continuous UV light exposure that had no effect on the shape of the contraction ($n = 6$).

High intracellular concentrations of the Ca^{2+} indicator can buffer intracellular Ca^{2+} (Rink & Pozzan, 1985; Ashley, 1986). To establish whether the time course or the magnitude of tension was modified in this manner, we determined the effect of loading with fura2 on contraction. Unchanged contractile amplitudes were found after loading the specimen with the dye. The amplitude of the carbachol-induced contraction after loading was 137 ± 35% of the unloaded control stimulation. The rate of force development was $0{\cdot}18 \pm 0{\cdot}05$ mN s^{-1} before and $0{\cdot}24 \pm 0{\cdot}06$ mN s^{-1} ($n = 7$) after loading with fura2AM. The amplitude of the high-K^+-induced contractions was, after loading, 122 ± 24% of the control and the rate of increase in force was $0{\cdot}12 \pm 0{\cdot}025$ mN s^{-1} before and $0{\cdot}16 \pm 0{\cdot}045$ mN s^{-1} after loading with 2 μM-fura2AM ($n = 8$). Thus, we conclude that the extent of dye loading required for our studies had no significant effect on the contractile parameters observed.

Spontaneous activity

Most of the experiments described below were performed at room temperature, in order to minimize the fura2 leakage that is significant at 37 °C. However, in order to study the changes in $[Ca^{2+}]_i$ during spontaneous rhythmic contractions, synchronized activity of the smooth muscle cells was optimized by performing these experiments at 37 °C and using a solution containing 5 mM-Ca^{2+} and 10 mM-K^+. Figure 2 shows an example of these measurements ($n = 15$). The two signals are displayed in the upper

part of the Figure, while the ratio of the two fluorescence signals is seen in the middle panel and the force is displayed in the lower panel: each spontaneous increase in $[Ca^{2+}]_i$ is followed by a force transient. The average peak to peak delay between the ratio and the force transient was 700 ± 80 ms ($n = 20$).

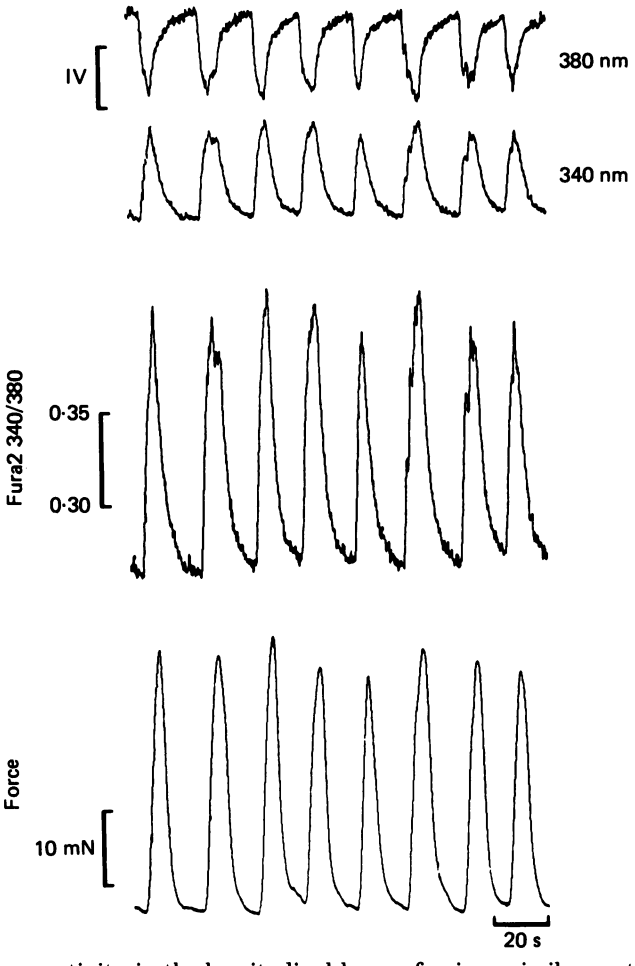

Fig. 2. Spontaneous activity in the longitudinal layer of guinea-pig ileum at 37 °C with a modified Krebs solution containing 10 mM-K^+ and 2·5 mM-Ca^{2+}. In the upper part of the Figure the original data obtained at 340 and 380 nm wavelengths are shown. The middle panel shows the ratio of the two signals while in the lower the accompanying force development is shown ($n = 15$).

In Fig. 3 slow rhythmic contractions of the longitudinal layer are shown with spontaneous rhythmic fluctuations in the fluorescence ratio signal that presumably reflect the firing rate of the action potentials by the pacemaker cells.

Changes in the resting $[Ca^{2+}]_i$ *and tension*

The resting fluorescence level during superfusion with a solution containing 1·2 mM-Ca^{2+} was constant. However, removal of the external Ca^{2+}, by replacing the

solution with one containing 0 mM-Ca^{2+} with 2 mM-EGTA, reduced both the tension and $[Ca^{2+}]_i$ ($n = 20$). In Fig. 4*A*, the external Ca^{2+} was replaced by a Ca^{2+}-free solution. Initially, sixteen out of twenty muscles underwent a transient increase in $[Ca^{2+}]_i$ and tension, followed by a gradual decline in both signals towards a new steady-state level. After reperfusing with the Ca^{2+}-containing solution, $[Ca^{2+}]_i$ and tension increased again. However, tension followed fluorescence rather slowly with a 150 ± 25 s ($n = 20$) delay between the onset of the fluorescence and the force. Much

