

CALCIUM TRANSIENTS EVOKED BY ELECTRICAL STIMULATION OF SMOOTH MUSCLE FROM GUINEA-PIG ILEUM RECORDED BY THE USE OF FURA-2

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SUMMARY

1. Intracellular free calcium levels were recorded in strips of longitudinal smooth muscle from guinea-pig ileum, by the use of the fluorescent calcium indicator Fura-2.

2. The resting intracellular free calcium concentration was estimated to be 210 nM. Many muscle strips showed spontaneous bursts of contractions, accompanied by bursts of calcium transients. Following these the calcium level often fell transiently below the resting level. The spontaneous transients were unaffected by tetrodotoxin (TTX) and atropine.

3. Field electrical stimulation of muscle strips evoked a series of calcium transients comprising: (i) an initial rise in free calcium, reaching a peak within 20–30 ms of stimulation, (ii) a second rise in calcium, beginning after a few hundred milliseconds, and finally (iii) a decline in calcium to below the resting level, persisting for a few seconds. The mean peak increase in free calcium above the resting level during components (i) and (ii) was, respectively, 130 and 200 nM. The mean decrease in free calcium during the third component was to 20 nM below the resting level.

4. The short-latency calcium transient required relatively long stimuli for activation, and was not blocked by TTX and atropine. The long-latency transient was selectively activated by brief stimuli, and was abolished by TTX and atropine. Thus, the short-latency component probably arose because of direct electrical stimulation of muscle fibres, while the long-latency component was due to stimulation of muscarinic nerves.

5. The first detectable increase in tension began about 100 ms after the peak of the initial calcium transient. Contractions associated with the long-latency calcium transient were much larger than those associated with the short-latency transient, even in muscle strips where the calcium levels were similar for both transients.

6. Removal of calcium in the bathing solution caused the resting intracellular calcium level to fall, following an initial rise accompanied by increased spontaneous transients. Electrically evoked contractions and calcium transients were abolished in

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calcium-free solution, and by the addition of verapamil or diltiazem to normal Krebs solution.

INTRODUCTION

Until recently, the only monitors suitable for recording rapid calcium transients in contractile tissues were aequorin and metallochromic dyes (Blinks, Wier, Hess & Prendergast, 1982; Thomas, 1982). Both of these probes are membrane impermeant, and therefore need to be physically introduced into cells, usually by microinjection. Although this procedure is possible with the small cells of smooth muscle (Fay, Shleven Granger & Taylor, 1979; Neering & Morgan, 1980), its difficulty seems to have deterred widespread use. Morgan and Morgan developed an alternative method, in which aequorin is introduced into smooth muscle cells following permeabilization of the cell membrane by a calcium-free solution (Morgan & Morgan, 1982, 1984*a, b*; DeFeo & Morgan, 1985; Bradley & Morgan, 1987). However, disadvantages of this technique include the drastic loading procedure, and a less than ideal sensitivity of the aequorin-calcium signals.

A more promising approach has recently become available with the development of a novel family of fluorescent calcium indicators (Grynkiewicz, Poenie & Tsien, 1985). These offer an important advantage in that they can be loaded as a membrane-permeant ester derivative, which is then hydrolysed in the cytoplasm, leaving the membrane-impermeant indicator trapped within the cell. Several reports already describe the application of these indicators to the study of free calcium in smooth muscle (Williams & Fay, 1985; Williams, Fogarty, Tsien & Fay, 1985; Ashley & Pritchard, 1985; DeFeo & Morgan 1986; Sumimoto & Kuriyama, 1986; Parker, Ito, Kuriyama & Miledi, 1987; Himpens & Somlyo, 1988). The most suitable indicator presently available is Fura-2, which offers improvements over its predecessor Quin-2 of an increased fluorescence brightness, and the possibility of calibration in terms of absolute free calcium levels by comparison of the fluorescence excited by different wavelengths (Grynkiewicz *et al.* 1985; Williams *et al.* 1985; Poenie, Alderton, Tsien & Steinhardt, 1985). We have used Fura-2 to measure free calcium levels, together with measurements of tension development, in strips of longitudinal smooth muscle from guinea-pig ileum. The present paper deals largely with changes in calcium evoked by electrical stimulation, and a preliminary report of some of the findings has been presented (Ito, Parker, Miledi, Itoh, Sumimoto, Fujiwara, Kubota & Kuriyama 1987). Himpens & Somlyo (1988) recently described the use of a similar system to study changes in contraction and intracellular calcium in ileum smooth muscle in response to agonists and KCl-induced depolarization.

METHODS

Preparation and Fura-2 loading

Strips of longitudinal smooth muscle were obtained from the ileum of adult guinea-pigs, killed by decapitation following anaesthesia with Nembutal (Rang, 1964). Muscle strips had a weight of about 1 mg, and dimensions of about 6 × 3 mm. Intracellular loading with Fura-2 was accomplished by loosely pinning the strips onto blocks of Sylgard, and incubating them for 1–2 h in Krebs solution including 5 μM-Fura-2 acetoxymethyl ester (Fura-2 AM; Molecular Probes Inc.), added as a 1 mM solution in dimethyl sulphoxide. Several strips were incubated each morning, and then stored in a large volume of Krebs solution, bubbled with 95% O₂, 5% CO₂, until required.

Recordings were not made until at least 1 h after loading, to facilitate the de-esterification of internalized Fura-2 AM, and removal of extracellular Fura-2 AM.

A rough estimate of the final intracellular concentration of Fura-2 was obtained by comparing the fluorescence (350 nm excitation) of loaded muscle strips with that of a sealed capillary tube containing Fura-2 (Molecular Probes Inc.) in a solution of 100 mM-KCl, 1 mM-EGTA and no added calcium. The fluorescence from a muscle strip weighing 1.3 mg (after blotting dry) matched that of 1 μ l of a solution of 100 μ M-Fura-2. Thus, the intracellular concentration of Fura-2 in the muscle must have been at least 100 μ M, and probably greater than this because of the presence of connective tissue and extracellular fluid, which would not trap the dye.

Recording of Fura-2 fluorescence signals

Two recording systems were used: one for simultaneous measurement of fluorescence and tension from muscle strips, and a second microscopic system to record fluorescence from small areas of a muscle strip.

