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CALCIUM–FORCE RELATIONSHIPS AS DETECTED WITH AEQUORIN IN TWO DIFFERENT VASCULAR SMOOTH MUSCLES OF THE FERRET

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SUMMARY

1. The bioluminescent calcium indicator aequorin was chemically loaded into isolated strips of ferret portal vein and ferrt aorta. Aequorin light emission (a function of $[Ca^{2+}]_i$) was recorded simultaneously with tension.

2. Assuming an $[Mg^{2+}]_i$ of 0·5 mM, $[Ca^{2+}]_i$ was $1{\cdot}8 \times 10^{-7}$ M in the unstimulated portal vein at 22 °C where there was negligible resting tone. In contrast, in the unstimulated aorta at 22 °C where there was significant basal tone, the $[Ca^{2+}]_i$ was $2{\cdot}7 \times 10^{-7}$ M.

3. In both portal vein and aorta, potassium depolarization caused a monophasic rise in intracellular Ca^{2+} in parallel with the rise in tension, whereas phenylephrine caused an initial spike of light during the period of the force development which then fell to a much lower plateau level during the period of force maintenance.

4. Calcium–force curves were generated by plotting calibrated aequorin light against force while intracellular $[Ca^{2+}]$ was made to change either by increasing degrees of potassium depolarization or decreasing extracellular $[Ca^{2+}]$.

5. The steady-state calcium–force curve in the presence of phenylephrine was shifted to the left of the curve in the presence of potassium depolarization in both the portal vein and aorta.

6. In the aorta there was a counter-clockwise hysteresis in the calcium–force relationship. In contrast, in the portal vein there was no demonstrable hysteresis, indicating that the apparent change in calcium sensitivity of the contractile apparatus in the presence of phenylephrine must be caused by a second messenger other than calcium.

INTRODUCTION

The present estimates of absolute ionized calcium levels ($[Ca^{2+}]_i$) in smooth muscle cells are generally based on either extrapolation from direct measurements in other muscle cell types or on indirect measurements. In skeletal muscle resting calcium levels have been measured both with calcium-sensitive electrodes and with calcium indicator dyes and range from $0{\cdot}5 \times 10^{-7}$ M to $1{\cdot}6 \times 10^{-7}$ M (Allen & Blinks, 1979; Tsien & Rink, 1980; Lopez, Alamo, Caputo, DiPolo & Vergara, 1983; Weingart &

Hess, 1984). In cardiac muscle the range is somewhat higher with resting $[Ca^{2+}]_i$ (assuming an activity coefficient of 0·32) reported to range from $1·2 \times 10^{-7}$ up to $3·5 \times 10^{-7}$ M (Lado, Sheu & Fozzard, 1982; Lee & Dagostino, 1982; Wier & Hess, 1984; Weingart & Hess, 1984; Cobbold & Bourne, 1984). Two reports have appeared in abstract form, attempting to measure $[Ca^{2+}]_i$ in relaxed isolated amphibian gastrointestinal smooth muscle. Yamaguchi (1982), using calcium-selective microelectrodes has reported a Ca^{2+} activity of 50–70 nM. Williams & Fay (1985) have reported a Ca^{2+} concentration of $1·2 \times 10^{-7}$ M using the fluorescent indicator quin 2. However, since many intact vascular smooth muscle preparations demonstrate an intrinsic resting tone, one might expect higher levels in vascular smooth muscle compared to these gastrointestinal muscle cells.

Quantitative calcium–force relationships have been obtained from skinned smooth muscle preparations, however, it is recognized that the position of the calcium–force relationship on the *X* axis in smooth muscle can be modulated by the quantity of calmodulin added to these preparations (Sparrow, Mrwa, Hoffman & Ruegg, 1981). Since it is not known how much calmodulin and other regulatory factors are lost from chemically skinned preparations it is difficult to extrapolate from calcium–force relationships in skinned muscles to the physiological relationship in intact muscle cells. With respect to the $[Ca^{2+}]_i$ necessary to produce force, early chemically skinned smooth muscle preparations suggested that 10^{-5}–10^{-4} M-intracellular Ca^{2+} was required (Saida & Nonomura, 1978) for maximal contraction. However, more recent skinned muscle studies indicate that the calcium–force curve reaches a maximum before 10^{-5} M and begins to rise around 10^{-7} M (Arner, 1982; Peterson, 1982; Ruegg & Paul, 1982; Itoh, Kuriyama & Ueno, 1983). Other studies in which a combination of skinned muscles and electron probe analysis in intact muscles was used have also suggested that free calcium concentrations in intact smooth muscle cells never reach 10^{-5} M (Somlyo, Somlyo, Shuman & Endo, 1982) under normal circumstances.

