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RELEASE AND RECYCLING OF CALCIUM BY THE SARCOPLASMIC RETICULUM IN GUINEA-PIG PORTAL VEIN SMOOTH MUSCLE

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SUMMARY

1. The amplitude of interrupted contractions evoked by noradrenaline or caffeine in Ca^{2+}-free, high-K^+ solutions containing EGTA or La^{3+} was determined in small (40–60 μm thick) bundles of guinea-pig portal anterior mesenteric vein. Interrupted contractions were produced by removing the stimulating agent as soon as the amplitude of the tension record reached its peak. The distribution of intracellular Ca^{2+} was determined, with electron probe X-ray microanalysis, in cryosections of preparations frozen in the relaxed state and at the peak of noradrenaline-induced contractions.

2. Interrupted contractions of maximal or near-maximal amplitudes could be evoked every 2 min for up to 15 min in the virtual absence of extracellular Ca^{2+}. If noradrenaline was allowed to remain in the solution throughout the period of spontaneous relaxation, a subsequent contraction could no longer be evoked in the absence of extracellular Ca^{2+}. Interrupted contractions, similar to those evoked by noradrenaline, could also be stimulated by caffeine.

3. The amplitude of reproducible interrupted contractions in Ca^{2+}-free, high-K^+ solution was graded with noradrenaline concentration.

4. The ability of these smooth muscles to contract repeatedly and maximally in Ca^{2+}-free solutions indicates the recycling of Ca^{2+} released from an intracellular store. The occurrence of these contractions in high-K^+ (depolarizing) solutions supports the conclusion (Devine, Somlyo & Somlyo, 1972) that the release of intracellular Ca^{2+} is one of the mechanisms of pharmacomechanical coupling.

5. The number of subplasmalemmal regions in which high Ca concentrations (> 10 mmol/kg dry wt.) were detected, with approximately 75 nm diameter electron probes, was reduced in muscles frozen at the peak of contraction, from 4·7/cell periphery in the relaxed to 1·4/cell periphery in the contracted preparations.

6. In freeze-substituted smooth muscles, in which the membranes of the junctional sarcoplasmic reticulum could be visualized, the regions containing high Ca were identified as part of the sarcoplasmic reticulum (s.r.), indicating that the s.r. is the store from which noradrenaline and caffeine release Ca^{2+}.

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INTRODUCTION

The sarcoplasmic reticulum (s.r.) in smooth muscle (Somlyo, Devine, Somlyo & North, 1971; Devine *et al.* 1972; Somlyo, 1980; Gabella, 1983) can accumulate Ca^{2+} or Sr^{2+} (Somlyo & Somlyo, 1971; Somlyo, Somlyo, Shuman & Endo, 1982), and smooth muscle can contract in the absence of extracellular Ca^{2+} (Bozler, 1969; Somlyo *et al.* 1971; Droogmans, Raeymaekers & Casteels, 1977). Neurotransmitters, such as acetylcholine or noradrenaline (Deth & Casteels, 1977; Endo, Kitazawa & Yagi, 1980; Itoh, Kuriyama & Suzuki, 1983) release the same pool of intracellular Ca^{2+} as caffeine, an agent known to release Ca^{2+} from the s.r. (Weber & Herz, 1968), and this caffeine-releasable pool of Ca^{2+} has properties similar to those of the s.r. in striated muscle (Endo, Yagi & Iino, 1982). Such observations lead to the view that the s.r. regulates intracellular Ca^{2+} in smooth, as in striated, muscle (for review see Johansson & Somlyo, 1980). However, after a single contraction in Ca^{2+}-free solutions, smooth muscle generally fails to respond or responds minimally to subsequent stimulation and, therefore, it has been questioned whether intracellular Ca^{2+}, once released, can be recycled (Casteels, Raeymaekers, Suzuki & van Eldere, 1981). Furthermore, high concentrations of Ca in the s.r. have been previously demonstrated only in Ca^{2+}-loaded smooth muscles (Popescu & Diculescu, 1975; Somlyo, Somlyo & Shuman, 1979; Somlyo *et al.* 1982), and the release of Ca^{2+} from the s.r. has not been directly demonstrated. In this study, we present evidence of recycling of intracellular Ca^{2+} indicated by repeated, near-maximal contractions in the absence of extracellular Ca^{2+} and show, through electron probe analysis of cryosections, the release of Ca from the s.r. Preliminary reports of some of these findings have been presented at the FASEB Symposium on Recent Advances in Arterial Wall Research, 1983 (Somlyo, Somlyo, Bond, Kitazawa, Shuman & Wasserman, 1984*a*; Somlyo, Somlyo, Kitazawa, Bond, Shuman & Kowarski, 1984*b*).

METHODS

Dissection

Male guinea-pigs (250–400 g) were stunned and bled. About 1 cm of the portal vein was dissected and cut longitudinally. The open segment was fixed with pins in a silicon-based chamber to visualize the directions of muscle bundles under a stereomicroscope equipped with dark field illumination. Strips of the longitudinal muscle bundles were cut and transferred to the Perspex dissecting chamber and, to facilitate diffusion, the adventitia was carefully removed by dissection with small razor knives. The final strips were 20–60 μm thick (Pl. 1), 100–250 μm wide and 1–2 mm in length (for the physiological experiments) or 3–4 mm long (for rapid freezing).

Contraction measurements

The preparations were transferred to a Perspex trough with a volume of 0·7 ml and the ends of the bundles were tied with monofilament silk to one end of the trough and to a force transducer (AE801, AME, Horten, Norway). Solutions were exchanged very rapidly by perfusion. The bundles were set in a slightly stretched state.

The temperature of the solutions was maintained at 25 °C by circulating water.

Solutions

The normal external solution contained (in mM): NaCl, 143·5; KCl, 4; Ca methanesulphonate, 2; Mg methanesulphonate, 2; glucose, 5·6 and HEPES$^-$ (*N*-2-hydroxyethylpiperazine-*N*′-2′-

ethanesulphonic acid), 5; neutralized with Tris (Tris-(hydroxymethyl)-aminomethane) to give a pH of 7·4 at 25 °C and a total osmolarity of 320 mosmol.

The depolarizing, high-K^+ or high-Rb^+, solutions were made by replacing all of the NaCl in the normal external solution with equimolar KCl or RbCl and adding 43·8 mM-sucrose, to increase the osmolarity to 363·3 mosmol, and prevent swelling of the smooth muscle cells (Jones, Somlyo & Somlyo, 1973). In the Ca^{2+}-free solution, Ca methanesulphonate was omitted and Tris–EGTA (ethyleneglycol-bis-(β-aminoethylether)*N-N'*-tetraacetic acid) or $LaCl_3$ was added, while the osmolarity was adjusted to that of the normal or depolarizing solution. $MgCl_2$ was substituted for Mg methanesulphonate in the La^{3+}-containing solutions. Caffeine was added to the solution without adjusting the osmolarity because the surface membrane is reasonably permeable to the drug.

Freezing, cryosectioning and electron probe analysis

3–5 mm long muscle bundles were mounted vertically with one end attached to a tension transducer and the other end tied to a small hook held by fine forceps. The muscle was incubated at 23–25 °C in a beaker of the appropriate solution which was removed just prior to freezing by shooting up a 200 ml beaker of rapidly stirring supercooled Freon (-164 ± 3 °C), at the desired moment while monitoring tension.

The experimental protocol followed that of the comparable tension measurements. Paired bundles were contracted for 10 min in 2 mM-Ca^{2+}, high-K^+ solution and relaxed for 5 min in 0 Ca^{2+}, 20 mM-La^{3+}, high-K^+ solution to obtain relaxed smooth muscles under standard conditions not affected by spontaneous activity. 4% polyvinylpyrrolidone (PVP), average mol.wt. 40000, was added as a cryoprotectant 4 min before freezing. The muscles were rapidly frozen for electron probe analysis, either in the resting state or at the peak (20–30 s after the addition of the drug) of a noradrenaline- (30×10^{-6} M) induced contraction. In some of the experiments, high Rb^+ was used instead of high K^+, to minimize measurement errors due to the overlap of the K^+K_β and the $Ca^{2+}K_\alpha$ peaks which could occur due to changes in detector resolution or calibration. A computer fitting routine was developed while these experiments were in progress to eliminate these errors (Kitazawa, Shuman & Somlyo, 1983), and consequently the later experiments were carried out in high-K^+ rather than Rb^+.

Transverse cryosections, approximately 100 nm thick, were cut at -130 °C, placed on carbon films supported on 200 mesh copper grids, freeze dried and carbon coated prior to analysis. The methods for freezing and for cryoultramicrotomy have been published in detail (Somlyo, Shuman & Somlyo, 1977; Somlyo & Silcox, 1979; Karp, Silcox & Somlyo, 1981).

The experimental apparatus used for electron probe analysis has been previously described (Kitazawa, Somlyo & Somlyo, 1984). Analysis was performed on a liquid nitrogen cooled stage (Gatan Inc.) at -100 to -105 °C, and the residual H_2O vapour in the column was monitored with a Spectramass 1000 mass spectrometer; the residual H_2O pressure ($3–5 \times 10^{-9}$ torr) was always below the vapour pressure of ice at the cold stage temperature. In this manner specimen etching due to the deposition of ice (Somlyo & Shuman, 1982) was eliminated. The method used for quantitation in thin cryosections was based on that proposed by Hall (for review, Hall & Gupta, 1983), and relates concentrations to the ratio of characteristic/continuum X-rays (Shuman, Somlyo & Somlyo, 1976; Kitazawa *et al.* 1983), and is corrected for errors due to K^+–Ca^{2+} peak overlap with changes in detector calibration (Kitazawa *et al.* 1983). All results are expressed in mmol/kg dry wt.