Fura-2-loaded muscle strips were positioned vertically in a glass chamber (volume *ca.* 3 ml), and tied with thread between a hook and an isometric tension transducer. After mounting, the strip was stretched to about 1.5 times rest length, and was continuously perfused with Krebs solution (composition in mM; NaCl, 122; KCl, 4.7; MgCl₂, 1.2; CaCl₂, 2.5; glucose, 11.5; NaHCO₃, 15.5; KH₂PO₄, 1.2). The solution was at room temperature (23–26 °C), and was bubbled with 95% O₂, 5% CO₂. Coils of Ag–AgCl wire above and below the muscle strip allowed electrical field stimulation, via a Grass pulse generator and stimulus isolation unit. Stimuli were unipolar pulses, of about 100 V amplitude and variable duration. Fluorescence excitation was made with light from a 100 W tungsten–halogen lamp (Zeiss), operated from a well-stabilized power supply. Although this gave much less output in the ultraviolet than an arc lamp source, it was preferred because of the greater stability of the light emission. Narrow band interference filters (Oriel Corp.; half-bandwidth, 10 nm) allowed excitation at either 350 or 373 nm. The filters were interchanged by hand. Screens in the light path prevented illumination of the stimulating wires and of components in the muscle chamber joined with (fluorescent) epoxy resin. Fluorescence emission was monitored at 510 nm, using an interference filter (half-bandwidth, 10 nm) and photomultiplier (EMI 9524B) positioned close to the chamber, and at right angles to the excitation beam. The optical system was designed to give even illumination over an area greater than that occupied by the muscle strip, and also to gather light evenly over a wide area. Thus, small movements of the muscle gave minimal changes in fluorescence signal. The photomultiplier output was measured through a current to voltage convertor, and low-pass filtered through a Bessel filter (Kemo Ltd), which was set to 10 Hz unless otherwise noted. Some photomultiplier signal was present in the absence of the muscle, largely due to incomplete blocking of the interference filters outside their bandpasses. Measurements of this background were taken after removing each muscle strip, and were subtracted from all measurements of fluorescence given here.

Recordings of Fura-2 fluorescence from small areas of muscle strips were obtained through a compound microscope (Zeiss Ergaval), fitted with an epi-fluorescence system and a 25 \times long working distance objective. Muscle strips were stretched, both along their length and across their width, and were pinned onto Sylgard blocks in a small dish filled with Krebs solution. Fluorescence excitation was made at 373 nm, and emission monitored at 510 nm, as in the whole-strip recording system. A mercury arc lamp (Zeiss) was used for fluorescence excitation. Ag–AgCl wires were placed on either side of the muscle strip, parallel to the long axis of the fibres, and were used for electrical stimulation.

Calibration of Fura-2 signals

Fura-2 fluorescence was calibrated in terms of absolute free calcium concentrations by introducing solutions of Fura-2, buffered to known free calcium levels, into the (empty) chamber used for muscle recording. The calibrating solution contained 1 μ M-Fura-2, 120 mM-KCl, 5 mM-HEPES at pH 7.2 and 10 mM-K₂H₂EGTA/K₂CaEGTA. Different levels of free calcium between pCa ∞ and pCa 6 were set by varying the ratio of K₂H₂EGTA/K₂CaEGTA, as described by Grynkiewicz *et al.* (1985, legend to their Fig. 3). The ratio of fluorescence excited by 350/373 nm light in solutions of different free calcium levels was used to plot a calibration curve. This measure is proportional to the free calcium concentration, but is independent of Fura-2 concentration or path length (Grynkiewicz *et al.* 1985).

Resting free calcium levels in the muscle strips were estimated by measuring the 350/373 nm fluorescence ratio, and reading off the corresponding calcium concentration from the calibration curve. However, it was difficult to measure the fluorescence ratio during brief calcium transients, as we were unable to swap rapidly the excitation filters. Instead, we measured the fractional change in fluorescence emission (ΔI) with 373 nm excitation, and calibrated these ΔI values in terms of changes in free calcium from a nominal resting level of 150 nM. The range of ΔI values recorded from the muscle was quite small (-0.15 to $+0.02$) and, over this restricted range, a ΔI of 0.1 corresponded to a change in free calcium concentration by a factor of nearly two.

RESULTS

Resting free calcium level

Muscle strips loaded with Fura-2 gave fluorescence signals which were 2–3 times greater than the background level of the recording system. In contrast, the fluorescence of control (non-Fura-2-loaded) strips was less than 10% of that in loaded strips. Some of this signal probably came from autofluorescence in the muscle, but it also arose from scattering of light incompletely blocked by the excitation and emission filters. Measurements of Fura-2 fluorescence were corrected by subtraction of background signals in the absence of the muscle, but we did not attempt to correct for the smaller intrinsic signal from the muscle strip.

Spontaneous calcium transients were seen in most muscle strips. These usually occurred in bursts, and fluorescence measurements to estimate the resting free calcium were made during 'silent' intervals between these bursts. The ratio of fluorescence excited by 350/373 nm light, measured at these times, was 0.65 ± 0.013 (S.E. of mean, twenty-four muscle strips), which corresponds to a mean intracellular free calcium concentration of 210 nM. The variation in the readings is equivalent to a standard error of only ± 10 nM, but it is likely that systematic errors in the calibration procedure would be greater than this.

Fura-2 signals at 373 and 350 nm

Fura-2 shows a large decrease in fluorescence emission on binding calcium when excited with 373 nm light, and a smaller increase at 350 nm (Gryniewicz *et al.* 1985; Poenie *et al.* 1985). As expected from this, the fluorescence signals recorded from muscle strips during spontaneous and evoked contractions showed transient decreases in fluorescence at 373 nm, and smaller increases at 350 nm (Fig. 1A–C). Control muscle strips without Fura-2 showed no detectable optical signals, even when examined with a high photomultiplier recording gain (Fig. 1D).

Recordings of Fura-2 calcium transients were usually made with 373 nm excitation, since this gave the larger signal. For convenience, therefore, decreases in fluorescence are shown as upward deflections, so as to correspond with increasing calcium. Tracings were also obtained at intervals using 350 nm excitation, mainly as a check that the fluorescence signals were not contaminated by movement artifacts. The Fura-2 fluorescence at this wavelength was about two-thirds that with 373 nm excitation, so that changes in light signal due to movement would be expected to be of the same direction with both wavelengths, but somewhat smaller (*ca.* 60%) with 350 nm excitation. Calcium-dependent changes, on the other hand, would be in opposite directions with the two wavelengths and much smaller (*ca.* 10%) with 350 nm light. As illustrated in Figs 1A–C, 2A and B and 8A this was generally the

case, indicating that the 373 nm signals were not appreciably contaminated by movement artifacts.

Spontaneous calcium transients

The pattern of spontaneous calcium transients varied considerably between muscle strips, and during recordings from a single strip. Some examples are shown