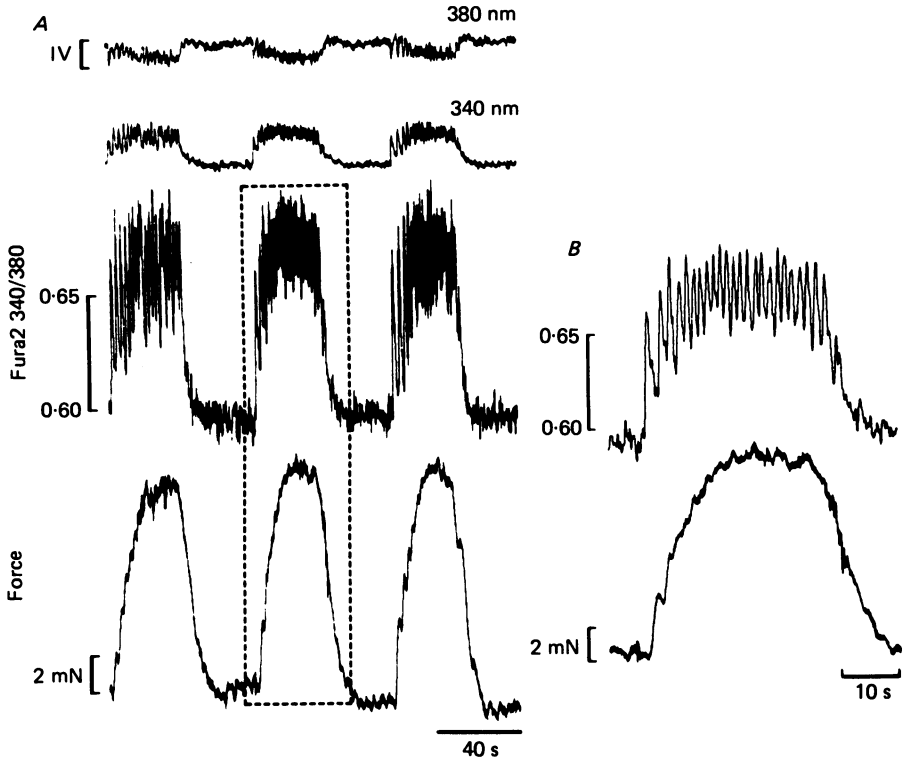

Fig. 3. *A*, spontaneous rhythmic contractions (lower trace) triggered by Ca^{2+} transients resembling action potentials superimposed on slow waves. The upper traces show fluorescence at 510 nm excited at, respectively 340 and 380 nm, above the ratio signal. The central wave shown in *A* is displayed on a faster time scale in *B*.

of this delay appears to be due to the time required for $[Ca^{2+}]_i$ to reach contractile threshold levels. Resumption of rhythmic spontaneous contractions, when they occurred, was accompanied by rhythmic $[Ca^{2+}]_i$ transients.

Figure 4*B* shows the effect of increasing extracellular Ca^{2+} to 15 mM on $[Ca^{2+}]_i$ and tension. This large increase in extracellular Ca^{2+} caused a drop in both the force and fluorescence ratio. Upon restoring the original (1·2 mM) Krebs solution, $[Ca^{2+}]_i$ increased to the basal level and the spontaneous contractions resumed.

Verapamil (10^{-5}M) abolished the spontaneous activity (Fig. 4*C*) and caused a fall in the fluorescence ratio and resting tension ($n = 5$).

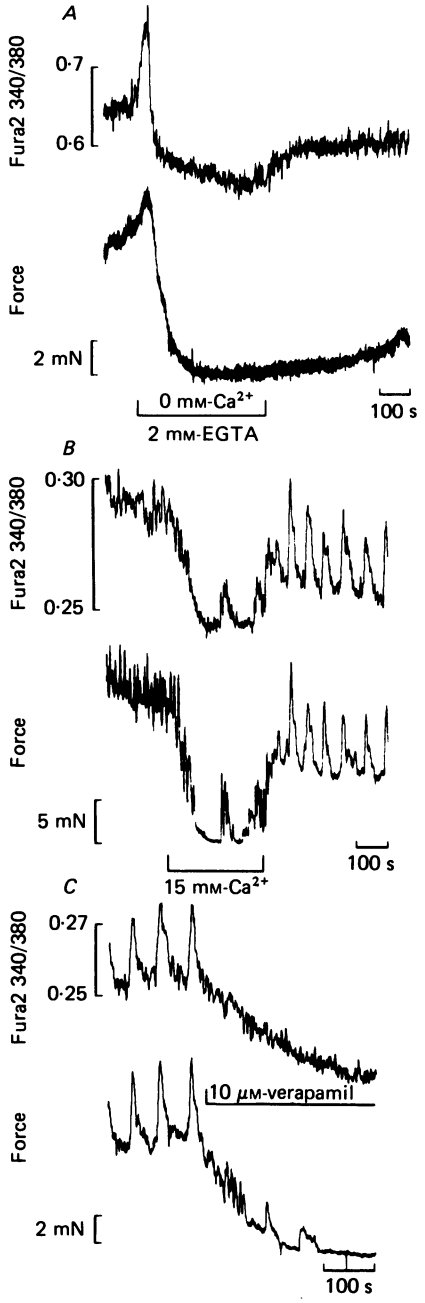

Fig. 4. Changes in $[Ca^{2+}]_i$ (upper trace) and force (lower trace) in fura2-loaded strips, in response to replacing the normal Krebs solution (1·2 mM-Ca^{2+}) by one containing 0 mM-Ca^{2+} and 2 mM-EGTA (*A*), 15 mM-Ca^{2+} (*B*) or verapamil 10^{-5} M (*C*). In *A* upon addition of Ca^{2+}-free solution for 7 min, a transient increase in $[Ca^{2+}]_i$ and tension occurs. This is followed by a decline in both signals during continued incubation in the Ca^{2+}-free solution. After reperfusion with normal Krebs solution, both signals slowly increase ($n = 20$). In *B* the two signals decline upon superfusing the muscle with 15 mM-Ca^{2+} ($n = 4$). In *C* the spontaneous activity ceases and there is a decline in tension and fluorescence after adding 10^{-5} M-verapamil ($n = 5$). Note also that two small spontaneous contractions are not accompanied by detectable fluorescence transients.

Effect of excitatory agents on $[Ca^{2+}]_i$

High K^+. The response to depolarization with high K^+ displayed several distinct patterns: (1) the most common response ($n = 27$) was a monophasic increase in the fura2 ratio signal consisting of a rapid spike and quasi-exponential decline. This was accompanied by a biphasic contraction: an initial phasic component followed by varying degrees of partial relaxation (Fig. 5*A*) ($n = 12$) and mostly a secondary, slow

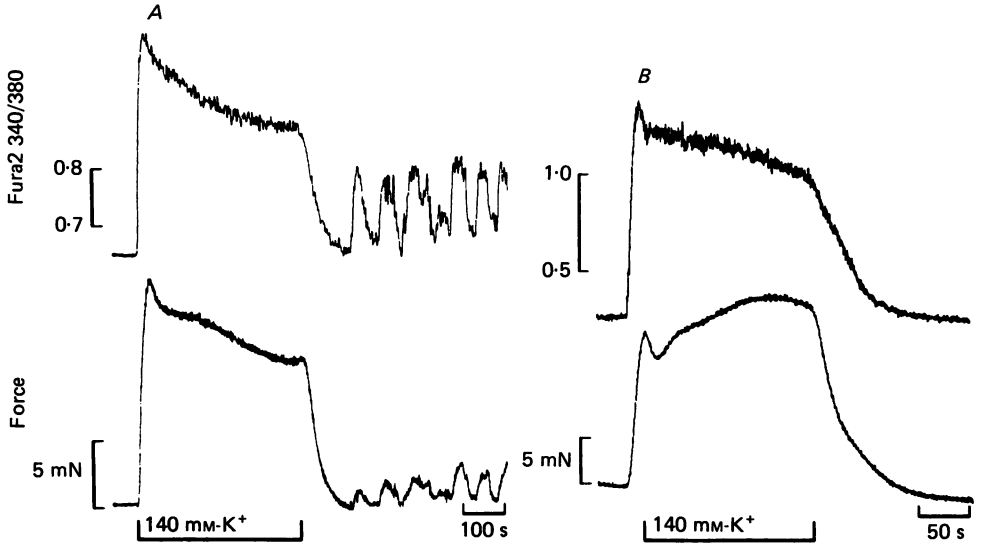

Fig. 5. Representative light (upper) and force (lower) recording during depolarization with high K^+. The most common response ($n = 27$) shows a monophasic increase in the fura2 ratio signal consisting of a rapid spike and quasi-exponential decline. This is accompanied by a biphasic contraction: an initial phasic component followed by varying degrees of partial relaxation with in *A* a slowly declining plateau phase ($n = 12$) or with a secondary slow rise in tension (*B*) ($n = 15$).

rise in tension. This second component of tension development (Fig. 5*B*) was not accompanied by a significant rise in the ratio signal ($n = 15$); (2) a monophasic increase in the fura2 ratio signal followed by a relatively maintained plateau, but with relaxation preceding the decline in the fura2 ratio signal.