Diffusion measurements

To ascertain that the time allowed for diffusion was sufficient to deplete the extracellular space of Ca^{2+} and to permit the entry of EGTA or La^{3+} throughout the fibre bundle, the diffusion constant for Ca^{2+} was estimated and La^{3+} entry was monitored. The diffusion constant for Ca^{2+} in the muscle sheet of guinea-pig portal vein was estimated in a manner similar in principle to that used by Kato, Ogasawara & Osa (1982) for determining the diffusion of Ca^{2+} in rat uterus. Briefly, the time course of the change in amplitude of twitches (Fig. 1) evoked by electrical stimuli (3 ms duration) and of K^+ contractures was followed after a sudden decrease in the external [Ca^{2+}]. The effective diffusion constant for Ca^{2+} was $1·1 \times 10^{-6}$ cm^2/s. This was estimated from the curve obtained by reducing Ca^{2+} to 0 (Fig. 2) and the Ca^{2+}–K^+ contraction relationship, using the theoretical curve of the diffusion of Ca^{2+} in a muscle sheet, as shown in fig. 10 of Kato *et al.* (1982), calculated according to eqn. 4.18 of Crank (1975) and is similar to the value reported ($1·7 \times 10^{-6}$ cm^2/s) by Keatinge

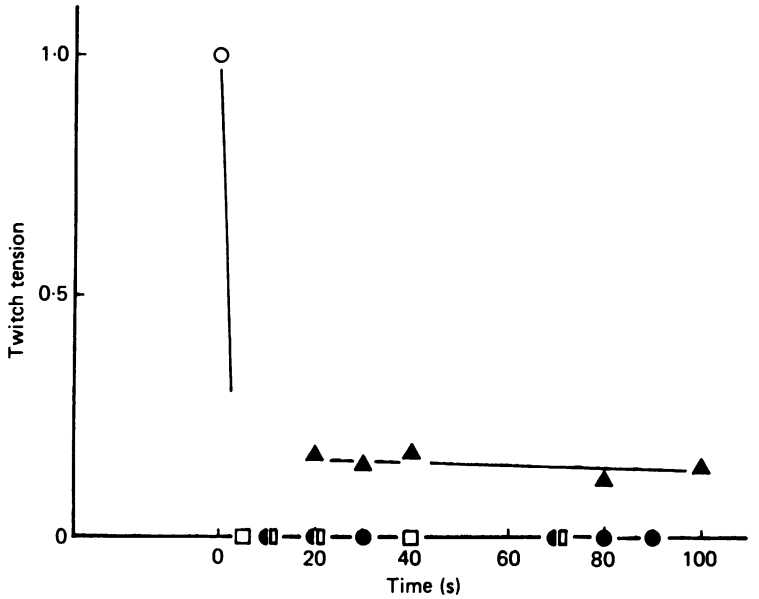

Fig. 1. The time course of the decline in twitch tension height of longitudinal muscle strips of portal vein after a sudden decrease in Ca^{2+} concentration. The bundle was stimulated by an electrical pulse of 3 ms duration at the times indicated after each change in Ca^{2+} concentration. Similar results were obtained in three experiments. The twitch height values are normalized to (1·0) that in the normal external solution containing 2 mM-Ca^{2+}. The thickness of the strip was 50 μm. ○, 2 mM-Ca^{2+}; ▲, 0·5 mM-Ca^{2+}; ●, 0 Ca^{2+}; □, 1 mM-EGTA.

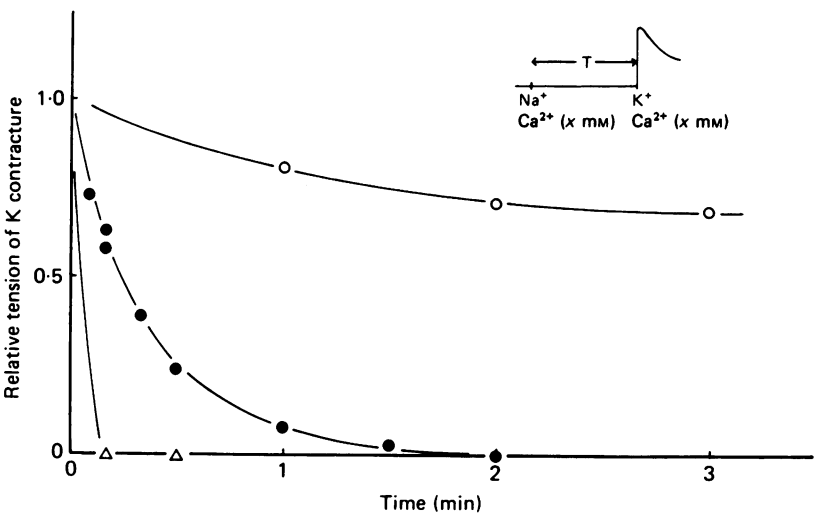

Fig. 2. The time course of the decline in size of the peak K^+ contracture amplitude following a rapid decrease in Ca^{2+}. K^+ contractions were developed by exchanging the normal high-Na^+ solution for the high-K^+ depolarizing solution. The Ca^{2+} concentration was decreased and K^+ contractions were produced as a function of time. K^+ contraction was normalized to the response at 2 mM-Ca^{2+} (1·0). The thickness of the strip was 60 μm. The effective diffusion constant of Ca^{2+} in the tissue was estimated as $1{\cdot}1 \times 10^{-6}$ cm^2/s, if the average Ca^{2+} concentration in the extracellular space was regarded as one-tenth of the normal concentration at 7 s after reduction of Ca^{2+}, as estimated from the curve at 0. ○, 0·2 mM-Ca^{2+}; ●, 0 Ca^{2+}; △, 1 mM-EGTA.

(1972), but considerably larger than that (3×10^{-7} cm^2/s) of Kato *et al.* (1982). We note, as a simpler test of the rapid diffusion of Ca^{2+} out of the extracellular space, that the addition of 1 mM-EGTA abolished the twitch tension and K^+ contracture within 10 s.

A second type of experiment was performed to determine the adequacy of La^{3+} diffusion into the extracellular space throughout the entire thickness of the strip. Vascular strips were exposed for 2 min to the identical solutions (containing La^{3+}) used for the experiments designed to demonstrate Ca^{2+} release, and then frozen. X-ray spectra of the extracellular space obtained from cryosections showed the presence of La^{3+} across the entire strip (Pl. 2). Therefore, we conclude that the 2 min interval in, respectively, Ca^{2+}-free (EGTA- or La^{3+}-containing) solutions was sufficient to deplete the extracellular space of Ca^{2+} and/or to deliver a high extracellular La^{3+} concentration throughout the preparation, while minimizing the loss of intracellular Ca^{2+} that occurs during longer incubation in Ca^{2+}-free solutions (Sparrow & Simmonds, 1965; Keatinge, 1972).

Conventional electron microscopy

Conventional fixation was performed by glutaraldehyde and osmium followed by block staining with aqueous uranyl acetate and embedding in Spurr's resin; sections were stained on the grid with aqueous Pb citrate.

The method for freeze substitution has been previously described (Franzini-Armstrong, Heuser, Reese, Somlyo & Somlyo, 1978). To avoid the loss of Ca^{2+} during sectioning, 100 nm sections were cut serially on glycerol (Ornberg & Reese, 1980; Chiesi, Ho, Inesi, Somlyo & Somlyo, 1981). Alternate sections were either kept unstained for electron probe analysis or stained with aqueous Pb citrate, to permit the identification of the s.r.

Electron micrographs were taken on a Philips EM400 or Zeiss 109 electron microscope.

RESULTS

Contractions of smooth muscle in the absence of extracellular Ca^{2+}

After evoking a K^+ contracture with Ca^{2+}-containing (2 mM) solution and replacing it for 2 min with a high-K^+, 10 mM-EGTA (Ca^{2+}-free) solution, noradrenaline (30×10^{-6} M) could still evoke a contraction of approximately the same size as the K^+ contracture (Fig. 3, upper trace) or the rapid component of a maximal noradrenaline-induced contraction in the presence of 2 mM-Ca^{2+} (not shown). If the drug was left in the solution throughout the period of spontaneous relaxation and then removed, its repeated addition, in the absence of extracellular Ca^{2+}, could not evoke a second contraction. This result is similar to that obtained in earlier studies of comparable design, in which drug-induced contractions in Ca^{2+}-free solutions were either non-repeatable, or repeated exposures to the drug produced only very small responses (Casteels *et al.* 1981).