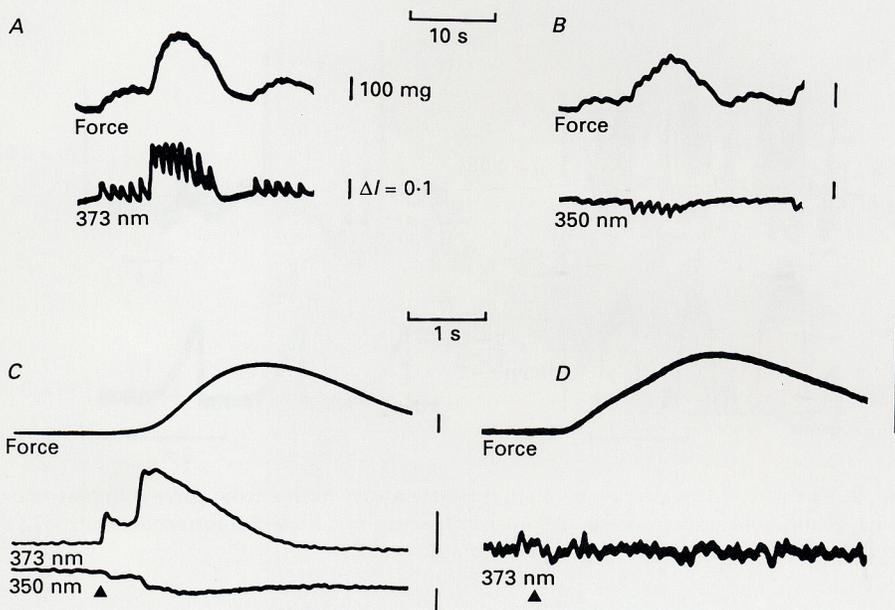


Fig. 1. Signals from Fura-2-loaded muscle strips during spontaneous contractions (*A* and *B*) and contractions evoked by electrical field stimulation (*C*). In this and other figures the upper trace in each frame is muscle tension, and the lower trace(s) fluorescence excited by 373 and/or 350 nm light. Decreasing fluorescence is shown as an upward deflection, since this corresponds to increasing Ca²⁺ with 373 nm excitation. Light signals are calibrated as the fractional change in fluorescence emission (ΔI) from the resting level. Calibration bars indicate 100 mg force and $\Delta I = 0.1$. *A* and *B*, force and fluorescence signals recorded sequentially at 373 and 350 nm from a muscle strip showing spontaneous activity. *C*, tension and fluorescence signals from a different muscle strip, in response to a single stimulus (100 V, 10 ms duration) applied at the arrow-head. Records with 373 and 350 nm excitation were obtained during successive stimuli; the tension records were nearly identical for both. *D*, control record from a muscle strip which was not loaded with Fura-2. Same stimulus as in (*C*). The recording gain from the photomultiplier was 20 times greater than in *C*.

in Figs 1*A* and *B* and 2. Individual transients lasted 1–2 s (Fig. 2*C*), but were often grouped into bursts lasting several seconds, interspersed by silent intervals (e.g. Figs 1*A* and 2*A*). The accompanying contractions had slower time courses than the individual calcium transients, so that each burst of transients usually gave rise to a maintained tension (Figs 1*A* and 2*A*). Addition of tetrodotoxin (TTX; 3×10^{-6} M) and atropine (10^{-6} M) to the bathing medium failed to alter the spontaneous activity, indicating that it does not depend upon nerve activity.

The spontaneous Fura-2 signals were not of constant size, even in the same muscle strip (e.g. Fig. 1*A*). Large transients gave a change in fluorescence emission ΔI of about -0.2 (373 nm excitation), which corresponds to an increase in free calcium level by about 400 nM above the resting level. An interesting feature was that the calcium signals often showed an 'undershoot' following each transient, falling transiently below the steady resting level observed between bursts (Fig. 2*A* and *B*). These undershoots also remained after addition of atropine and TTX.

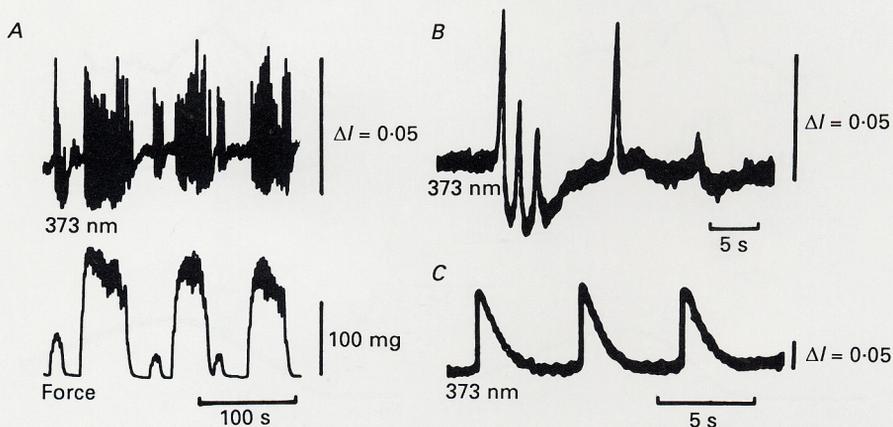


Fig. 2. Examples of spontaneous calcium transients, recorded from three different muscle strips at different sweep speeds. All optical traces show Fura-2 fluorescence with 373 nm excitation. The lower trace in *A* shows muscle tension.

Calcium transients evoked by electrical stimulation

A supramaximal electrical pulse delivered to the muscle strip evoked a complex Fura-2 signal (Fig. 3*A*), which was made up from three distinct components. The first was a rapid rise in calcium, beginning nearly coincidentally with the pulse, and reaching a peak within about 10 ms. The calcium level then declined for several hundred milliseconds, until interrupted by a second rise in calcium, which was often greater than the first (e.g. Fig. 3*E*). After this, the calcium level again declined, and usually fell below the initial resting level 1–2 s after the peak. Subsequent recovery back to the baseline took several seconds. All of these components of the Fura-2 signal appeared to be a true indication of changes in calcium, since control records with 350 nm excitation showed little evidence of movement artifact (Figs 1*A* and 3*A*).

The rise in tension accompanying the calcium transients had a slower time course, reaching a maximum 1 s or more after stimulation, at which time the calcium signal had declined close to, or below, the resting level (Fig. 3*A* and *E*). The biphasic nature of the rise in calcium was not usually evident in the tension trace, which was usually monophasic, although an inflexion was sometimes visible on the rising phase, corresponding to the second rise in calcium (e.g. Fig. 3*A*).