The purpose of the present study was to attempt a quantitation of calcium-dependent signals from aequorin-loaded vascular smooth muscle cells. Aequorin is a bioluminescent calcium indicator whose properties have been studied in detail (Blinks, Wier, Hess & Prendergast, 1982). The ferret portal vein was used as a source of vascular smooth muscle because we have performed previous qualitative studies on this vessel and because of technical advantages of using aequorin in this vessel. Additionally, the values in the phasically active ferret portal vein were compared with those of a tonically active, slower muscle, the ferret aorta. Finally, an attempt was made to directly measure a hysteresis in the calcium–force relationships in these two muscle types. Such a hysteresis has been predicted from previous skinned smooth muscle studies (Chatterjee & Murphy, 1983).

METHODS

Adult ferrets of either sex were anaesthetized with chloroform; the abdomen was opened and either the portal vein or aorta was removed to a dissection dish filled with oxygenated physiological saline solution. Longitudinal strips of portal vein and circular strips of aorta (1 cm × 0·5–1·0 mm) were dissected. The cross-sectional area (as determined by the length and weight of the tissue) ranged from $1·0 \times 10^{-3}$ to $2·5 \times 10^{-3}$ cm^2 in portal vein and from $1·2 \times 10^{-3}$ to $5·0 \times 10^{-3}$ cm^2 in aorta. However, it should be pointed out that these values are not corrected for the percentage of cell

content of the tissue and thus were not used to calculate values for tension since this would considerably underestimate the true cellular values. The endothelium was removed by gentle rubbing of the inside surface of the vessel with a rubber policeman. The strips were placed in the bath with one end held in a clamp and the other end attached to a Gould UC2 transducer. For light recording the muscle was lowered into a pair of ellipsoidal mirrors within a light-tight enclosure. For further details see Blinks (1982) and Morgan & Morgan (1984*b*). The composition of the physiological saline solution was (mM): NaCl, 120; KCl, 5·9; dextrose, 11·5; $NaHCO_3$, 25·0; $MgCl_2$, 1·2; NaH_2PO_4, 1·4; $CaCl_2$, 2·5; equilibrated with 95% O_2/5% CO_2 and had a pH of 7·4. Solutions of elevated potassium were obtained by equimolar replacements of NaCl with KCl. Aequorin was loaded into cells by a chemical loading procedure consisting of incubating the muscle in a series of four solutions at 2 °C for approximately 30 min each. The compositions of the solutions were (mM): solution I: EGTA, 10; Na_2ATP, 5; KCl, 120; $MgCl_2$, 2; *N*-tris(hydroxymethyl)methyl-2-aminoethanesulphonic acid (TES), 20; solution II: EGTA, 0·1; Na_2ATP, 5; KCl, 120; $MgCl_2$, 2; TES, 20; aequorin, 0·4 mg/ml; solution III: EGTA, 0·1; Na_2ATP, 5; KCl, 120; $MgCl_2$, 10; TES, 20; solution IV: NaCl, 120; KCl, 5·9; dextrose, 11·5; $NaHCO_3$, 25·0; $MgCl_2$, 10; NaH_2PO_4, 1·4. Calcium was gradually re-added up to 2·5 mM in solution IV and then the muscle was transferred to normal physiological saline solution. The muscle was allowed to re-equilibrate for several hours (until basal light had become absolutely stable) before the start of the experiment.