Two modifications of the experimental design led to repeated, large contractions in the absence of extracellular Ca^{2+}. The first was to remove the drug at the peak of the induced contraction, rather than allowing it to remain in contact with the muscle throughout relaxation. This procedure is analogous to the interrupted contractions induced by caffeine in skinned skeletal muscle fibres in which re-uptake of Ca^{2+} into the s.r. occurs, if caffeine is removed before the released Ca^{2+} is lost to the bathing solution (Ford & Podolsky, 1972). The second modification was to include La^{3+} in the solution, instead of 10 mM-EGTA to further reduce the loss of cellular Ca^{2+} into the extracellular medium (Deth & van Breemen, 1977). Under these conditions, near-maximal contractions could be evoked repeatedly by noradrenaline, as shown in Fig. 3. Similar results were obtained with caffeine as a stimulating agent (Fig. 3, lower trace). Further increasing the concentration of extracellular La^{3+} to

10 mM resulted in the first several interrupted noradrenaline contractions being greater than the K^+ contracture in the presence of Ca^{2+} and in the long persistence of, reproducible, interrupted contractions of undiminished amplitude in the absence of extracellular Ca^{2+} (Fig. 4). A summary of six similar experiments (Fig. 5) shows the slow decline of the interrupted contractions in Ca^{2+}-free, 10 mM-La^{3+}-containing

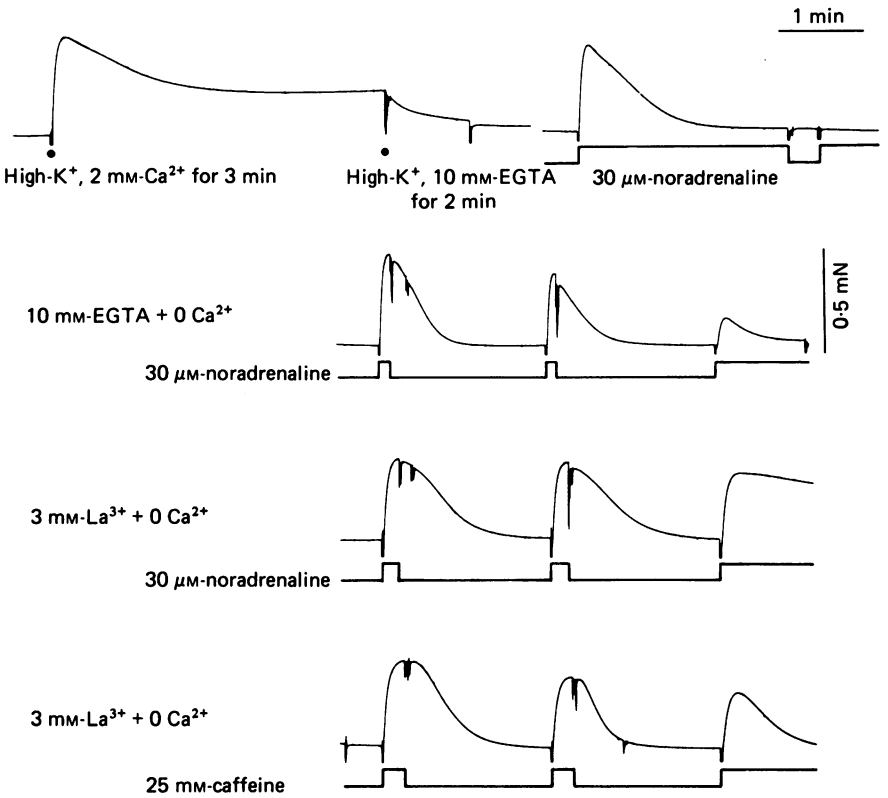

Fig. 3. Internal recycling of Ca^{2+} indicated by repeatable near maximal contractions in Ca^{2+}-free solution when noradrenaline or caffeine are removed at the peak of the force transient. The muscles were first contracted in a high-K^+, 2 mM-Ca^{2+} solution for 3 min, followed by high-K^+, 0 Ca^{2+} for 2 min before the first addition of noradrenaline. In the two upper traces, the high-K^+ solution contained 10 mM-EGTA, whereas in the two lower traces, 3 mM-La^{3+} was added to retard Ca^{2+} efflux from the cells.

solutions, and indicates that contractions of greater than or the same size as the normal K^+ contracture (in 2 mM-Ca^{2+}-containing solutions) can be elicited for approximately 15 min when the strips are stimulated every 2 min with noradrenaline.

The lack of effect of the interval between stimuli on contractions in Ca^{2+}-free solution

Because the previous experimental protocol involved 2 min intervals between repeated exposures to stimuli, it seemed possible that this interval was necessary for the return of intracellular Ca^{2+} to the release sites. That this was *not* the case is indicated in Fig. 6 which shows that repeated contractions, comparable in amplitude

to those evoked every 2 min, can be observed even when the time between stimuli is reduced to 36 s or less, and even before complete relaxation of the muscle has taken place.

Relationship between the extent of loading of the Ca store and the subsequent contraction in the absence and presence of extracellular Ca^{2+} and Na^{+}

The amount of Ca^{2+} released from the Ca store in the absence of extracellular Ca^{2+} could be graded, as shown by the sigmoid noradrenaline dose–response curve (Fig. 7). At concentrations of less than 10^{-5} M, the store was not depleted, as indicated

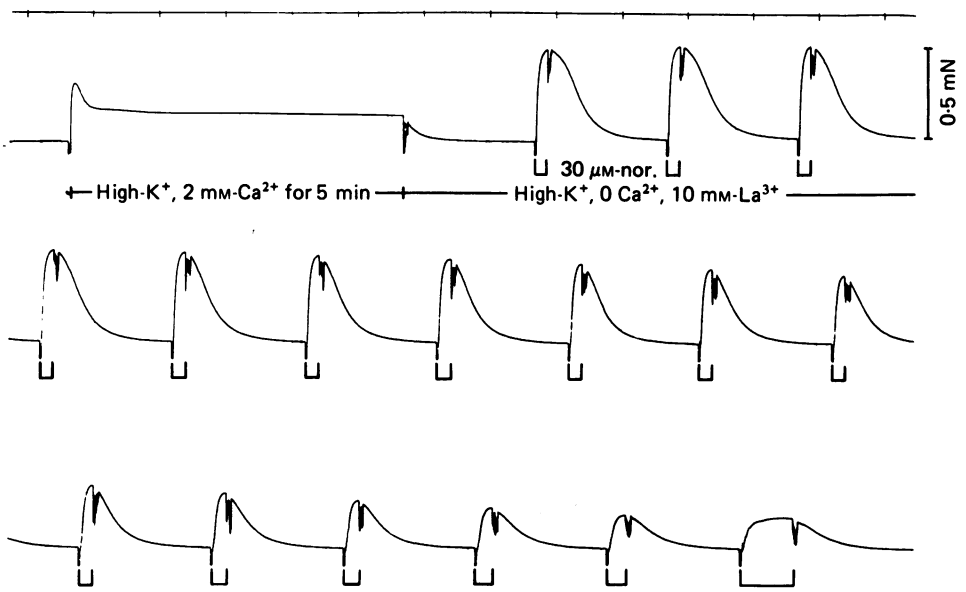

Fig. 4. Repeated contractions induced by noradrenaline in Ca^{2+}-free, 10 mM-La^{3+} solution. The longitudinal muscle of guinea-pig portal vein was first contracted by the high-K^{+}, 2 mM-Ca^{2+} solution for 5 min, and then relaxed by removal of Ca^{2+} and simultaneous addition of 10 mM-La^{3+}. The fibres were contracted by 30 μM-noradrenaline (nor.). The drug was then rapidly removed, as soon as the contraction reached its peak. Several interrupted contractions induced by the repetitive application of noradrenaline every 2 min were larger than the K^{+} contraction in the presence of 2 mM-Ca^{2+}. Time scale: 1 min interval.

by the size of a subsequent contraction elicited by a maximal dose of noradrenaline (3×10^{-5} M). The curve showing the remaining Ca^{2+} in the store is a mirror image of the noradrenaline dose–response curve. Submaximal concentrations of caffeine or histamine used for the first contraction could substitute for noradrenaline and partially deplete the Ca^{2+} store.

The noradrenaline dose–response curve for muscles exposed for 15–20 s to a normal solution (high-Na^{+}, 2 mM-Ca^{2+}) prior to noradrenaline is plotted in the lower portion of Fig. 7 (●), and the Ca^{2+}-store loading procedure is shown on the right. The amplitude of the noradrenaline-induced contractions was slightly greater in the

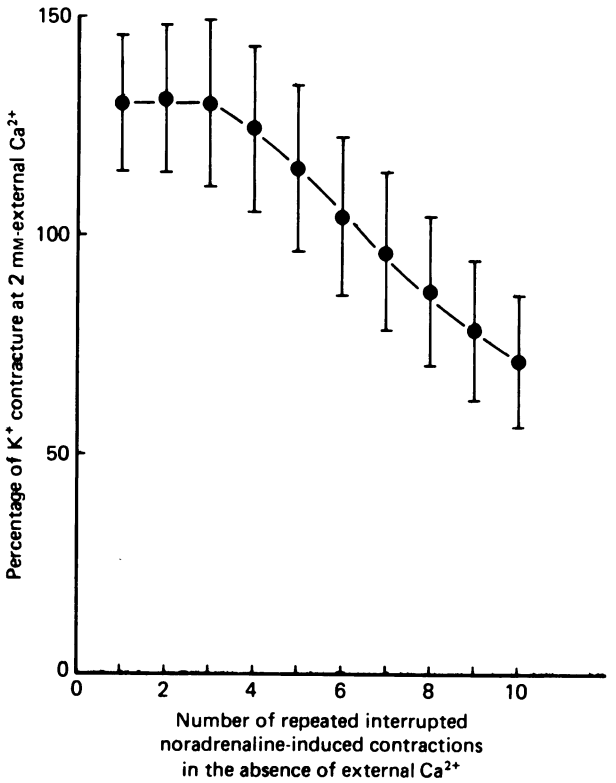

Fig. 5. The time course of the decline in size of repeated, interrupted noradrenaline-induced contractions in Ca^{2+}-free, 10 mM-La^{3+} solution. The results shown were obtained with longitudinal strips of portal vein muscle strips and show the average of data obtained from six animals. The experimental conditions were the same as in Fig. 4. The strips were stimulated with noradrenaline every 2 min. In only one strip out of the six was the first 30 μM-noradrenaline-induced contraction smaller than the K^+ contraction.