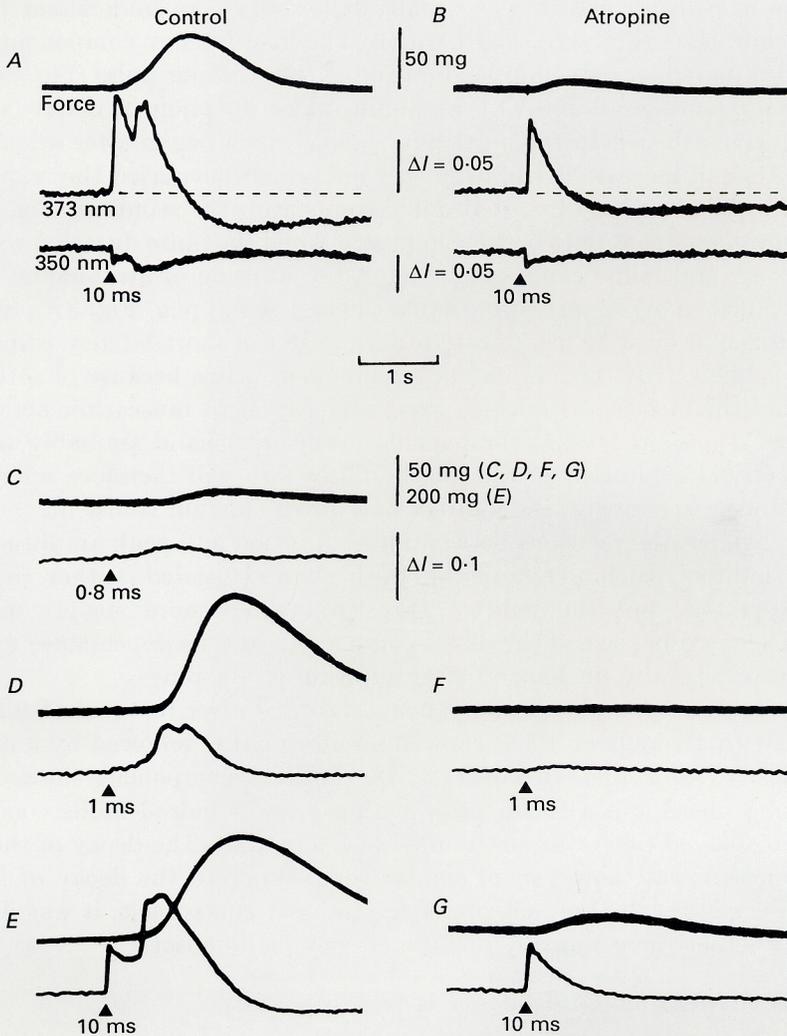


Fig. 3. Direct and nerve-evoked calcium transients and contractions in response to field electrical stimulation. *A* and *B*, records from a Fura-2-loaded muscle strip before (*A*) and after (*B*) addition of 10^{-6} M-atropine to the bathing solution. The upper trace is tension, middle trace, Fura-2 fluorescence with 373 nm excitation, and lower trace, fluorescence with 350 nm excitation. Records at 373 and 350 nm were obtained with successive stimuli. Pulse was 100 V and 10 ms duration. Dashed lines indicate baselines. *C–E*, records from a different muscle strip before (*C–E*) and after (*F* and *G*) addition of 10^{-6} M-atropine. In each panel, the upper trace is tension and the lower fluorescence. Stimulus pulses (100 V) were of the durations indicated. Note that the tension recording gain in (*E*) was 4 times lower than for the other panels.

Direct and indirect calcium signals

A clue as to the origin of the two peaks of the calcium signal was provided by the observations that the long-latency component could be selectively abolished by addition of atropine (10^{-6} M) or TTX (3×10^{-6} M) to the bathing solution. In the presence of either of these drugs, supramaximal stimuli evoked only the short-

latency rise in calcium, which was usually followed by an undershoot below the resting calcium level (Fig. 3*B*). Furthermore, the long-latency component could be selectively activated by reducing the duration of the stimulus pulse (Fig. 3*C* and *D*). For a stimulus voltage of 100 V, a minimum pulse duration of about 0.8 ms was required to give a just-detectable calcium signal, which began after a long latency (Fig. 3*C*). A small increase in duration to 1 ms greatly increased the response size, but this still peaked several hundred milliseconds after the stimulus (Fig. 3*D*). The short-latency component only became apparent when the pulse duration was further increased to several milliseconds (Fig. 3*E*). After addition of atropine or TTX, the 1 ms pulse failed to evoke any appreciable calcium signal (e.g. Fig. 3*F*), and longer pulses of 10 ms, or even 50 ms, duration gave only the short-latency response.

Thus, it is likely that the long-latency component arises because of activation of nerves within the muscle strip, which exert an excitatory, muscarinic action on the muscle fibres. The short-latency component, on the other hand, probably arises from a direct electrical excitation of the muscle fibres. We will therefore refer to these components as, respectively, the indirect and direct calcium transients.

Figure 4*A* shows the relations between pulse duration and peak amplitudes of the direct and indirect calcium transients. Both showed graded, rather than all-or-nothing, responses, but the indirect transient varied more steeply with pulse duration. The maximal size of the direct component, and its dependence upon pulse duration, were virtually unchanged after addition of atropine.

The time course of the direct component, recorded after removal of the indirect component with atropine or TTX, showed an abrupt rise, followed by a monotonic decline (Figs 3*G* and 7*A*). In contrast, the indirect component showed a more gradual rising phase and a broad peak lasting a few hundred milliseconds, which sometimes displayed two or more 'humps' (e.g. Fig. 3*D*). The decay of the indirect calcium transient was, however, of similar time course to the decay of the direct transient. Because of the gradual rise of the indirect component, it was difficult to determine whether there was any definite latency to its onset.

Contractions activated by the direct and indirect transients

Although the peak size of the direct calcium transient was little altered by atropine and TTX, tension development was strongly reduced following abolition of the indirect component (Fig. 3*A, B, E* and *G*). Similarly, activation of the indirect component alone, using a brief stimulus duration in normal Krebs solution, gave a contraction of almost the same size as that produced by a longer pulse which activated both direct and indirect components (Fig. 2*D* and *E*). Thus, almost all of the contractile activation appeared to result from the indirect calcium transient, even in muscle strips where the peak level of free calcium during this transient was less than that during the direct component (e.g. Fig. 3*A*).

The relationships between the amplitude of the calcium transients and the size of the associated contractions are shown in Fig. 4*B*, for responses elicited by stimulating pulses of different durations. Measurements were made of the peak size of the indirect calcium transient in normal Krebs, and of the direct transient after adding atropine. Both of these showed a smoothly graded increase in contractile force with increasing peak free calcium, but for a given calcium level, the indirect