Nevertheless, in these instances the change from high-K^+ to normal Krebs solution resulted in accelerated decline of both the fura2 signal and tension. In a few instances, such dissociation between the fluorescence signal and tension could be ascribed to a probable artifact in the fura2 ratio signal, indicated by non-reciprocal changes in the fluorescence emitted in response to excitation at the two (340 and 380 nm) wavelengths. However, excluding these cases and restricting our observations to instances in which the changes in fluorescence excited at 340 and 380 nm were reciprocal, in six of eight muscles the tension still declined faster than the fura2 signal; (3) monophasic, relatively slow increases in the ratio signal were followed by parallel changes in tension in ten muscles.

The responses to three different extracellular K^+ concentrations (140, 70 and

35mM; equimolar replacement of Na^+ by K^+) were also compared (Fig. 6). The pronounced phasic component of the force and fluorescence to 140 mM-K^+ was very much reduced or absent when the same preparations were stimulated with lower (70 or 35 mM) K^+. During the superfusion with 70 mM-K^+, the phasic force component and the fluorescence ratio were, respectively, 77 and 75% of the values obtained during stimulation with 140 mM-K^+ while during exposure to 35 mM-K^+ the values were 47 and 56% ($n = 15$). The tonic components expressed as the fractions of the peak tension and fluorescence response to the 140 mM-K^+ were not significantly different for the three elevated K^+ solutions.

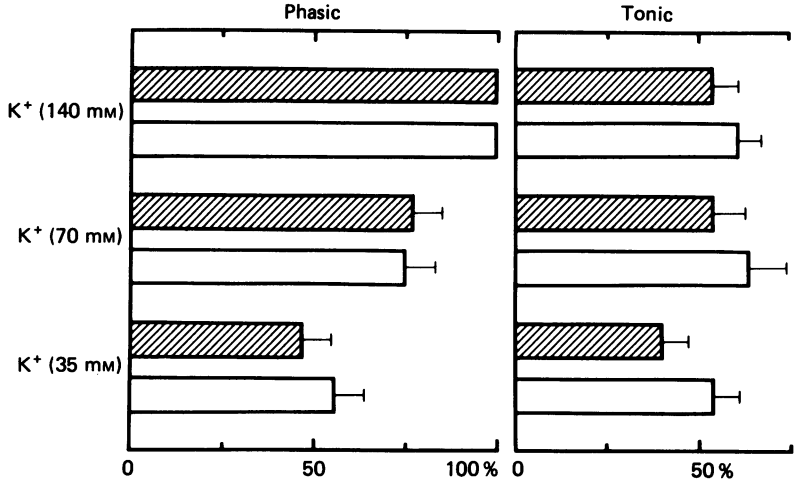

Fig. 6. Peak contractions elicited by different external K^+ concentrations in ileal smooth muscle. The bars show the magnitude of the fluorescence (open) and tension (shaded) responses to different external K^+ concentrations (140, 70 and 35 mM-K^+) ($n = 15$) expressed as percentage of the respective maximal phasic response (100%) to 140 mM $[K^+]_o$. In the left part the effect of different external K^+ concentrations on the phasic component are shown, while the right part shows the responses of the tonic phase. In each instance, the gradations in, respectively, $[Ca^{2+}]_i$ and force are proportional.

The effect of replacing a 140 mM-K^+ solution containing 1·2 mM-Ca^{2+} by a 0·2 mM-Ca^{2+}-containing high-K^+ solution was also determined. On replacing the high-K^+, 1·2 mM-Ca^{2+} solution with a high-K^+, low-Ca^+ solution, there was a small but detectable decline in both the fluorescence signal and tension ($n = 10$). Readmission of the high-K^+, 1·2 mM-Ca^{2+} solution caused another increase in force and in fluorescence.

Carbachol. Continued stimulation with carbachol (10^{-4} M) caused a more transient (phasic) force and fluorescence response ($n = 30$) than did stimulation with K^+. Thus, during continuous exposure to the drug, the half-life ($t_{\frac{1}{2}}$) of the fluorescence decay was 120 ± 33 s compared to the 163 ± 38 s decay of tension relaxation. The initial rapid contractile response was invariably preceded by a similarly rapid rise in $[Ca^{2+}]_i$ (Fig. 7). Following variable partial relaxation of tension, an 'after-contraction' frequently (twenty-five out of thirty-one muscle strips) occurred, separated by variable time from the initial phasic contraction. In a few instances (Fig. 7*A*) this 'after-

contraction' was associated with a similar rise in the fura2 ratio signal but in other experiments (fourteen out of twenty-five experiments) the second phase of the rise in tension was not accompanied by a detectable change in fluorescence (Fig. 7*B*).

We also wished to determine whether in smooth muscles depolarized with high K^+, the contraction produced by carbachol is also associated with a rise in cytoplasmic Ca^{2+}. Figure 8 shows the response to carbachol (10^{-4} M) during the sustained phase of contraction stimulated with 140 mM-K^+ ($n = 6$). The drug caused

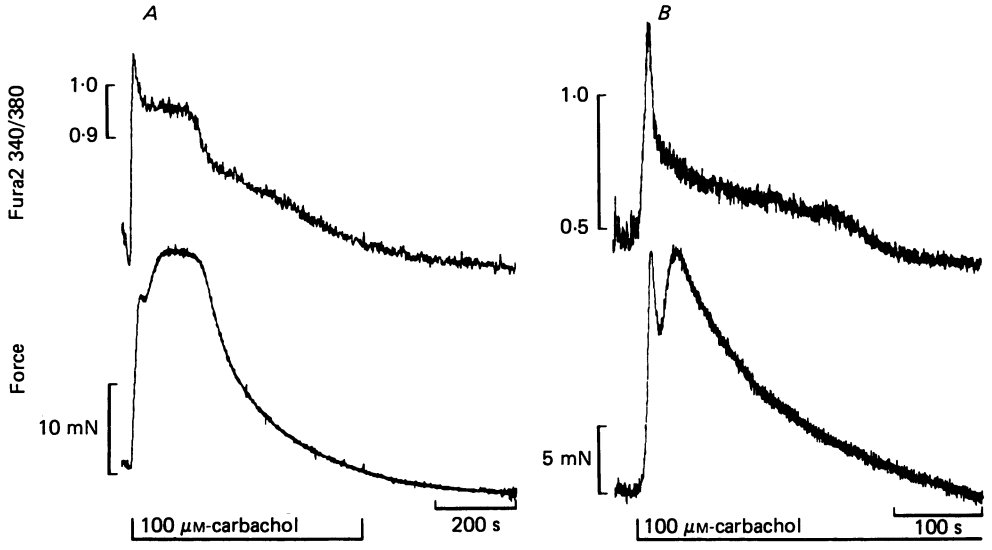

Fig. 7. The effect of carbachol (10^{-4} M) on fluorescence (upper trace) and on the tension (lower trace). Note the presence of a definite fluorescence signal accompanying the 'after-contraction' in *A* but not in *B*.