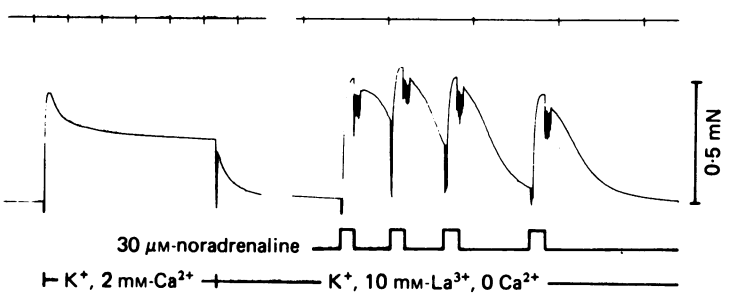

Fig. 6. Repeated contractions induced by noradrenaline in Ca^{2+}-free, 10 mM-La^{3+} solution with reduced interval between noradrenaline stimuli. The amplitude of the interrupted contractions was not greatly influenced by the interval between repetitive applications of 30 μM-noradrenaline nor by the degree of relaxation of the preceding contraction. Time scales: 1 min intervals.

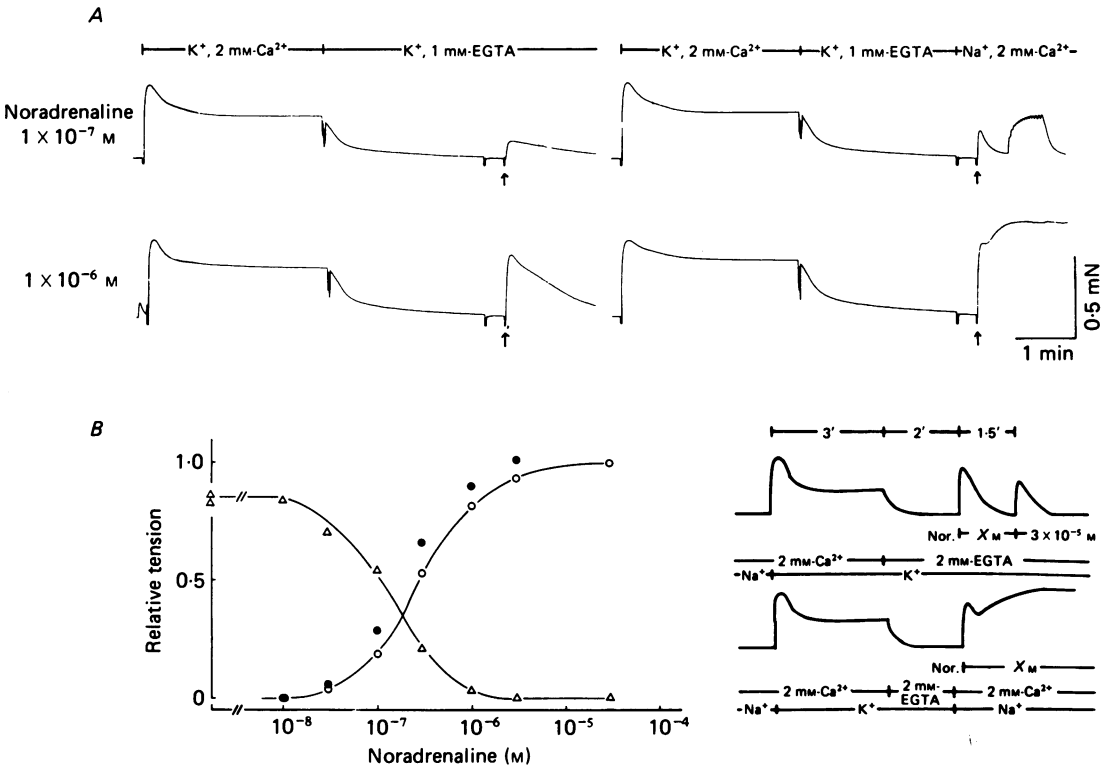

Fig. 7. *A*, the ability of increasing concentrations of noradrenaline to release more Ca^{2+} from the Ca^{2+}-loaded store in the absence of external Ca^{2+} (left panel) and in the presence of 2 mM-Ca^{2+} and high-Na^+ (right panel). Before the traces shown, Ca^{2+} was depleted from the store with high concentrations of noradrenaline or caffeine in Ca^{2+}-free solution. The muscles were subsequently immersed in the normal Ca^{2+}-containing external solution for 10 min and then a K^+ contracture (illustrated) was produced by exposure to high-K^+, Ca^{2+}-containing solution for 3 min in order to load the store. The muscles were relaxed by exposure to high-K^+, 0 Ca^{2+} (1 mM-EGTA) solution and 3 min later exposed to various concentrations of noradrenaline. In the right-hand panel, high-Na^+, Ca^{2+}-containing solution was introduced 20 s before addition of the drug. *B*, dose–response curves in high-K^+, 0 Ca^{2+}, 2 mM-EGTA (○) or high-Na^+, 2 mM-Ca^{2+} (●) for noradrenaline-induced contractions and resulting depletion of the Ca^{2+} store (△). The same procedures were used as described in *A* and shown on the right in *B*, except that a higher (2 mM) concentration of EGTA was added. As shown on the right, after each exposure to various concentrations of noradrenaline (nor.), 30 μM-noradrenaline was applied to measure the remaining Ca^{2+} in the store and the size of the resulting contraction was plotted, producing a curve (△) which is approximately a mirror image of the curve for the preceding contraction. The dose–response curve for the first (rapid) component of the noradrenaline-induced contraction in muscles exposed to high-Na^+, Ca^{2+}-containing solution for 15 s prior to addition of the drug is also plotted (●) and the protocol is shown on the right.

normal, than in the depolarizing, high-K^+, 0 Ca^{2+}-containing solution. The rate of spontaneous relaxation in the presence of the low noradrenaline concentration was slower in the high-K^+, 0 Ca^{2+}-containing solution than in the normal solution (Fig. 7, top trace). When recorded at a high gain, the second component of the (low and high concentration) noradrenaline-induced contraction in the high-Na^+,

Ca^{2+}-containing solution exhibited small oscillations of tension superimposed on the tonic contracture (Fig. 7, upper right trace).

Ca^{2+} release from the junctional s.r. demonstrated by electron probe analysis

In guinea-pig portal vein, as in other smooth muscles (Somlyo *et al.* 1971; Devine *et al.* 1972; Somlyo, 1980), a proportion of the s.r., the junctional s.r., is in close proximity to the plasma membrane, as shown in a transverse section of a conventionally

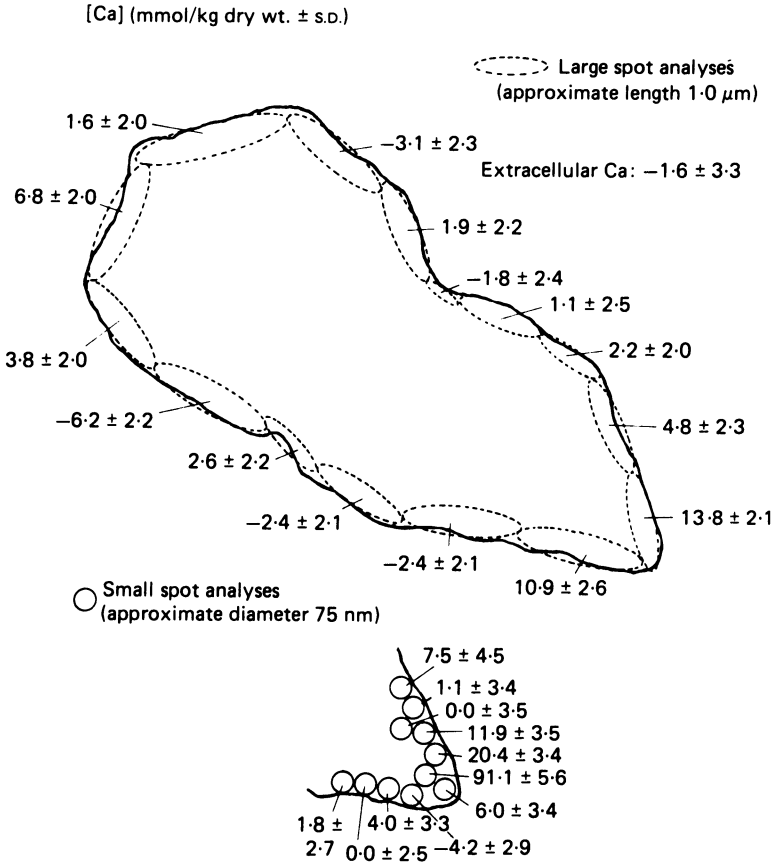

Fig. 8. Tracing of a transverse section of a smooth muscle cell from a cryosection, illustrating the size and locations of analyses done to measure Ca in the junctional s.r. The three areas with the highest Ca concentrations detected with the large astigmated spots were subsequently entirely analysed using small (75 nm diameter) probes. An example is shown in the inset that includes two regions localized with the astigmated probes, at the pointed end of the cell, which contained two 'hot spots' with Ca concentrations greater than 15 mmol/kg dry wt.

fixed and stained preparation (Pl. 3). An unstained cryosection (Pl. 4), of the type used for electron probe analysis, illustrates that while cell borders, nuclei and mitochondria can be readily visualized in these sections, the s.r. is not identifiable, due to its low contrast and the unavoidable presence of small ice crystals. Therefore, the experimental design illustrated in Fig. 8 was adopted for identifying regions

containing high concentrations of Ca (presumably the junctional s.r.) in the subplasmalemmal region of cryosections of resting and contracted smooth muscle. An initial survey of the Ca concentrations in the peripheral region of such cross-sections was made with large, astigmated probes of approximately 1 μm length. X-ray spectra were collected with these probes to an instrumental precision (S.D.) of approximately 2 mmol/kg dry wt. Any region that contained a Ca concentration of 8 mmol/kg or higher was identified as a 'high-Ca region' or, if no region in the periphery contained Ca at 8 mmol/kg or higher, the two or three regions showing the highest Ca concentration were explored. Small circular probes (approximately 75 nm in diameter) were next used to explore the entire length of the regions identified by the astigmated probes as containing (relatively) high concentrations of Ca. In this manner, 'hot spots' of Ca ($\geqslant$10 mmol/kg dry wt.) were identified around the periphery. For example, in the two regions in Fig. 8, identified with astigmated large probes as containing relatively high concentrations of Ca, two 'hot spots' containing more than 15 mmol Ca/kg were identified using the small diameter probes; one of these contained approximately 90 mmol Ca/kg. The average number of s.r. elements indicated by Ca 'hot spots' within a distance of 50 nm from the plasma membrane and Ca $\geqslant$ 15 mmol/kg was 2·9/cell, and for Ca $\geqslant$ 10 mmol/kg it was 4·7/cell. This incidence is in good agreement with the average number of junctional elements of s.r. (4·7/cell, $n = 60$ cells) found in conventionally fixed and stained preparations.