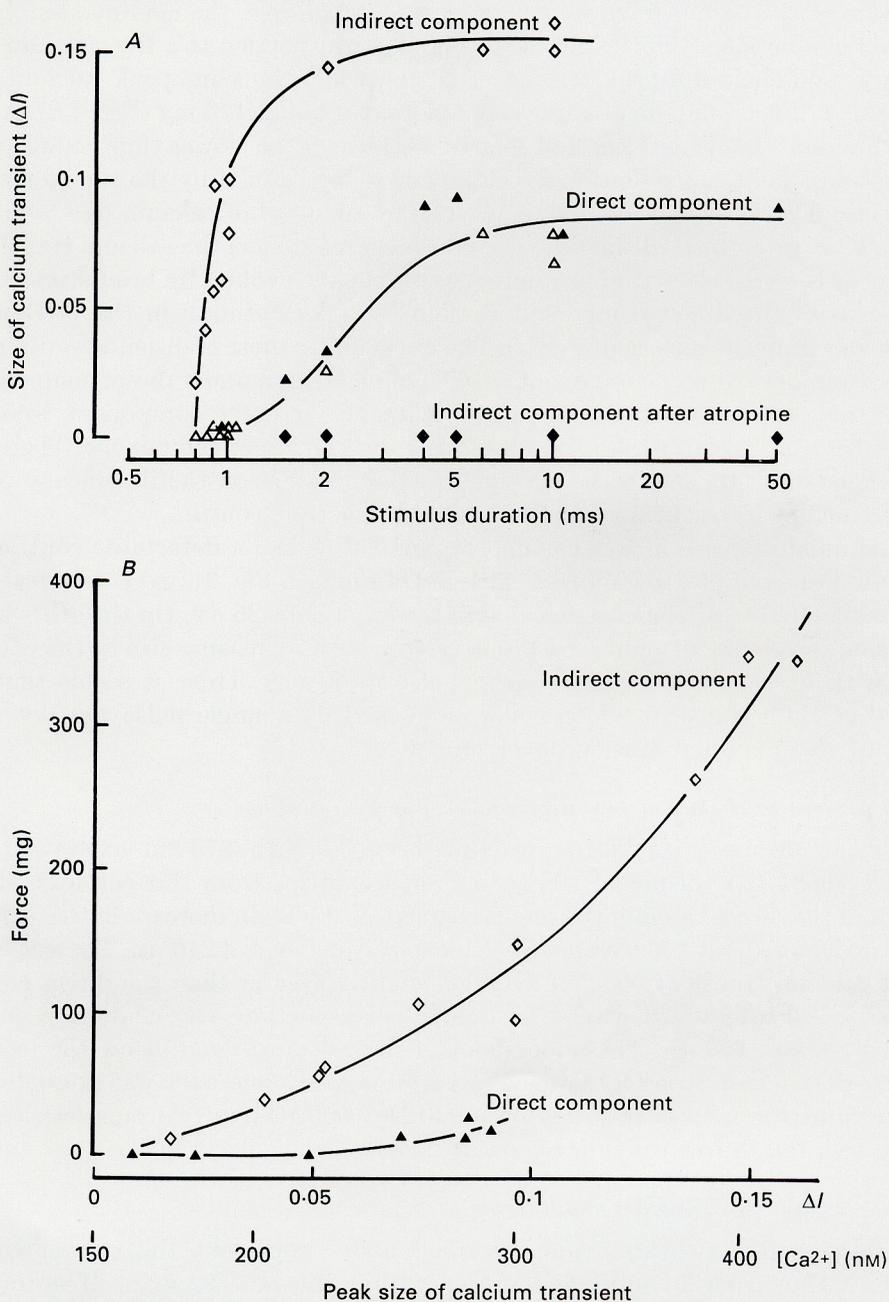


Fig. 4. *A*, variation in peak size of Fura-2 calcium signal with duration of stimulus pulse, measured from the same muscle strip as Fig. 3C-G. Points show sizes of the short-latency (triangles) and long-latency (diamonds) components of the response, recorded before (open symbols) and after (filled symbols) addition of atropine (10^{-6} M). Stimulus voltage was constant at a nominal 100 V. *B*, relation between size of the calcium transient and amplitude of the accompanying contraction, measured from the same muscle strip as Fig. 3C-G. Open symbols represent the peak size of the indirect calcium transient, and filled symbols the size of direct component after addition of atropine. Scales on the abscissa indicate fractional change in fluorescence and the estimated intracellular free calcium level at the peak of the response.

component evoked a much larger contraction. For example, the maximal amplitude of the direct component in the muscle of Fig. 4 corresponded to a free calcium level of 290 nM, and elicited a peak tension of 20 mg, while the same peak calcium level during the indirect component gave a contraction of about 110 mg (Fig. 4B). A part of this difference might be expected to arise because of the slower time course of the indirect component, since tension development is determined by the time for which the calcium level remains elevated, as well as by the level of calcium (see later). To test this idea, we estimated the time integral (i.e. area under) the calcium transients. Three records were selected of the indirect component evoked by brief stimuli, and five records of the direct component evoked by longer stimuli in the presence of atropine (all from the muscle of Fig. 4). The areas under these transients were similar (mean area under indirect component = 97% of mean area under direct component). Despite this, the contractions associated with the indirect component were, on average, 3.6 times greater than those produced by the direct component. Therefore, some other factor appears to be involved in the greater contractile efficacy of the indirect calcium transient, in addition to its slower time course.

The minimal increase in free calcium required to evoke a detectable contraction was small. For example, the indirect calcium transient in Fig. 3C gave a contraction, but corresponded to an increase in calcium by only about 25 nM. On the other hand, the tension development increased progressively with increasing size of the calcium signal, with no saturation even at long pulse durations. Thus, it seems that the maximal calcium transient which could be evoked by a single pulse was not great enough to give maximal tension development.

Changes in free calcium concentrations following stimulation

The mean decrease in fluorescence intensity ΔI with 373 nm excitation was -0.072 ± 0.0034 (S.E. of mean, thirty-four muscle strips from thirteen animals) at the peak of the direct calcium transient, corresponding to an increase in free calcium concentration of about 130 nM above the mean resting level of 210 nM. The size of the indirect calcium transient was, on average, slightly greater than the direct component ($\Delta I = -0.101 \pm 0.018$, twelve muscles), corresponding to an increase in free calcium by about 200 nM. The undershoot of the calcium signal below the baseline following the calcium transients was very variable, and sometimes was not detected. A mean value for ΔI was recorded of $+0.012 \pm 0.003$ (thirty-two muscles), corresponding to a fall in free calcium by about 20 nM.

Microscopic observations from small areas of muscle

The fluorescence measurements described above represent the summated behaviour of thousands of individual fibres within the muscle strip. If individual fibres, or fibres in different regions of the muscle strip showed different properties, the averaged Fura-2 signal might not reflect faithfully the calcium transients occurring in each individual fibre. To obtain more information about this, we made recordings through a compound microscope from small areas of muscle (*ca.* $2 \times 10^4 \mu\text{m}^2$), which comprised less than 0.01% of the total area of muscle strips used in the experiments described above.

Figure 5 shows recordings made in this way, using different stimulus durations to

selectively activate the direct and indirect calcium transients, in the same way as in Fig. 3C-G. In this example, TTX, rather than atropine, was used to block the indirect component. These traces were in most respects like those obtained from the whole-strip preparation; the times to peak of the direct and indirect components

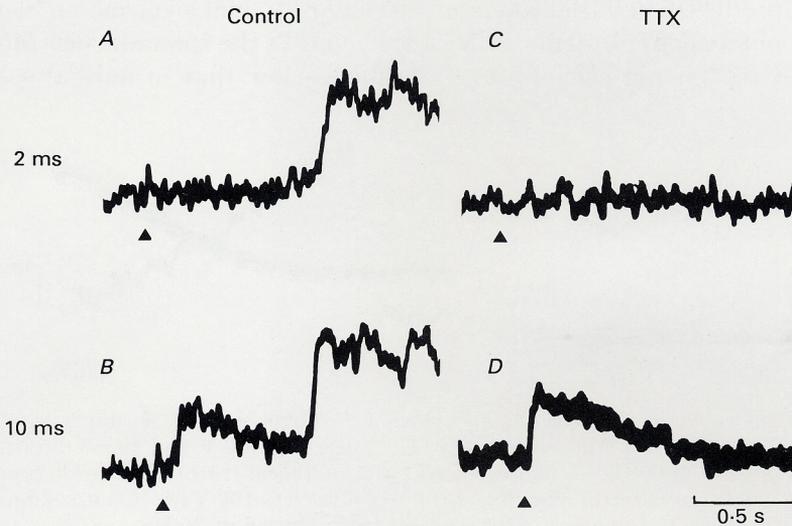


Fig. 5. Fura-2 fluorescence signals recorded through the microscope from a small region of a muscle strip. Diameter of the light spot was about 150 μm . Fluorescence excitation at 373 nm. Stimuli of 100 V and 2 ms (*A* and *C*) or 10 ms (*B* and *D*) duration were applied at the arrow-heads. Records on the left (*A* and *B*) were obtained in normal Krebs solution, and those on the right (*C* and *D*) after addition of 300 nM-TTX. Fluorescence signals were filtered at 30 Hz.