We have previously shown (Morgan & Morgan, 1982, 1984*a*) that the chemical loading procedure does not change the maximal force of contraction, the speed of development of the contraction, or the sensitivity to agonists; and, that the resulting aequorin signals are qualitatively similar to those obtained by microinjection of aequorin into *Amphiuma* aorta smooth muscle cells. A similar method has been used to load aequorin into platelets (Johnson, Ware, Cliveden, Smith, Dvorak & Salzman, 1985) and the method caused no detectable release of dense granules, loss of [^{14}C]5-hydroxytryptamine or lactate dehydrogenase, or change in platelet function. These findings indicate that cells are not made grossly permeable by this method, but the actual mechanism of aequorin loading is still unknown.

The possibility must be considered that aequorin might be loaded by this method into some subcellular compartment other than the cytoplasm. Most subcellular compartments which are ionically isolated from the cytoplasm (such as the sarcoplasmic reticulum or the mitochondria) would be expected to have a much higher [Ca^{2+}] than the cytoplasm and thus any aequorin trapped in these compartments would be expected to be consumed (and thus give no further signals) over the course of the hours that the preparation is allowed to stabilize before the beginning of the experiment. The available evidence indicates that aequorin is evenly distributed in the cytoplasm (Blinks *et al.* 1982), but this has not been closely examined. One exception is the report by Fabiato (1985) suggesting 'an accumulation of aequorin in the myofilament space ... without a tight binding to the myofilaments'. This situation probably does not apply to intact smooth muscle cells (with no organized myofilament lattice) but if it did it would be an experimental advantage to the investigation of calcium–force relationships.

Light emitted from aequorin was detected with an EMI 9635A photomultiplier tube specially selected for low dark current and is reported as nanoamps of anode current (a function of $[Ca^{2+}]_i$). The aequorin used in this study was purchased from the laboratory of J. R. Blinks and prepared by the methods described in Blinks *et al.* (1982). The aequorin light was calibrated in terms of absolute $[Ca^{2+}]_i$ by the method of Allen & Blinks (1979). This method involves discharging the aequorin in the cells at the end of the experiment by exposing it to saturating calcium concentrations by the addition of Triton X-100 (0·5%) to the bath. The total quantity of light emitted can be multiplied by the rate constant for consumption of aequorin at the appropriate temperature to obtain the value of 'L_{max}'. Light levels recorded during the experiment are then expressed as L/L_{max} (fractional luminescence). Fractional luminescence was converted to $[Ca^{2+}]_i$ by the use of an appropriate *in vitro* calibration curve. The *in vitro* calibration curves were performed in a solution containing 150 mM-KCl, 5 mM-piperazine-*N*,*N*′-bis(2-ethanesulphonic acid) (PIPES) and MgCl at 3·0, 1·0 or 0·5 mM at a pH of 7·0 and a temperature of 7, 22 or 35 °C and were essentially identical to those previously published by Blinks *et al.* (1982), Allen & Orchard (1983) and Wier & Hess (1984).

RESULTS

Calculation of $[Ca^{2+}]_i$

Basal $[Ca^{2+}]_i$, as determined by aequorin luminescence was measured at both 22 and 35 °C in the ferret aorta and in the ferret portal vein. These results are summarized in Table 1. The calibration curve for aequorin is shifted to the right with increasing assumed values for $[Mg^{2+}]_i$ (Blinks *et al.* 1982). Preliminary nuclear magnetic resonance studies suggest that $[Mg^{2+}]_i$ is around 0·5 mM in smooth muscle (Dillon, Meyer, Kushmerick & Brown, 1982; M. J. Kushmerick, personal communi-