The frequency distribution of subplasmalemmal Ca concentrations in relaxed and contracted smooth muscles is shown in Fig. 9 and indicates a marked reduction in the number of 'hot spots' in cells of tissue frozen at the peak of a noradrenaline contraction as compared with the relaxed tissue. In the relaxed muscles there were 133 'hot spots' (4·8/cell) with a [Ca] $\geqslant$ 10 mmol/kg and 81, or 2·9/cell, with a [Ca] $\geqslant$ 15, while in the 23 contracted cells there were only 33 (1·4/cell) 'hot spots' with a [Ca] $\geqslant$ 10 mmol/kg or 9 (0·4/cell) with a [Ca] $\geqslant$ 15 mmol/kg.

The elemental concentrations measured in the 'hot spots' with small probes are shown in Table 1, and indicate that the average Ca content of these regions was approximately 28 mmol/kg dry wt. under conditions of high K^+ or high Rb^+. The few 'hot spots' found in the contracted cells had similar elemental concentrations to those in relaxed cells.

The Ca concentrations in the mitochondria and the central cytoplasm were not different in the relaxed and the contracted cells (Table 2). The precision of the cytoplasmic Ca measurements (about 0·6 mmol/kg dry wt.) is, however, insufficient to detect small changes in cytoplasmic Ca.

Because of the presence of caveolae (invaginations of the surface membrane) on the smooth muscle membrane and the necessity of locating the probes as close to the surface membrane as possible, the inclusion of some extracellular contribution to the X-ray spectra was unavoidable. This relationship is readily apparent upon inspection of conventionally fixed electron micrographs such as Pl. 3, and is also indicated by the presence of detectable concentrations of La in the analysis of the cell peripheries (Table 2) and in some junctional s.r. measurements. However, in the cytoplasm farther removed from the surface membrane, La was below detectable levels (Table 2), confirming the assumption that it is an extracellular marker. No correlation was found ($r = 0·09$) between La and Ca concentrations in any of the spectra obtained from the cell peripheries ($n = 863$). To further test whether the Ca 'hot

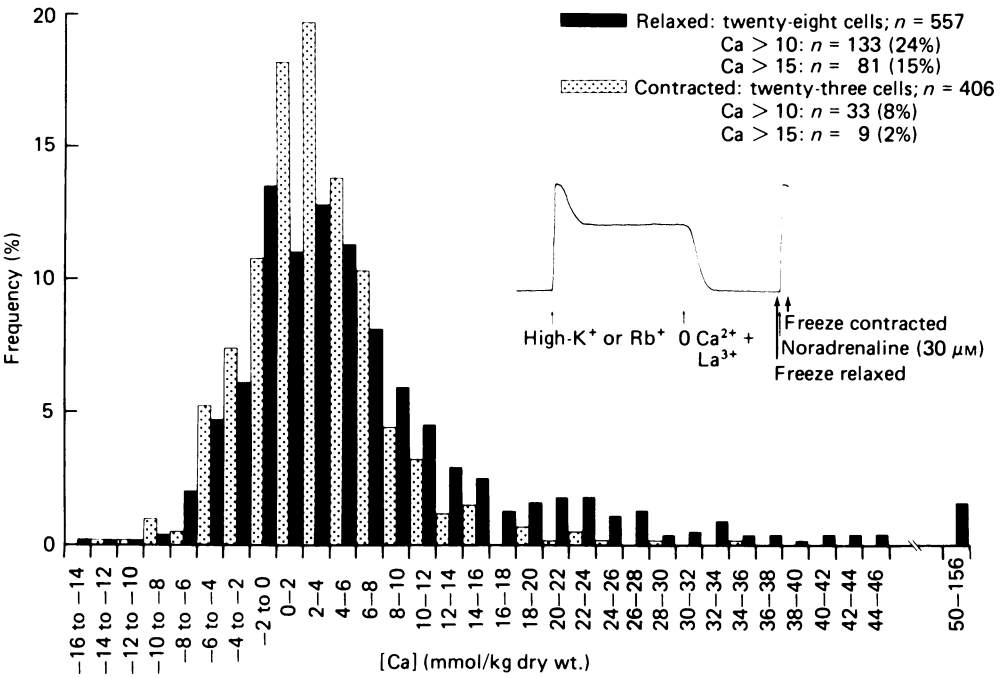

Fig. 9. Histogram showing the distribution of Ca concentrations measured with the small diameter probe analyses of the cell peripheries in the relaxed and contracted guinea-pig portal veins. The tension trace and the freezing protocol for the relaxed and contracted states are shown in the inset. Negative values reflect statistical fluctuations that are relatively large. In order to make the large number of measurements required, spectra were acquired for only 100 s each and the S.D. of the earlier (individual) measurements reached 3·9 mmol/kg dry wt.

TABLE 1. Elemental composition of the 'hot spots' in the junctional s.r. region in relaxed guinea-pig portal vein smooth muscle (±S.E. of mean mmol/kg dry wt.)

Element	Ca^{2+}-free, La^{3+}, high-K^+ soln. ($n = 37$)	Ca^{2+}-free, La^{3+}, high-Rb^+ soln. ($n = 44$)
Na	16·2±4·2	49·3±7·2
Mg	67·0±3·7	60·0±3·5
P	737·0±34·9	523·6±35·0
S	270·5±8·0	371·9±11·5
Cl	363·3±22·9	367·0±16·6
K	924·8±37·3	450·1±19·7
Ca	28·6±2·8	27·7±2·4
Rb	—	360·9±24·6

Muscles were contracted with 147·5 mM-K^+ or Rb^+ solution in the presence of 2 mM-Ca^{2+} for 10 min and relaxed by removal of Ca^{2+} and addition of 20 mM-La^{3+} for 5 min prior to freezing. Junctional s.r. included all analyses of the cell peripheries which had $[Ca^{2+}] \geqslant 15$ mmol/kg dry wt. measured with small diameter probes.

spots' were associated with the cell interior or the extracellular space only the [Ca] $\geqslant$ 15 mmol/kg, $n = 81$ in the relaxed muscles were plotted against the [La], and again the correlation coefficient ($r = 0{\cdot}01$) showed that there was no relation between the concentrations of the two elements. Consistent with an intracellular location, over 50% of the 'hot spots' did not contain detectable La.

In order to further verify that the 'hot spots' of Ca in the subplasmalemmal region represented the Ca content of the junctional s.r., we have performed electron probe analysis on unstained, freeze-substituted preparations from which alternate

TABLE 2. Elemental composition of mitochondria and cytoplasm in relaxed and in contracted guinea-pig portal vein

	Na	Mg	P	S	Cl	K+Rb	Ca	La
			(±S.E. of mean mmol/kg dry wt.)					
				Mitochondria				
Relaxed ($n = 59$)	18±1·7	58±1·7	623±26	314±11·1	132±9·2	544±18·0	0·7±0·3	0·5±0·3
Contracted ($n = 64$)	18±1·5	61±2·1	646±19	344±9·6	115±8·3	610±18·7	1·1±0·3	0·4±0·3
				Central cytoplasm				
Relaxed ($n = 67$)	27±1·6	56±1·8	252±8·8	285±10·3	286±10·3	759±15·7	0·8±0·2	1·0±0·5
Contracted ($n = 70$)	26±1·3	60±1·8	283±13·4	277±8·4	292±7·8	839±16·8	1·1±0·2	1·0±0·4
				Peripheral cytoplasm (Ca $\leqslant$ 15 mmol/kg dry wt.)				
Relaxed ($n = 467$)	37±1·2	59±0·9	411±7·8	338±3·8	383±5·7	783±10·8	2·8±0·2	24±1·6
Contracted ($n = 397$)	33±1·3	54±1·0	367±9·3	316±5·6	323±5·5	798±9·3	2·6±0·2	20±1·5

Paired muscles from five animals were contracted with 147·5 mM-K^+ or Rb^+ solution in the presence of 2 mM-Ca^{2+} for 10 min and relaxed by removal of Ca^{2+} and addition of 20 mM-La^{3+} for 5 min prior to freezing for the relaxed samples, or contracted with 30 μM-noradrenaline and frozen when maximum force was achieved (after 20–30 s).

sections were obtained and stained on the grids to permit the visualization of the membraneous structures in cells from particularly well-frozen regions. Although freeze substitution removes the diffusible elements, it permits the retention of at least some of the Ca in membrane-bound compartments, such as s.r. of striated muscle (Ornberg & Reese, 1980; Chiesi *et al.* 1981; Somlyo *et al.* 1982). A Pb-stained transverse section of a freeze-substituted, resting portal vein smooth muscle cell is illustrated in Pl. 5, and shows the presence of a membrane-bound tubule, i.e. the s.r. Electron probe analysis of the same region in the consecutive section that was not stained (to avoid the leeching out of Ca by the aqueous stain), shows the presence of a significant Ca peak, completing the identification of the 'hot spots' as elements of s.r. in smooth muscle. Similar experiments were done on thirteen subsarcolemmal tubules of s.r., and a significantly greater number of Ca counts was obtained from these regions compared with the adjacent cytoplasm or extracellular space.