were similar, as were their decay time courses and dependence upon stimulus duration. However, one difference was that the rising phase of the indirect component appeared more abrupt when recorded from a small area of muscle, and multiple peaks could be more easily discerned within the transient. For example, in Fig. 5 there were two peaks with latencies of about 700 ms and 1.1 s, and the longer-latency component could be selectively activated by brief (2 ms or less) stimuli.

Thus, the Fura-2 recordings from the whole strip probably give an accurate reflection of the time course of the calcium transients occurring in individual cells, with the exception that the indirect component may be 'smeared out' if it peaks at different times in different cells or areas of the muscle.

Latencies to onset of calcium signal and tension development

Figure 6A shows a record at fast sweep speed, and reduced filtering, of the directly evoked calcium transient in a whole muscle strip. The Fura-2 signal showed an abrupt onset, beginning about 10 ms from the onset of the stimulus pulse. The calcium level then rose steeply, reaching a peak after a further 25 ms.

Tension development began much more slowly than the calcium signal (Fig. 6B). The initial rise in tension showed a curved foot, making it hard to determine any

definite latency to the onset. However, from records like those in Fig. 6B it appeared that tension development did not begin until at least 100 ms after the rise in calcium.

Dependence upon extracellular calcium

Superfusion of muscle strips by a Krebs solution in which calcium was substituted by magnesium produced an initial rise in intracellular calcium level and an increased frequency of spontaneous transients. After a few minutes the spontaneous transients died away, and the resting calcium level declined below that in normal solution.

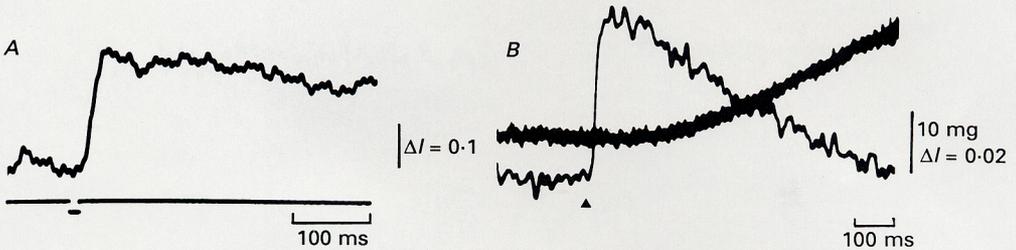


Fig. 6. Onset of calcium transient (A) and tension development (B). A, upper trace is fluorescence, and lower is a stimulus marker. The pulse was 100 V and 10 ms duration. Optical trace filtered at 60 Hz. B, fluorescence at 373 nm (lower trace) and tension (upper trace) from a whole muscle strip recording. Stimulus pulse was 100 V for 10 ms, beginning at the time indicated by the arrow-head. Optical trace filtered at 30 Hz.

Electrical stimuli given at this time failed to evoke direct (e.g. Fig. 7A and B) or indirect calcium transients, and tension development was suppressed. After returning to normal Krebs solution, the resting calcium level increased, spontaneous transients returned, and electrical stimulation evoked calcium signals of normal size.

The organic calcium antagonists verapamil and diltiazem also reduced the sizes of the evoked calcium transients and contractions. In the muscle strip illustrated in Fig. 7C and D, verapamil at a concentration of 10^{-4} M abolished the indirect calcium transient and reduced the size of the direct component to less than 10% (Fig. 7C and D). Both components recovered partially after washing for several minutes. In a different muscle strip, treated with atropine to block the indirect component, diltiazem had no effect on the direct calcium transient at concentrations below about 2×10^{-6} M, but reduced the signal to about 50% at 2×10^{-5} M and to about 20% at 10^{-4} M.

Paired and tetanic stimuli

Figure 8 shows direct calcium transients and contractions evoked by paired stimuli given at different intervals, in a muscle strip treated with atropine. A striking feature was that the additional calcium signal elicited by the second stimulus was much reduced at short pulse intervals, but recovered gradually as the time between stimuli was lengthened. This effect was such that the peak calcium level following the second stimulus never rose appreciably above the peak of the first response, and instead remained at about the same level as the initial transient for all pulse intervals. Despite this, paired stimuli evoked larger contractions than a single

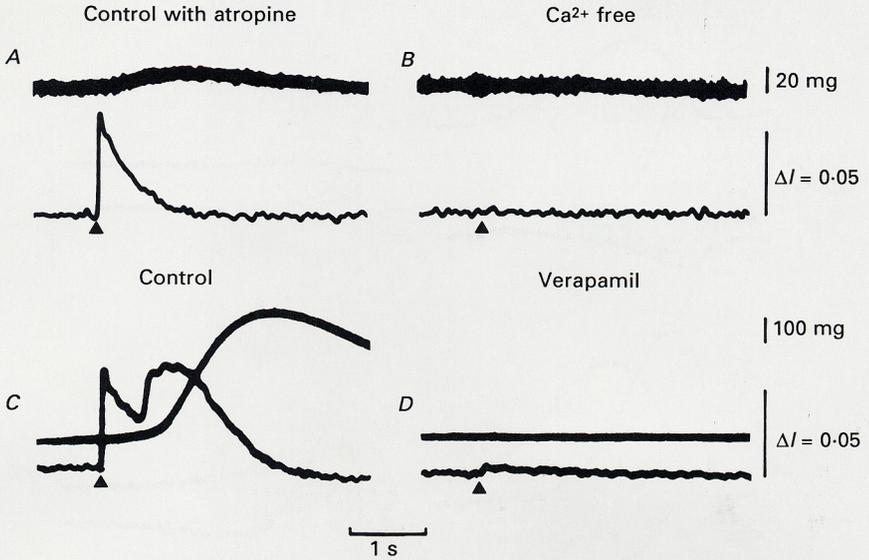


Fig. 7. Calcium transients depend upon extracellular calcium. *A* and *B*, force (upper traces) and Fura-2 fluorescence (lower traces) records from a muscle strip bathed in normal Krebs solution including atropine (10^{-6} M). *A*, control record. *B*, record 10 min after changing to a Krebs solution containing no added calcium and 3.7 mM-magnesium. *C* and *D*, records from a different muscle strip in normal Krebs solution (*C*) and after addition of 10^{-4} M-verapamil (*D*). Stimuli (marked by arrow-heads) were single pulses of 100 V and 10 ms duration.