significant 'spikes' in both the cytoplasmic Ca^{2+} and the tension record. This phasic increase in cytoplasmic Ca^{2+} and tension induced by carbachol was present in each of the six strips depolarized with high K^+. Interestingly, partial relaxation of tension was also here followed by an 'after-contraction' that was accompanied by a much smaller and slower Ca^{2+} transient.

The intracellular Ca^{2+} store

The effect of intracellular Ca^{2+} on excitation–contraction coupling was studied by superfusion over short periods with Ca^{2+}-free solution, since the loss of tension is known to be very rapid in phasic (such as ileum) compared to tonic smooth muscle (Devine, Somlyo & Somlyo, 1972; Casteels & Raeymaekers, 1979). After loading for 2 min in a high-K^+, 1·2 mM-Ca^{2+}-containing solution, this solution was replaced by a Ca^{2+}-free, high-K^+ solution (Bond *et al.* 1984). After 2 min, carbachol (10^{-4} M) was added, which triggered a transient, nearly maximal peak in tension and in fluorescence (Fig. 9). Despite continuous presence of carbachol, tension and $[Ca^{2+}]_i$ declined in the Ca^{2+}-free medium. Reincubation in Ca^{2+}-containing solution caused a transient overshooting of tension and fluorescence above their original levels.

The effect of the Ca^{2+} antagonist verapamil on $[Ca^{2+}]_i$

The possible role of Ca^{2+} influx during agonist stimulation was tested by studying the effect of blockers of the Ca^{2+} channels. Verapamil was used to test the effect of a Ca^{2+}-entry blocker on the tension transients, because it is not photolabile, unlike some dihydropyridines (Ebel, Schutz & Hornitschek, 1978). We determined the effect of 10^{-5} M-verapamil on the K^+- and carbachol-induced contractions. After recording control responses to 140 mM-K^+ and carbachol (10^{-4} M), the strips were pre-incubated for 10 min with 10^{-5} M-verapamil and the responses to carbachol and

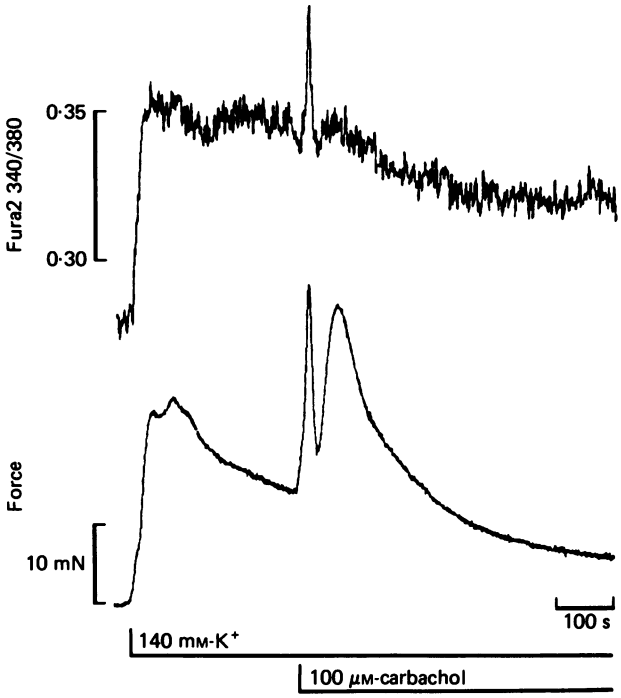

Fig. 8. The effect of adding carbachol during a maximal K^+ contraction on force (lower trace) and the fluorescence ratio (upper trace). After continuous perfusion with high K^+ for 5 min, carbachol (10^{-4} M) is added.

to high K^+ were determined in the presence of verapamil. Verapamil reduced the fluorescence and the force response to carbachol to, respectively, $70 \pm 10{\cdot}5$ and $30 \pm 4{\cdot}5\%$ ($n = 8$) of the control values. The K^+-induced fluorescence signal and contraction were reduced by verapamil to respectively, 21 ± 6 and $13 \pm 5\%$ of the control values ($n = 10$).

Stretch

Smooth muscle can respond actively ('myogenic response') to an imposed stretch (Bulbring, 1955; Burnstock & Prosser, 1960; Johnson, 1980). Figure 10 illustrates the response of fluorescence and force to an imposed stretch. After 4 s this was followed by an increase in $[Ca^{2+}]_i$ and contraction. It can also be seen that the ratio

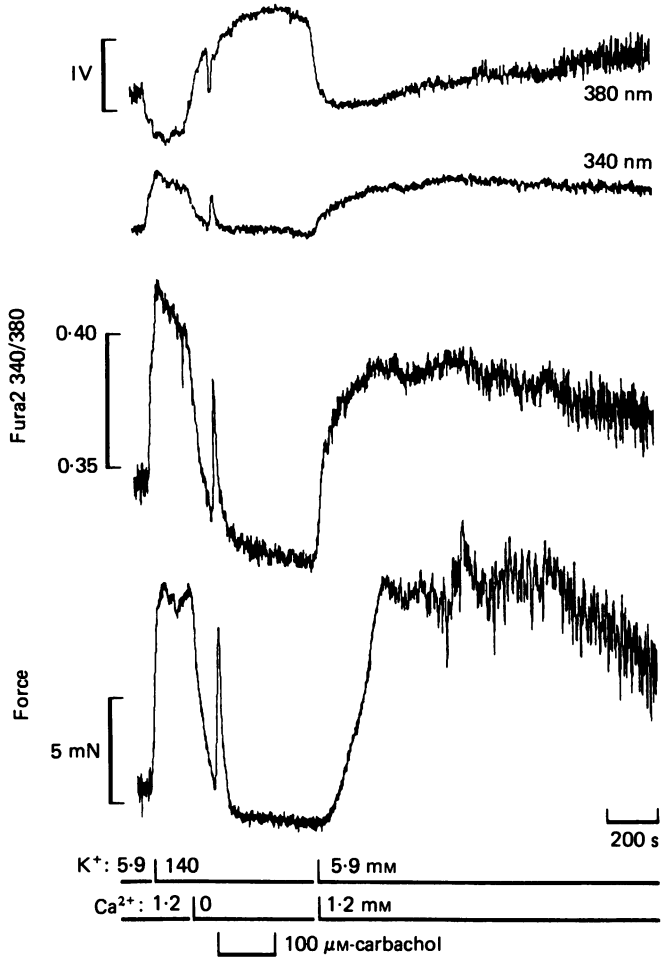

Fig. 9. Intracellular Ca^{2+} released by carbachol in depolarized smooth muscle. After 120 s in high K^+, 1·2 mM-Ca^{2+}, the Ca^{2+} was removed by perfusion with a high-K^+, Ca^{2+}-free solution. After 2 min, carbachol (10^{-4} M) was added, which triggered a transient increase in fluorescence and in force. After 6 min Ca^{2+} was restored by a 5·9 mM-K^+, 1·2 mM-Ca^{2+} solution.

signal was undisturbed during the stretch. Active myogenic responses were obtained in three out of eight strips.