DISCUSSION

Our major observation about the physiological properties of smooth muscle is that under appropriate experimental conditions, repeated, maximal or near-maximal contractions, indicative of recycling of intracellular activator Ca^{2+}, can be evoked by noradrenaline in the absence of extracellular Ca^{2+}. In earlier studies only single phasic contractions, or very small tonic contractions could be evoked in Ca^{2+}-free solutions (Devine *et al.* 1972; Deth & van Breemen, 1977; Casteels *et al.* 1981). The occurrence of the repeated contractions in Ca^{2+}-free solutions (this study) in a smooth muscle in which the volume of the s.r. is relatively small (approximately 2% of cell volume, unpublished observations), contrasts with expectations based on earlier studies which showed that relatively large (single) contractions in Ca^{2+}-free solutions occurred in those smooth muscles in which the s.r. volume was larger (5–7·5% of cell volume, Devine *et al.* 1972). Our conditions for producing large, reproducible contractions in Ca^{2+}-free solutions were the removal of the stimulating agent at the peak of the contraction ('interrupted contractions') and the inhibition of Ca^{2+} efflux by Na^+-free, La^{3+}-containing external solutions. It is probable that the retention of cellular Ca was also facilitated by conducting the experiments at room temperature, rather than at 37 °C (Somlyo *et al.* 1971; Keatinge, 1972). Such interrupted contractions were repeatable, whereas the continuous presence of noradrenaline prevented subsequent contractions, suggesting that prolonged exposure to the stimulating agent may cause a loss of cellular Ca, even in the presence of La^{3+}, and/or prevent the return of Ca^{2+} to the release sites. After an initial prolonged exposure to noradrenaline ($\sim$8 min) causing ^{45}Ca release (Deth & van Breemen, 1977), a noradrenaline contraction in the presence of ^{40}Ca is not accompanied by ^{45}Ca efflux on reapplication of the drug. This is consistent with the interpretation that the presence of the drug inhibits accumulation of Ca^{2+} into the release sites. In contrast, depolarization with high K^+ does not interfere with the uptake of Ca^{2+} into the internal store in rabbit ear artery (Casteels & Droogmans, 1981) or in the portal vein (this study).

The diversity of smooth muscles, in addition to experimental conditions, probably also influences the facility with which reproducible contractions can be evoked in the absence of extracellular Ca^{2+}. Some smooth muscles develop spontaneous, rhythmic contractions in Ca^{2+}-free (EGTA- and Na^+-containing) solutions in the absence of La^{3+} (Somlyo *et al.* 1971; Mangel, Nelson, Conner & Prosser, 1979). In contrast, even with the experimental protocol employed in the present study (10 mM-La^{3+}, K^+-substituted solution), the amplitude of cholinergically stimulated interrupted contractions of guinea-pig taenia coli did not persist as long in Ca^{2+}-free solutions (unpublished observations), although in this tissue the amount of intracellular Ca is also sufficient for a single, near-maximal contraction (Casteels & Raeymaekers, 1979). These differences may be due to the variable ability of different smooth muscles to conserve cellular Ca. The decline of contractions in Ca^{2+}-free solutions may also be due to failure of drug–receptor interactions and/or inactivation of the excitation–contraction coupling mechanism (for review, Johansson & Somlyo, 1980). On the other hand, the ability of La^{3+} to block noradrenaline-stimulated ^{45}Ca efflux is also variable: negligible in the rabbit ear artery (Droogmans *et al.* 1977) but effective in the rabbit aorta (Deth & van Breemen, 1977). Therefore, the La^{3+}-sensitive Ca^{2+} efflux pathways may also differ in different smooth muscles.

The repeated contractions observed in Ca^{2+}-free, high-K^+ solutions (Figs. 3–7) in which smooth muscles are depolarized, further show that pharmacomechanical coupling (activation of smooth muscle independent of changes in membrane potential, Somlyo & Somlyo, 1968), can involve the release of internal Ca^{2+} as well as Ca^{2+} influx (for review, see Johansson & Somlyo, 1980). This pharmacomechanical Ca^{2+} release in the absence of Ca^{2+} influx suggests that Ca^{2+}-induced Ca^{2+} release utilizing extracellular Ca^{2+} need *not* play a major role in excitation–contraction coupling, and indeed does *not* play a role in pharmacomechanical coupling in smooth muscle.

The question arises as to whether the repeated, interrupted contractions in the absence of Ca^{2+} represent a physiological mechanism of activation through the release of intracellular Ca^{2+} or, having been observed in La^{3+}- or EGTA-containing solutions, are only an extreme example of a 'laboratory phenomenon'. We consider that they reflect a physiological process, for the following reasons. First and foremost, although not exposed to La^{3+}, smooth muscle normally does not function in a Ca^{2+}-free environment. Physiological solutions (body fluids) contain approximately 1·2 mM-Ca^{2+}, that would reduce or inhibit net cellular Ca^{2+} loss. Secondly, the influx of Ca^{2+} during an action potential is insufficient to activate the contractile apparatus (Johansson & Somlyo, 1980; Itoh, Kuriyama & Suzuki, 1981), and contractions associated with rhythmic electrical activity are probably mediated by the release of intracellular Ca^{2+}. Thirdly, the sensitivity of the guinea-pig portal vein smooth muscle to noradrenaline, when in depolarizing high-K^+, 0 Ca^{2+} solution, was very similar to that observed in a normal physiological solution (high-Na^+, 2 mM-Ca^{2+}) (Fig. 7). Finally, physiologically released noradrenaline is also subject to rapid removal, and hence its effects are similar to our 'interrupted contractions'. Therefore, we believe that the type of excitation–contraction coupling mechanism evidenced by interrupted contractions in Ca^{2+}-free solutions reflects a physiological process that is at least capable of near-maximal activation of smooth muscle. We know of no evidence indicating whether Ca^{2+} influx also contributes to activation during brief or sub-maximal contractions.

Electron probe analysis of the intracellular source of activator Ca^{2+}: the s.r.

Electron probe analysis revealed, in relaxed smooth muscle, localized subplasmalemmal 'hot spots' containing Ca (approximately 28 mmol/kg dry wt.) far in excess of normal cytoplasmic concentrations (Somlyo *et al.* 1979; Bond, Somlyo, Shuman & Somlyo, 1983; present study). The number of such Ca sites was markedly reduced in contracted smooth muscles (Fig. 9). The high-Ca sites were localized in the subplasmalemmal region that contains the junctional s.r. and occurred in comparable numbers/cell periphery as the elements of the s.r. This, and the visualization of junctional s.r. containing high-Ca in freeze-substituted smooth muscles (Pl. 5), warrant the conclusion that the junctional s.r. is the compartment from which noradrenaline releases Ca^{2+}. The fact that the s.r. acts as a high-affinity sink for Ca^{2+} and similar cations (e.g. Sr^{2+}) has been previously demonstrated by the localization of these cations in the s.r. *in situ* (Somlyo & Somlyo, 1971; Popescu & Diculescu, 1975), by the isolation of an actively Ca^{2+}-accumulating s.r. fraction from smooth muscle (Raeymaekers & Hasselbach, 1981), and by the identification, in saponin-skinned smooth muscle, of a compartment that accumulates Ca^{2+} by an MgATP requiring mechanism and releases it under the influence of caffeine (Endo *et al.* 1982). The

detailed verification that the s.r. is the structure from which Ca^{2+} is released during contraction was considered necessary in this study, in order to exclude the possibility entertained by some authors (Deth & van Breemen, 1977; Haeusler, Richards & Thorens, 1981; for review, Daniel & Kwan, 1981), that the non-specialized plasma membrane may be the source of activator Ca^{2+} in smooth muscle.

The concentration of Ca measured with small diameter probes in the s.r. of smooth muscle (28 mmol/kg dry wt.) was considerably lower than the Ca content (approximately 110–120 mmol/kg dry wt.) in the terminal cisternae of frog s.r. (A. V. Somlyo, Gonzalez-Serratos, Shuman, McClennan & A. P. Somlyo, 1981). Some of the difference may reflect the lack of the Ca^{2+}-binding protein calsequestrin, in the s.r. of smooth muscle. This is unlikely to be the complete explanation, particularly since we have noted a proteinaceous content in the lumen of the s.r. in saponin-skinned smooth muscle (A. V. Somlyo, unpublished observation). It is probable, however, that our measurements underestimated the concentration of Ca in the s.r., because the size of the probes (approximately 75 nm) used and the 100–200 nm thickness of the cryosections would result in the analysis of a larger volume than that occupied by an s.r. tubule having the dimension (about 30–40 nm diameter) found in smooth muscle. The error due to the overlap of the probe with non-membraneous regions (i.e. cytoplasm) may be less than suggested by the difference between the probe and the s.r. diameters, because tubules of s.r. aligned along the plasma membrane in transverse sections are frequently longer than 75 nm. On the other hand, because the s.r. could not be directly visualized in the unstained cryosections, we cannot exclude the possibility that there are also other regions of s.r. that, like the longitudinal reticulum of skeletal muscle (A. V. Somlyo *et al.* 1981), contain lower concentrations of Ca than the 'hot spots'.

The mitochondrial Ca concentration in the resting smooth muscles was low, and it did not change significantly during contraction (Table 2). Because mitochondria can avidly accumulate Ca^{2+} when the free Ca^{2+} concentration rises to 10^{-5} M or higher, the present results support the conclusion (Somlyo, Somlyo, Shuman, Scarpa, Endo & Inesi, 1981; Somlyo *et al.* 1982) that cytoplasmic free Ca^{2+} normally remains below 10^{-5} M even in maximally contracting smooth muscle.