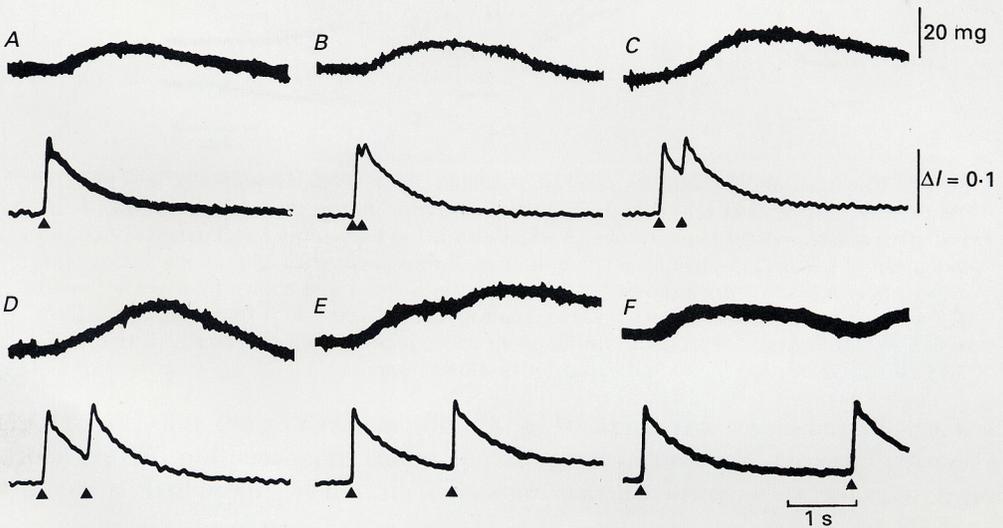


Fig. 8. Calcium transients and contractions evoked by paired stimuli. *A*, responses to a single stimulus (100 V, 10 ms duration). *B-F*, responses to paired stimuli applied at intervals of, respectively, 100 ms, 300 ms, 600 ms, 1.5 s and 3 s. Pulse parameters were the same as in (*A*). In each panel the upper trace is tension, and the lower, Fura-2 fluorescence with 373 nm excitation. Arrow-heads mark stimuli. All records from a single muscle strip, bathed in Krebs solution including 10^{-6} M-atropine.

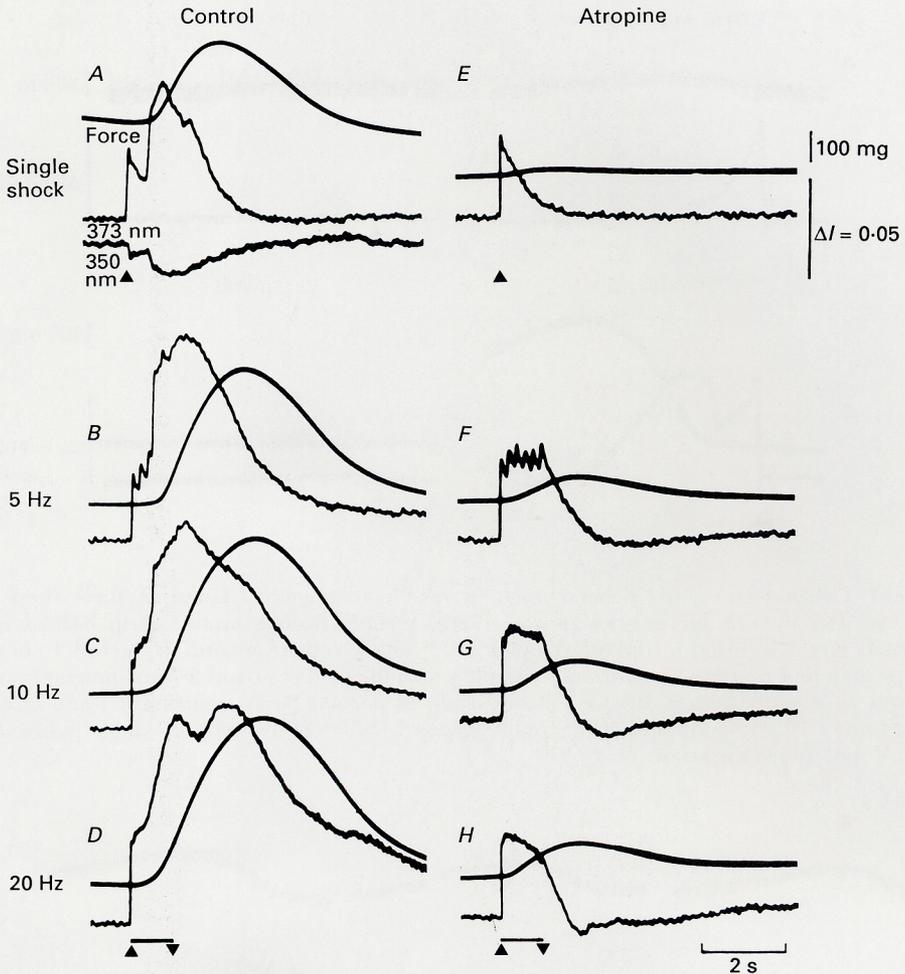


Fig. 9. Calcium signals and contractions evoked by tetanic stimulation at different frequencies. All records are from a single muscle strip, in normal Krebs solution (*A–D*) and after addition of 10^{-6} M-atropine (*E–H*). Calibration bars apply to all panels. With the exception of (*A*), which includes a trace showing fluorescence with 350 nm excitation, the upper trace in each frame shows tension, and the lower trace shows fluorescence with 373 nm excitation. *A* and *E*, responses to single stimuli of 100 V and 10 ms duration. *B–D* and *F–H*, responses to repetitive stimuli given at frequencies of 5 Hz (*B, F*), 10 Hz (*C, G*) and 20 Hz (*D, H*) for 1 s, as indicated by the lower bars.

stimulus. Maximal force was produced by stimuli separated by 0.3–1.5 s (Fig. 8 *C–E*). At shorter intervals the contraction was not much enhanced (Fig. 8 *B*), while at longer intervals two separate contractures were seen, which showed little summation (Fig. 8 *F*).

Tetanic stimulation of muscle strips treated with atropine or TTX evoked a series of direct calcium transients, which showed little or no summation above the peak level achieved following a single stimulus (Fig. 9 *E–H*). At a repetition rate of 5 Hz, individual transients could be seen following each stimulus, but the additional increments in calcium level during the second and subsequent responses were small,

and did not rise much above the peak level of the first response (Fig. 9F). Higher frequencies of stimulation (10 and 20 Hz) produced a 'fused' calcium signal, in which individual transients were not easily discerned (Fig. 9G and H). Although the calcium levels during tetanic stimulation were similar to the peak level following a single shock, the contractions evoked by tetani of 1 s duration were larger than the single-shock response, and increased slightly with increasing frequency of stimulation from 5 to 20 Hz (Fig. 9E-H).