Osmolarity

The osmolarity of the solution was changed during superfusion with high K^+ to determine the influence of the ionic strength on the fura2 signal, because the affinity of the dye to Ca^{2+} is sensitive to ionic strength (Grynkiewicz *et al.* 1985). Changes in tonicity of the bathing medium are known to modify considerably the contractile activity of the ileal smooth muscle (Syson & Huddart, 1973). Hypertonic solutions which shrink the cells and distort the extracellular spaces depress the high-K^+ contraction.

Figure 11*A* illustrates that when the depolarizing solution was made hypertonic by adding 200 mM-NaCl, the force and fluorescence signal declined. However, the decline in the 340 nm signal was much less than the increase in the 380 nm signal, resulting in an artificially low ratio. Control experiments in a specimen not loaded with fura2 (Fig. 11*C*) showed a significant increase in the 380 nm signal, with no change or a small increase in the 340 nm signal, under the same conditions as used for the fura2-loaded strips. Therefore, in addition to Ca^{2+}-sensitive components (the effect of ionic strength on the K_D of the Ca^{2+}–fura2 complex and a possible change in $[Ca^{2+}]_i$), this ratio signal also includes a Ca^{2+}- and fura2-independent component due to a rise in the 380 nm wavelength-

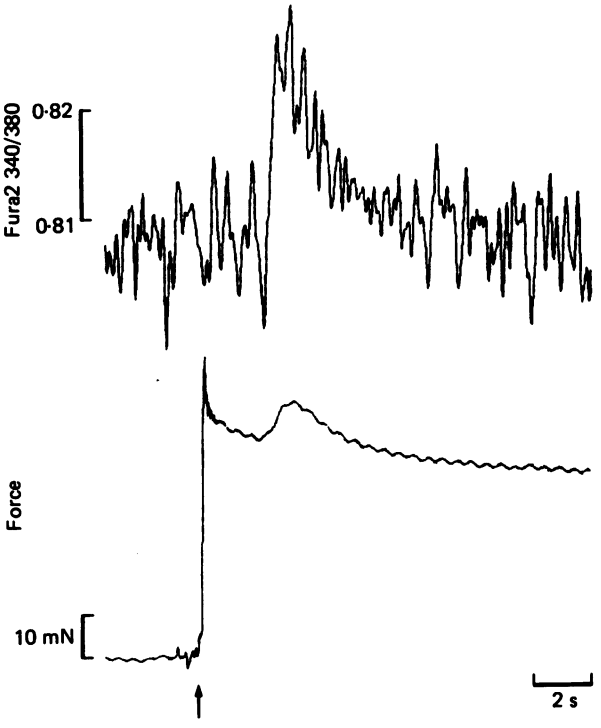

Fig. 10. Effect of stretch on $[Ca^{2+}]_i$ and contraction. A 40 mN stretch (arrow) is applied causing, after a long (4 s) latency a rise in $[Ca^{2+}]_i$ and accompanying myogenic contraction.

signal. In normally polarized smooth muscle, changing the normal Krebs solution to a hypertonic (200 mM-NaCl) solution also induced anomalous changes in the 340 and 380 nm signal, resulting in an increase in force while the fluorescence ratio meanwhile decreased (Fig. 12).

Hypotonic solutions increase the cell volume and strongly increase the size of the high K^+-induced contraction. Figure 11*B* shows that perfusion with hypo-osmotic (0·5) solution caused a symmetrical increase in the 340 and decrease in the 380 nm signal: the resultant increase in the ratio signal was associated with a parallel force transient. Control experiments with strips not loaded with fura2 (Fig. 11*D*) showed no significant change in the different signals.

DISCUSSION

Methodology

We have demonstrated that it is possible to record Ca^{2+} transients in intact, contracting smooth muscle with fura2, without movement artifacts, by using an