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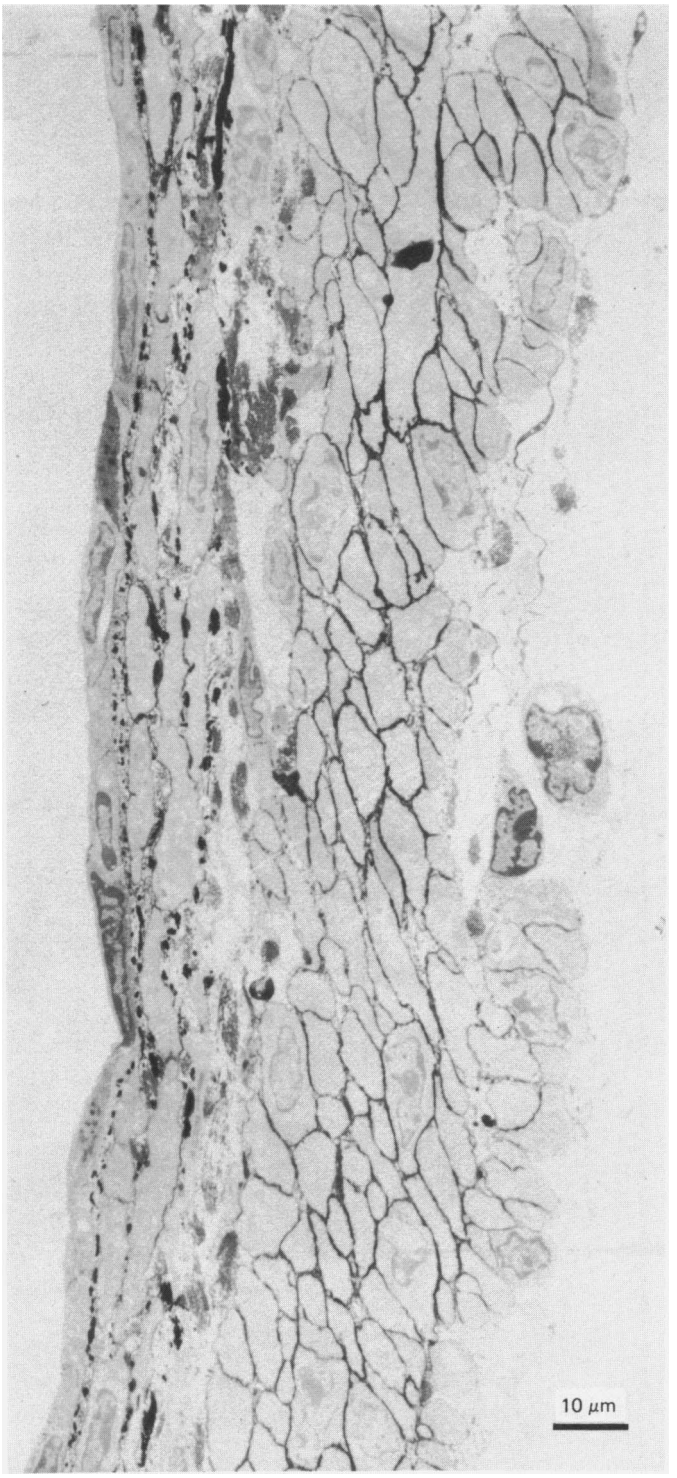

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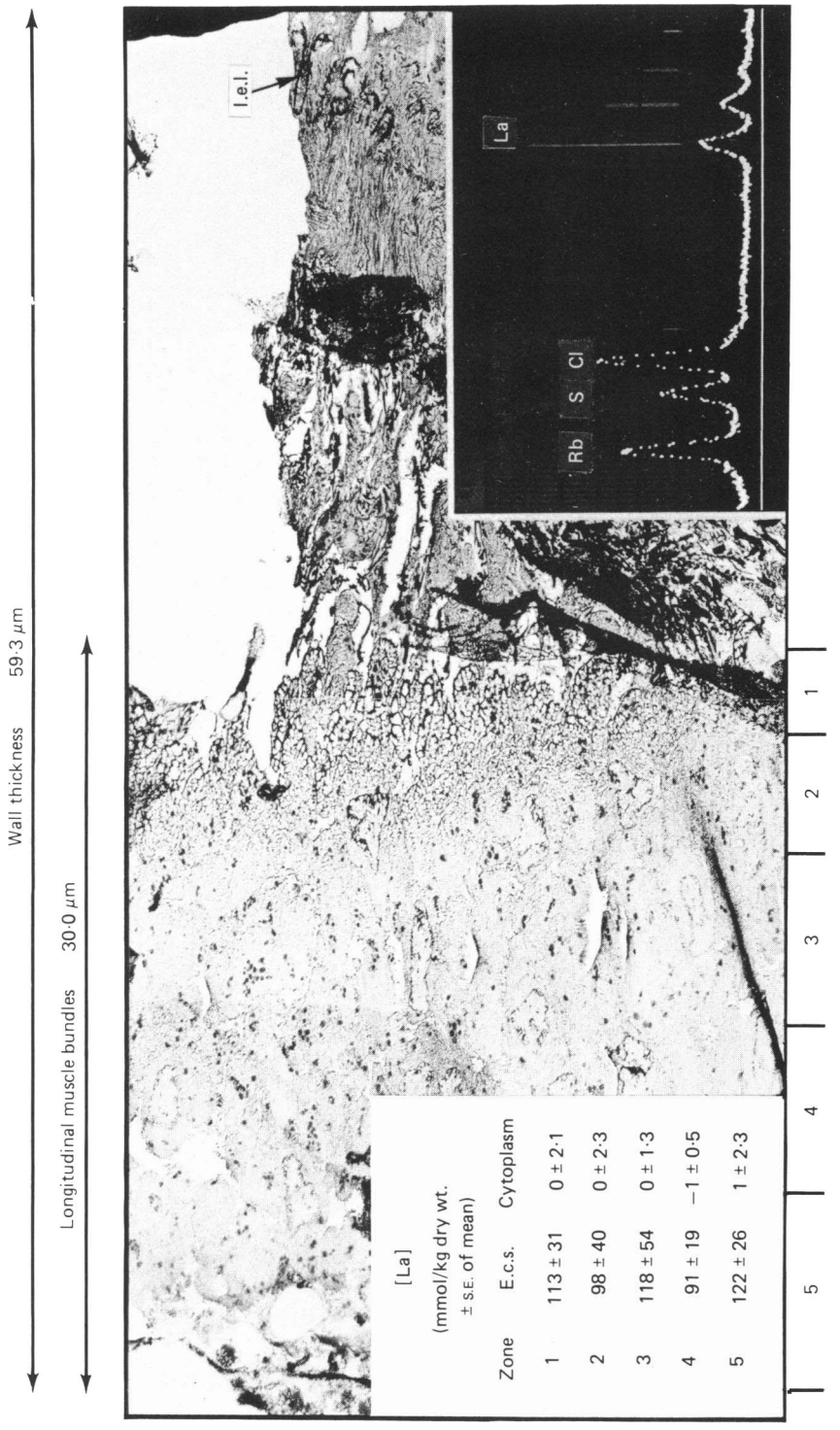