Tetanic stimulation of muscles in normal Krebs solution produced a more complex series of calcium transients, due to the summation of directly and indirectly evoked responses (Fig. 9A-D). During the first few hundred milliseconds of stimulation a series of direct calcium transients were evoked, which, as in atropine-treated muscles, showed little summation. However, a large increase in calcium then occurred, presumably because of summation between the indirect transient evoked by the first impulse in the train and the direct responses evoked by subsequent stimuli. The peak calcium level during this second phase of the tetanic response was greater than the peak level during the indirect response to a single shock, but did not increase with increasing frequency of stimulation between 5 and 20 Hz (Fig. 9A-D). The contractions associated with tetanic stimuli were much larger when the indirect calcium transient was present, as compared to when it was blocked by atropine (compare Figs 9B-D and F-H).

DISCUSSION

Previous estimates of the resting level of free intracellular calcium in a variety of smooth muscle cells, measured by the use of Quin-2, Fura-2, aequorin and calcium-selective electrodes, have ranged between about 100 and 300 nM (Morgan & Morgan, 1985; Williams & Fay, 1985; Williams *et al.* 1985; Pritchard & Ashley, 1986; Sumimoto & Kuriyama, 1986; Yamaguchi, 1986; Parker *et al.* 1987; Bradley & Morgan, 1987). Our estimate of 210 nM, measured between spontaneous burst contractions, is therefore consistent with these other values, although it may have been affected by increase in muscle tone produced by stretch, and by the low temperature (25 °C) at which the experiments were done. Himpens & Somlyo (1988) report a resting calcium level of 135 nM in guinea-pig ileum longitudinal smooth muscle, using Fura-2 under conditions similar to those used by us. It should be noted that both these latter measurements represent an average value over a muscle strip, and that variations in calcium level may exist between different cells, and within a single fibre (Williams *et al.* 1985; Williams, Becker & Fay, 1987). An interesting point is that the 'resting' level of free calcium was not the lowest level recorded; following both spontaneous and evoked calcium transients the free calcium fell transiently to concentrations a few tens of nanomoles per litre below the resting level, and we had previously shown that β -adrenergic agonists produce a maintained fall in free calcium (Parker *et al.* 1987).

Following electrical stimulation by a single or repetitive depolarizing pulses, the intracellular calcium level rose to a maximum of 400-500 nM, although levels several times greater than this were seen during depolarization with 128 mM-KCl (Y. Ito & I. Parker, unpublished data). This relatively modest increase in peak calcium

following electrical stimulation contrasts with the levels of around $10 \mu\text{M}$ seen during twitches in skeletal muscle fibres (e.g. Miledi, Parker & Zhu, 1982; Maylie, Irvine, Sizto & Chandler, 1987). The ability of smooth muscle fibres to contract at relatively low free calcium levels may arise in part from a higher affinity of the calcium regulatory sites. Also, the size of contraction may depend upon the duration for which the calcium remains elevated, and this is much greater in smooth muscle (time at half-maximal amplitude about 1 s at 25°C) than in skeletal muscle (about 20 ms at 25°C ; Miledi, Parker & Zhu, 1982).

Electrical field stimulation evoked a biphasic rise in intracellular calcium. The first component began after a short latency and was seen only with stimuli longer than a few milliseconds, while the second began after several hundred milliseconds and could be evoked by briefer stimuli. Addition of TTX or atropine to the bathing solution abolished the second, but not the first component. Thus, the initial calcium transient probably arises through direct excitation of muscle fibres, and the second through excitation of peripheral cholinergic nerves. This interpretation is supported by earlier work recording membrane potential in ileum longitudinal muscle (Holman, 1981; Bauer & Kuriyama, 1982). Field stimulation with single pulses evoked an action potential and excitatory junction potential (EJP) when the pulse was over 3 ms duration, but pulses shorter than about 1 ms evoked only the EJP. When the amplitude of the EJP exceeded the electrical threshold required for spike generation, single or multiple spikes were superimposed on it. Atropine blocked the EJP and its superimposed action potentials, but not the action potential evoked by direct muscle stimulation. The direct electrical muscle response (action potential) consistently occurred with shorter latency than the EJP.

The latency for the direct component of the calcium transient was 10 ms or less, and the peak calcium level from this component was reached after a further 25 ms. These parameters correspond fairly well with the action potential evoked by field stimulation, as the peak depolarization occurs after several milliseconds and the duration of the action potential (measured at half-height) is 10–20 ms (Holman, 1981; Bauer & Kuriyama, 1982). The latency of several hundred milliseconds for the indirect component of the calcium transient is much too long to be accounted for by nerve conduction time or synaptic delay in release of transmitter. Instead it probably arises from the second messenger pathway activated in the muscle cell by muscarinic receptors (Hartzell, 1981). Pharmacomechanical coupling in smooth muscle is thought to be mediated through phosphoinositide signalling (Somlyo, Bond, Somlyo & Scarpa, 1985; Hashimoto, Hirata, Itoh, Kanmura & Kuriyama 1986; Walker, Somlyo, Goldman, Somlyo & Trentham, 1987), and a long latency between receptor binding and liberation of intracellular calcium appears to be a characteristic feature of this messenger pathway (I. Parker & R. Miledi, unpublished data). Recordings of EJPs in the same muscle preparation have also shown an appreciable latency (Bauer & Kuriyama, 1982), and although this was not as long (100–250 ms) as the latency for the calcium transient, the difference may be accounted for by the lower temperature at which we worked.

The peak level of free calcium attained during the direct and indirect transients were roughly the same, and yet the contraction associated with the indirect transient was greater. Thus, the extent of force generation appears not to be determined solely

by the intracellular free calcium concentration, and a similar lack of correlation between intracellular calcium and force has been noted before during agonist- and K⁺-induced activation of smooth muscle (Morgan & Morgan, 1984*b*; DeFeo & Morgan, 1985; Himpens & Somlyo, 1988). One possibility is that diacylglycerol, which is formed together with inositol trisphosphate (IP₃) as a result of muscarinic stimulation (Berridge, 1987), increases the sensitivity of the contractile apparatus to calcium via the activation of protein kinase C. In support of this, the activation of protein kinase C by phorbol esters has been found to enhance K⁺-induced contractions without altering intracellular free calcium (Itoh, Kanmura, Kuriyama & Sumimoto, 1986).

An interesting feature was that 'undershoots' of calcium below the resting level were often seen following spontaneous and evoked calcium transients. One explanation for the undershoot might be that it arises from activation of adrenergic nerves, since we have shown that β -adrenergic agonists produce a fall in intracellular free calcium below the resting level (Parker *et al.* 1987). However, this seems unlikely, because undershoots were still seen following spontaneous and evoked transients in the presence of TTX, which is expected to block nerve activity. Instead, the undershoot probably arises from intrinsic properties of the muscle fibres, and might be due to an increased re-uptake of calcium into intracellular stores, increased extrusion of calcium from the fibres, or a decrease in the 'resting' influx of calcium into the fibres.

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