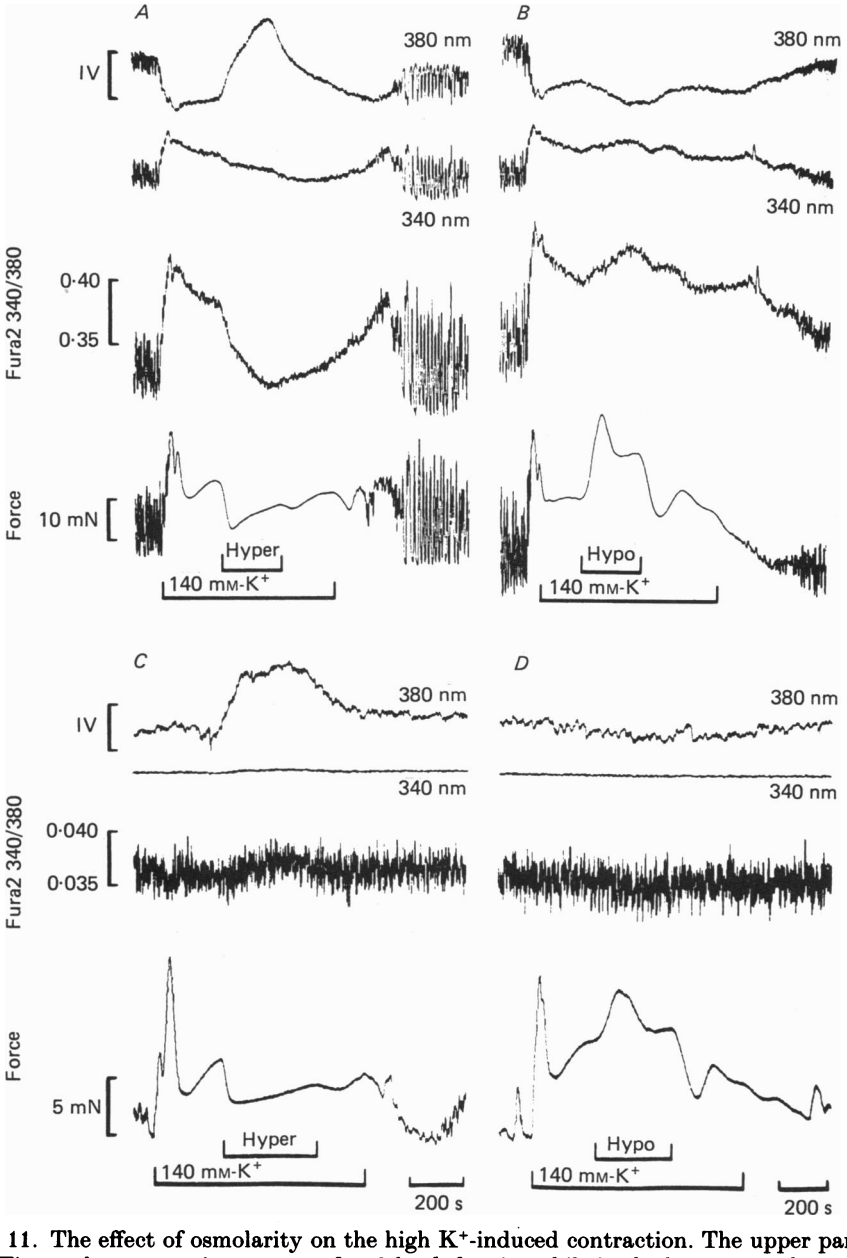

Fig. 11. The effect of osmolarity on the high K^+-induced contraction. The upper part of the Figure shows experiments on a fura2-loaded strip, while in the lower part the results obtained with an unloaded strip are shown, recorded under identical conditions as for fura2-loaded strips. In *A* and *C* after 200 s high-K^+ solution 200 mM-NaCl is added to the high K^+ for another 200 s after which the isotonic high K^+ is reperfused. The 380 nm signal increases in both loaded as well as in the unloaded strips significantly. In *B* and *D* the same protocol is performed for a solution made hypotonic by adding the same amounts of distilled water to the normal high-K^+ solution. In the fura2-loaded strip this results in comparable changes of the two signals, while in the unloaded strip no significant changes can be found.

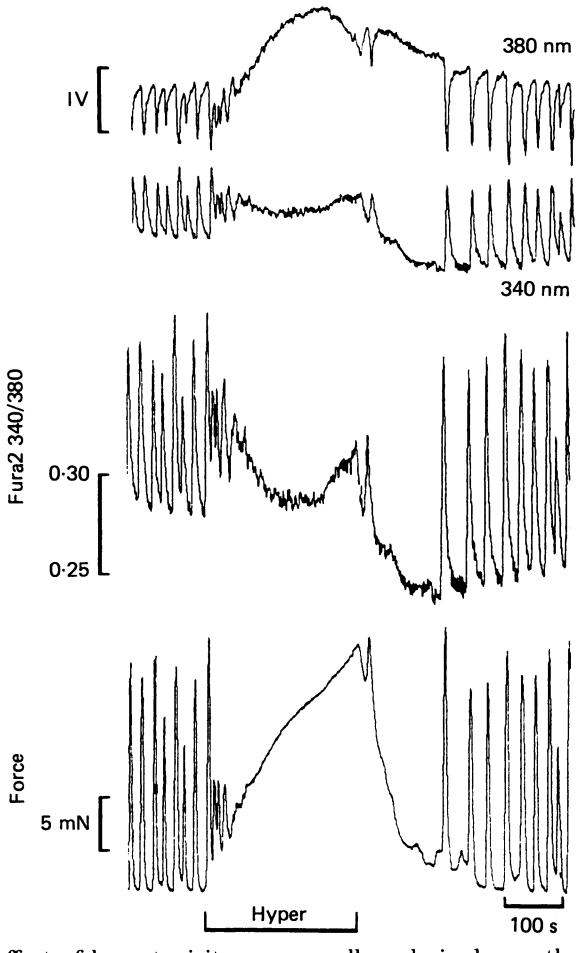

Fig. 12. The effect of hypertonicity on normally polarized smooth muscle loaded with fura2. The normal Krebs solution was made hypertonic for 6 min by adding 200 mM-NaCl to the solution. The set of raw data are shown in the upper part, while the fura2 ratio signal and the force are seen in the lower part. Note the decline in the fura2 ratio with the increase in tension. In this case, the change in light emission during excitation at 380 nm that occurs independently of the presence of fura2 (Fig. 11) causes the artificial dissociation between the fluorescence ratio and the force development.

experimental configuration that detects fluorescence with bifurcated fibre optics in a thin sheet of muscle from the region excited, and by immobilizing the small, optically unavailable region of the muscle. The sensitivity of the recording system and the quantum efficiency of fura2 (Grynkiewicz *et al.* 1985) were sufficient to obtain good signal–noise with low concentrations of fura2 that did not significantly buffer cytoplasmic free Ca^{2+} (Ashley, 1986) and, therefore, did not affect either spontaneous activity, the rate of force development or maximum tension. Neither the dye nor exposure to the UV light (Furchgott *et al.* 1961) had a detectable effect on any of these parameters, and the intracellular Ca^{2+} signal was not contaminated by extracellular leakage of fura2 acid that was removed by the continuous perfusion of the chamber. That extracellular fura2 did not contribute to the ratio signal was

indicated by the opposite direction in which $[Ca^{2+}]_i$ changed when extracellular Ca^{2+} was decreased or increased (see below).

The simultaneous recording of the fluorescence excited at, respectively, 340 and 380 nm with a time-sharing spectrophotometer is, in our experience advantageous. Thus, although transients in the ratio generally reflected changes in the 340 and 380 nm signals in opposite directions, one decreasing while the other increased, in several instances the fluorescence excited at the two wavelengths moved in the same direction, albeit at different amplitudes. The resultant changes in the ratio signal could have been interpreted as being due to fluctuation in free Ca^{2+}. Their artifactual origin was revealed by inspection of the non-ratio (340 and 380 nm) signals and confirmed by the behaviour of the force trace. Hypertonic solutions generally caused such artifacts in the ratio signal, but, occasionally, we have also recorded similar traces during drug-induced contractions. The changes induced by hypertonicity are not explained by the increased ionic strength decreasing the affinity of fura2 for Ca^{2+} (Grynkiewicz *et al.* 1985) and changes in the 380 nm signal were also present in smooth muscle not loaded with fura2 (Fig. 11).

The advantage of recording free-Ca^{2+} transients in intact sheets of smooth muscle is that such records can be correlated with force transients under more physiological conditions than attainable in dissociated or cultured cells. The correlation is easier in smooth muscles, such as the ileum, that can support synchronous conducted activity. Thus, given a 6 cm/s conduction velocity in intestinal smooth muscle (Bozler, 1948; Abe & Tomita, 1968; Brading, Bülbring & Tomita, 1969) and the 4 mm lengths of our muscle strips, 66 ms is the unlikely, high upper limit of error due to conduction time. Nevertheless, interpretation of our results still must take into account that both fluorescence and tension were recorded from a very large fibre population in which heterogeneity of physiological properties and/or loading with dye has not been excluded; consequently some of the questions raised in this study merit further exploration in single smooth muscle cells.