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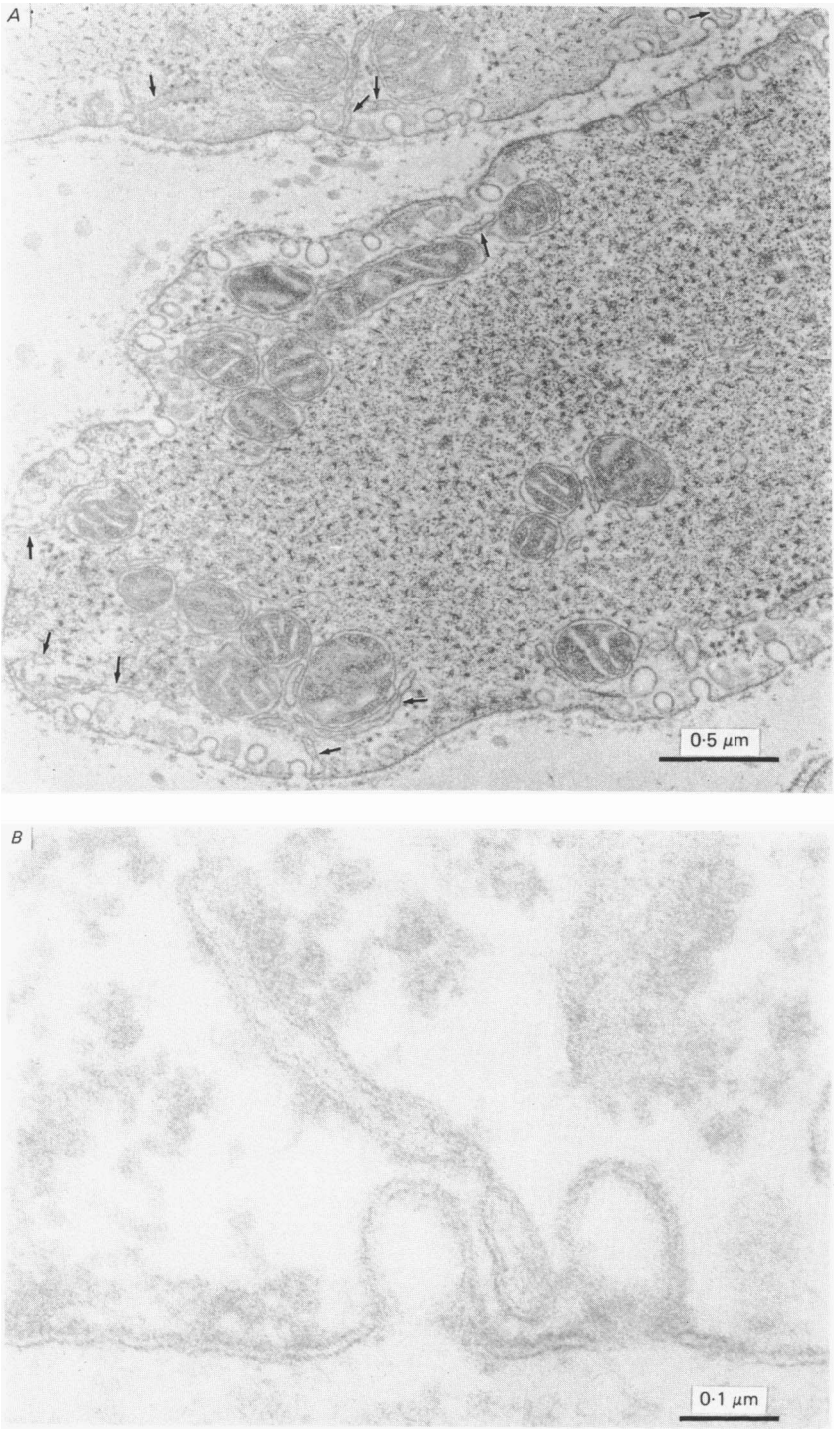
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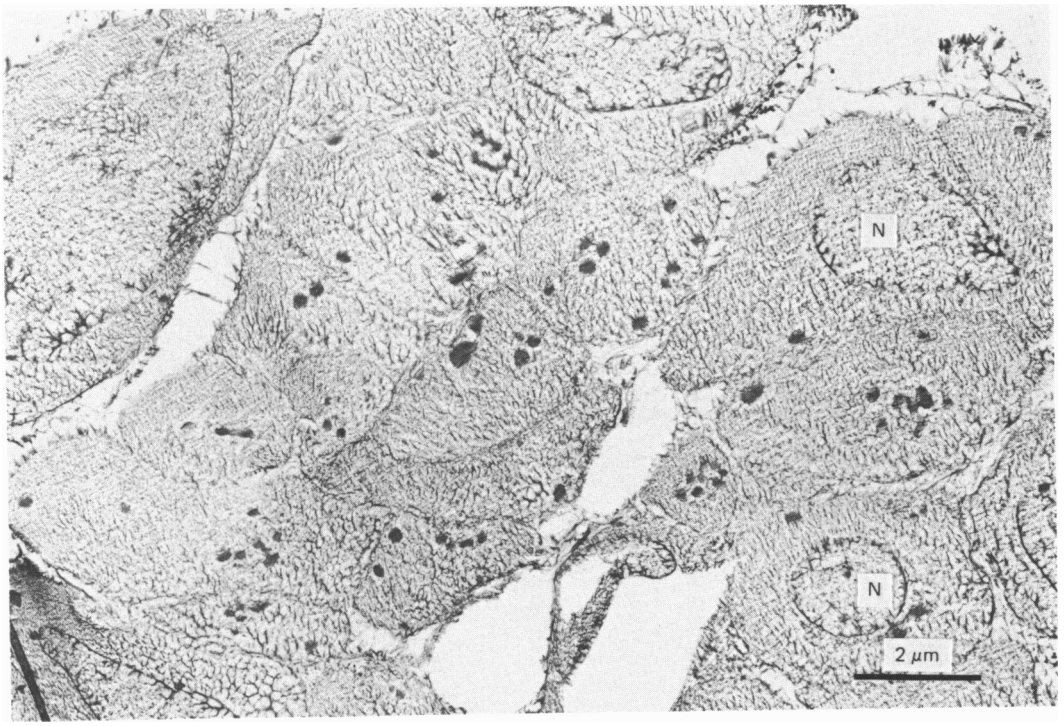
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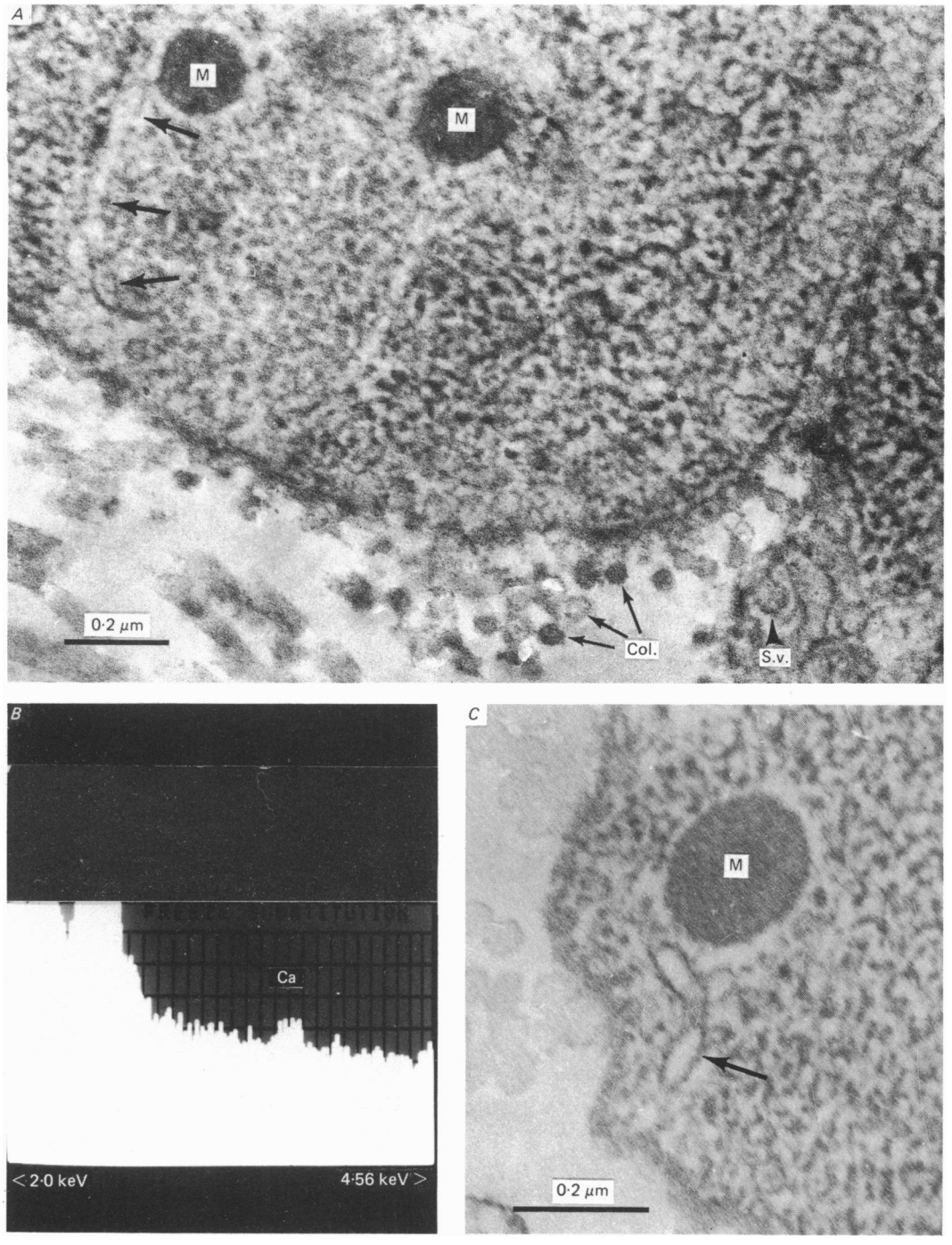
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EXPLANATION OF PLATES

PLATE 1

Low-magnification view of a transverse section of one of the longitudinal muscle strips of the portal vein used for the determination of the Ca^{2+} diffusion constant as shown in Fig. 1. The wall thickness is approximately 60 μm. The total force developed by this strip, based on the total number of cells, was 1·15 μN/cell cross-section or 1·2 kg/cm^2 cell. Tannic acid was used following osmium fixation to enhance the staining of the extracellular matrix.

PLATE 2

Transverse cryosection of a longitudinal strip of guinea-pig portal vein which, following a high-Rb^+, 2 mM-Ca^{2+} contracture, was relaxed in high-Rb^+, 0 Ca^{2+} containing 20 mM-$LaCl_3$ for 2 min and frozen. La penetration across the wall was followed by X-ray microanalysis of the extracellular space (E.c.s.) in the regions indicated on the abscissa. The mean concentrations of La across the tissue, as obtained from three different sections, are shown in the inset. Penetration of La into the extracellular space across the entire vessel wall was complete by 2 min. A typical X-ray spectrum of the extracellular space of a Rb^+-depolarized tissue shows large characteristic La X-ray peaks. Analysis of the cells indicated no significant uptake of La. I.e.l., internal elastic membrane.

PLATE 3

Transverse sections of conventionally fixed guinea-pig portal vein showing (arrows) the peripheral location of the sarcoplasmic reticulum (s.r.) in portions of two cells shown in *A*, and a surface coupling of s.r. approaching the cell membrane between two caveolae in *B*.

PLATE 4

Typical cryosection of a rapidly frozen longitudinal strip from the guinea-pig portal vein. The dark structures are mitochondria. N, nucleus.

PLATE 5

Sections of frozen guinea-pig portal vein processed by freeze substitution and cut on glycerol. In *A* the section was Pb stained to visualize the membranes and an element of s.r. is indicated by the arrows. An adjacent serial section was not Pb stained in order to retain Ca^{2+}, and an analysis of the same area as shown in *A* resulted in a significant Ca^{2+} peak as shown in *B*. The membranes in the unstained section are very faint. Another element of peripheral s.r. from freeze-substituted Pb-stained material is shown in *C*. M, mitochondria; Col., collagen; S.v., surface vesicle.