Resting cytoplasmic Ca^{2+}, spontaneous activity and myogenic response

The fall in $[Ca^{2+}]_i$ upon the removal of extracellular Ca^{2+} has been observed in cell suspensions by some (e.g. Sumimoto & Kuriyama, 1986), but not by other (e.g. Bitar, Bradford, Putney & Maklouf, 1986) investigators. However, none of these authors found any effect of Ca^{2+} blockers on the resting tension. Our results showing that verapamil decreases $[Ca^{2+}]_i$ and basal tension in intact smooth muscle support the role of a direct *in vivo* effect of Ca^{2+} blockers on smooth muscle. The fall in free intracellular Ca^{2+} following removal of extracellular Ca^{2+} indicates an increase in the gradient against which the sarcoplasmic reticulum pump has to maintain its internal, stored Ca^{2+} content. Therefore, the removal of extracellular Ca^{2+} could limit the ability of the sarcoplasmic reticulum to maintain its Ca^{2+} content, and cause a greater or lesser loss of stored Ca^{2+}, depending on the leak–pump ratio of the sarcoplasmic reticulum membrane. Muscle-specific differences in Ca^{2+} retention by the sarcoplasmic reticulum have been demonstrated: the sarcoplasmic reticulum in skinned cardiac muscle is less able to retain its Ca^{2+} content than the sarcoplasmic reticulum of frog skeletal muscle (Kitazawa, 1984). Therefore, our results contribute to the suspicion that the loss of contractility due to the removal of extracellular Ca^{2+} is not unambiguous evidence of extracellular Ca^{2+} being a significant source of activator Ca^{2+} in normal Ca^{2+}-containing solution (for review, Johansson & Somlyo, 1980; Somlyo, 1985).

A very transient increase in cytoplasmic Ca^{2+}, preceding its subsequent decline below relaxed levels, was triggered by the removal of extracellular Ca^{2+} in smooth muscle (Fig. 4*A*). The accompanying phasic force transient has been previously decribed (Hurwitz & Joiner, 1969), and could be due to either transient depolarization of the surface membrane, increased Ca^{2+} permeability and Ca^{2+} influx during the transition from 1·2 mM to nominally Ca^{2+}-free condition or both. That the 'membrane stabilizing effect' of Ca^{2+} also controls the permeability of the surface membrane to Ca^{2+} itself in smooth muscle (for early speculation see Somlyo & Somlyo, 1968*a*, *b*), is also indicated by the fall in the cytoplasmic Ca^{2+} caused by increasing extracellular Ca^{2+} to 15 mM (Fig. 4*B*).

The spontaneous fluorescence signals accompanying tension fluctuations are the first, albeit not unexpected, evidence showing that spontaneous contractions in smooth, as in cardiac, muscle are due to intracellular Ca^{2+} transients triggered by spontaneous electrical activity. We have observed two types of Ca^{2+} waveforms in this study: (1) relatively rapid Ca^{2+} 'spikes' (Fig. 2) that presumably represent more rapid single cell transients convoluted by cell to cell conduction velocity, and less frequently, (2) the superposition of rapid transients on 'slow waves' of Ca^{2+} (Fig. 3). We are unable to determine whether such waves were due to the incomplete fusion of rapid Ca^{2+} transients or the result of true electrical 'slow waves' and superimposed action potentials (Connor, Kreulen, Prosser & Wiegel, 1977). Spontaneous Ca^{2+} transients were invariably associated with contractions, and the rise of Ca^{2+} preceded the onset of force. The occasional presence of small spontaneous force transients not preceded by detectable increases in $[Ca^{2+}]_i$, (e.g. Fig. 4*C*) suggests that the sensitivity of our systems is lower for recording cytoplasmic Ca^{2+} than force, because it is highly unlikely that such spontaneous contractions, normally associated with Ca^{2+} transients, were triggered by something other than an increase in cytoplasmic Ca^{2+}. Hence, caution should be exercised in evaluating the significance of small and slow contractions unassociated with detectable changes in cytoplasmic free Ca^{2+}.

The peak to peak time between fluorescence signal and force during spontaneous contractions was relatively long: 700 ms. Accurate assessment of the, more relevant, foot to foot delay was not obtained, due to the relatively high noise level of the signals, but records such as Fig. 3*C* were consistent with the several hundred milliseconds latency observed in amphibian single smooth muscle cells (Yagi, Becker & Fay, 1987) and the delay between stimulus and the onset of phosphorylation in intact bovine tracheal smooth muscle (Kamm & Stull, 1985).

The increase in the fura2 ratio signal following rapid stretch suggests that at least some of this myogenic response is activated by an increase in cytoplasmic Ca^{2+}, but the possibility of additional, myofibrillar responses cannot be excluded. An interesting feature of such myogenic responses, observed in force records of various smooth muscles (Burnstock & Prosser, 1960), is the long (several seconds) latency, between the applied rapid stretch and the subsequent rise in cytoplasmic Ca^{2+} and force development.

Response to depolarization with high K^+

Depolarization with high K^+ increased cytoplasmic free Ca^{2+}, as previously reported (Morgan & Morgan, 1984*a*, *b*; De Feo & Morgan, 1985; Sumimoto & Kuriyama, 1986; Williams & Fay, 1986; Himpens & Casteels, 1987); graded

concentrations of K^+ caused graded changes in both fluorescence and tension. Pronounced phasic components of the fluorescence signal and tension were frequently, though not invariably, evoked by 140 mM-K^+, but usually not by lower concentrations (35 or 70 mM) of K^+. The origin of the phasic component of tension induced in other smooth muscles by agonists (Bohr, 1963; Steinsland, Furchgott & Kirpekar, 1973) or by high K^+ (Urakawa & Holland, 1964), has been ascribed to the intracellular release of Ca^{2+}, and may well have had the same origin in our experiments, since depolarization can release intracellular Ca^{2+} (Kobayashi, Kanaide & Nakamura, 1985; for review see Somlyo, 1985). The absence of the phasic component in response to lower concentrations of K^+ may have been due to accommodation of electromechanical coupling to slower, diffusion-limited rates of depolarization.

Transient reduction of (extracellular) Ca^{2+} during high K^+-induced contractions resulted in transient decreases of both cytoplasmic Ca^{2+} and force. We did not observe the large dissociation between Ca^{2+} and force found by Remboldt & Murphy (1986) who stimulated the swine carotid artery with high K^+, and on lowering the K^+ concentration observed a substantial decline in $[Ca^{2+}]_i$ (measured with aequorin) while force remained high. However, on the steep part of the pCa-tension curve (Endo, Kitazawa, Yagi, Iino & Kakuta, 1977), small and undetectable changes in free Ca^{2+} could cause easily detectable changes in force. This, among other considerations, complicates the interpretation of experiments in which the signals reporting, respectively, cytoplasmic Ca^{2+} and tension diverge, such as the occasionally faster decline of the force than the fluorescence ratio or the increase in tension during the decline in the fura2 ratio (Fig. 5*B*). It remains to be determined whether Ca^{2+}-independent changes in the light chain kinase–phosphatase equilibrium can account for these phenomena.

The effect of carbachol on polarized smooth muscle

Carbachol caused a transient increase in the fura2 ratio and force, consistent with much other evidence showing that excitatory agonists activate smooth muscle primarily by increasing cytoplasmic free $[Ca^{2+}]$. The decline of both $[Ca^{2+}]_i$ and force during the maintained presence of carbachol is probably due to the well-known desensitization of receptors to cholinergic agents (Paton, 1961). An interesting feature of carbachol-induced contractions was the delayed, slow rise of force after partial decline of the initial, phasic response (Fig. 7). This 'after-contraction' was occasionally accompanied by a similar, secondary rise in the fluorescence signal (Fig, 7*A*). However, even in such instances, the rise in the fura2 signal seemed disproportionately smaller than the magnitude of associated force, compared to the preceding, phasic force component. In numerous instances the fluorescence transient was barely or not at all detectable during the after-contraction (Fig. 7*B*); several mechanisms could be responsible for this phenomenon. 'After-contractions' could be the expression of a heterogenous cell population of phasic and tonic smooth muscle fibres (reviewed in Somlyo & Somlyo, 1968*b*), if the tonic fibres were developing force more slowly and were also more Ca^{2+} sensitive. In this case, a small rising fura2 signal of tonic fibres could be buried in the larger declining signal originating from phasic fibres.

An operationally equivalent, although functionally quite different, mechanism

giving rise to the after-contraction would be the presence of a second, slower regulatory mechanism operating in each smooth muscle fibre independently of light chain kinase, either more sensitive to Ca_4^{2+}–calmodulin or activated through another pathway. Submaximal concentrations of Ca_4^{2+}–calmodulin (Wagner & Ruegg, 1986) or phorbol ester (Chatterjee & Tejada, 1986) can induce, in skinned smooth muscles, slowed submaximal contractions accompanied by disproportionately low levels of light chain phosphorylation. Similar, slow mechanisms have been proposed in the context of a 'latch' mechanism (Murphy, Aksoy, Dillon, Gerthoffer & Kamm, 1983).

Myogenic stretch could also cause the after-contraction, the stretch in this instance being brought about by the initial, phasic contraction. This mechanism, although most highly developed in insect fibrillar muscle (Ruegg & Stumpf, 1969) has also been observed in mammalian cardiac and skeletal muscle (Bozler, 1982).

Lower sensitivity of fura2, perhaps due to its subcellular distribution, to Ca^{2+} influx than to Ca^{2+} release, could also account for the low or absent fluorescence signal associated with after-contractions, if the after-contractions were due to Ca^{2+} influx. We have no evidence of heterogeneous distribution or sensitivity of the dye, but we cannot dismiss this possibility, because after-contractions were absent in carbachol-stimulated muscles in Ca^{2+}-free depolarizing solutions (see below) and were inhibited by verapamil. Furthermore, although excitatory agents can open Ca^{2+} channels of smooth muscle in a voltage-independent manner (Somlyo & Somlyo, 1971; Tsien, Benham, Fox, Hess, Lansman, Lipscombe, McCleskey, Madison & Rosenberg, 1987), Ca^{2+} influx due to such channel opening may be delayed and persist longer than intracellular Ca^{2+} release.

Finally, we also have to entertain the possibility that the low fluorescence signal accompanying the after-contractions is an artifact of the experimental set-up, and the result of delayed diffusion of the drug to a region of the muscle sheet that contributes to force, but less, or not at all, to fluorescence. We consider this possibility unlikely, because similar after-contractions occur in conventionally mounted ileum smooth muscles, and, furthermore, would not have been eliminated by applying the drug in Ca^{2+}-free, depolarizing solution or by verapamil.

Pharmacomechanical coupling: the effect of carbachol in depolarized smooth muscle

Pharmacomechanical coupling is defined as the membrane potential-independent process mediating contraction or relaxation induced by drugs and transmitters. Initially demonstrated in fully depolarized smooth muscle (Evans, Schild & Thesleff, 1958), it was subsequently shown to operate in normally polarized smooth muscles (Somlyo & Somlyo, 1968*a*). The contribution of intracellular Ca^{2+} release to pharmacomechanical coupling was suggested by the drug-induced contractions in Ca^{2+}-free, high-K^+ solutions (Devine *et al.* 1972). The sarcoplasmic reticulum was subsequently shown to be the source of intracellular Ca^{2+} (Bond *et al.* 1984; Kowarski, Shuman, Somlyo & Somlyo, 1985) and recently inositol 1,4,5-trisphosphate has been implicated as the messenger of pharmacomechanical Ca^{2+} release from the sarcoplasmic reticulum (Somlyo, Bond, Somlyo & Scarpa, 1985; Walker, Somlyo, Goldman, Somlyo & Trentham, 1987). Ion flux and electrophysiological experiments suggest that, at least under certain conditions, Ca^{2+}

influx can also contribute to the rise in cytoplasmic Ca^{2+} in depolarized smooth muscles.

Carbachol increased cytoplasmic Ca^{2+} in depolarized smooth muscle, both in the presence and in the absence of extracellular Ca^{2+}. A rapid, initial fura2 transient and contraction were triggered under both conditions; in the presence of extracellular Ca^{2+}, this was followed by an after-contraction and, occasionally, a small and slow fura2 transient. We attribute the initial, rapid phase of the Ca^{2+} transient primarily to the release of intracellular Ca^{2+} from the sarcoplasmic reticulum. The fura2 signals induced by carbachol in high-K^+, Ca^{2+}-free solution also support the conclusion (Bond *et al.* 1984) that the release of intracellular Ca^{2+} by drugs is a major mechanism of pharmacomechanical coupling.

The large, rapid component of Ca^{2+} release seen in our experiments was not detected, with aequorin as the Ca^{2+} indicator, in depolarized ferret portal vein smooth muscle stimulated with phenylephrine (Morgan & Morgan, 1984*a*). However, in dispersed smooth muscle cells of the porcine coronary artery Sumimoto & Kuriyama (1986), using quin2, also noticed a similar increase in $[Ca^{2+}]_i$ upon adding acetylcholine to depolarized cells. It remains to be determined whether the different results are (1) species or smooth muscle specific, (2) related to the different agonists used (carbachol and phenylephrine) or (3) due to the properties of the different Ca^{2+} indicators.

The sources of the second component of the fura2 signal and the possible causes of its being frequently small or undetectable are likely to be the same in depolarized as in polarized smooth muscles. The presence of this component in depolarized smooth muscles indicates that it is not due to depolarization of the surface membrane and Ca^{2+} influx through voltage-operated channels. In view of the abolition of both the delayed fura2 signal and the accompanying after-contraction by Ca^{2+}-free solutions and by verapamil, it is tempting to infer that it is due to the influx of extracellular Ca^{2+}. However, rapid depletion of Ca^{2+} from some regions of the junctional sarcoplasmic reticulum or uncoupling of a mechanism responsible for slow, intracellular release could also abolish the after-contraction.

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