TABLE 1. Calculated $[Ca^{2+}]_i$, assuming various values for $[Mg^{2+}]_i$

	$[Mg^{2+}]_i$		
	0·5 mM	1·0 mM	3·0 mM
Unstimulated f.p.v., 22 °C	$1{\cdot}8 \times 10^{-7} \pm 0{\cdot}16 \times 10^{-7}$ (13)	$3{\cdot}0 \times 10^{-7} \pm 0{\cdot}22 \times 10^{-7}$ (13)	$5{\cdot}5 \times 10^{-7} \pm 0{\cdot}40 \times 10^{-7}$ (13
Unstimulated f.a., 22 °C	$2{\cdot}7 \times 10^{-7} \pm 0{\cdot}24 \times 10^{-7}$ (6)	$4{\cdot}5 \times 10^{-7} \pm 0{\cdot}38 \times 10^{-7}$ (6)	$8{\cdot}3 \times 10^{-7} \pm 0{\cdot}78 \times 10^{-7}$ (6)
Unstimulated f.p.v., 35 °C	$2{\cdot}0 \times 10^{-7} \pm 0{\cdot}23 \times 10^{-7}$ (7)	$3{\cdot}6 \times 10^{-7} \pm 0{\cdot}31 \times 10^{-7}$ (7)	$6{\cdot}6 \times 10^{-7} \pm 0{\cdot}71 \times 10^{-7}$ (7)
Unstimulated f.a., 35 °C	$2{\cdot}8 \times 10^{-7} \pm 0{\cdot}14 \times 10^{-7}$ (8)	$4{\cdot}9 \times 10^{-7} \pm 0{\cdot}22 \times 10^{-7}$ (8)	$8{\cdot}8 \times 10^{-7} \pm 0{\cdot}39 \times 10^{-7}$ (8)
Maximum K^+ f.p.v., 35 °C	$3{\cdot}8 \times 10^{-7} \pm 0{\cdot}19 \times 10^{-7}$ (6)	$6{\cdot}2 \times 10^{-7} \pm 0{\cdot}31 \times 10^{-7}$ (6)	$1{\cdot}1 \times 10^{-6} \pm 0{\cdot}064 \times 10^{-6}$ (6
Maximum K^+ f.a., 35 °C	$3{\cdot}8 \times 10^{-7} \pm 0{\cdot}25 \times 10^{-7}$ (6)	$6{\cdot}1 \times 10^{-7} \pm 0{\cdot}4 \times 10^{-7}$ (6)	$1{\cdot}1 \times 10^{-6} \pm 0{\cdot}09 \times 10^{-6}$ (6)

F.p.v., ferret portal vein; f.a., ferret artery.

cation). Since an assumed value of 0·5 mM also gives more reasonable resting $[Ca^{2+}]_i$ values than do values such as are reported in skeletal and cardiac muscle (i.e. 3–4 mM-intracellular Mg^{2+}), 0·5 mM-Mg^{2+} was used in constructing the subsequent graphs. However, since there is still a fair amount of controversy surrounding $[Mg^{2+}]_i$ values in smooth muscle, the range which might be expected is shown in the three columns of Table 1. It is interesting to note that even if $[Mg^{2+}]_i$ is as high as 3 mM, $[Ca^{2+}]_i$ during a 66 mM-K^+ contracture (the agonist which gives the largest $[Ca^{2+}]_i$–force ratio (Morgan & Morgan, 1984*a*)) can only reach $1{\cdot}1 \times 10^{-6}$ M. We have previously shown (Morgan & Morgan, 1984*a*) that greater amounts of potassium depolarization can produce higher $[Ca^{2+}]_i$ but no further force.

At 22 °C, resting $[Ca^{2+}]_i$ is significantly higher in the aorta compared to the portal vein ($P < 0{\cdot}01$). This is consistent with the finding that portal vein at 22 °C has negligible active tone but the aorta begins to pick up significant active tone as low as 10 °C (see 'Calcium–force relationships'). In both types of muscles warming of the muscle from 22 to 35 °C caused a rise in tone but in neither case was the increase in $[Ca^{2+}]_i$ statistically significant.

Calcium–force relationships

Calcium–force curves in intact aequorin-loaded cells were generated by causing graded degrees of potassium depolarization of the muscle and plotting the calibrated aequorin signal against the force observed in each case. Points for sub-basal Ca^{2+} and

force were generated by decreasing the extracellular Ca^{2+} (nominally Ca^{2+}-free, then nominally Ca^{2+}-free plus 2 mM-EGTA) in the presence of normal (5·9 mM) K^+. In each case steady-state force was plotted against steady-state calcium. The mean curve for ferret portal vein preparations at 35 °C is shown in Fig. 1. The point of the curve at

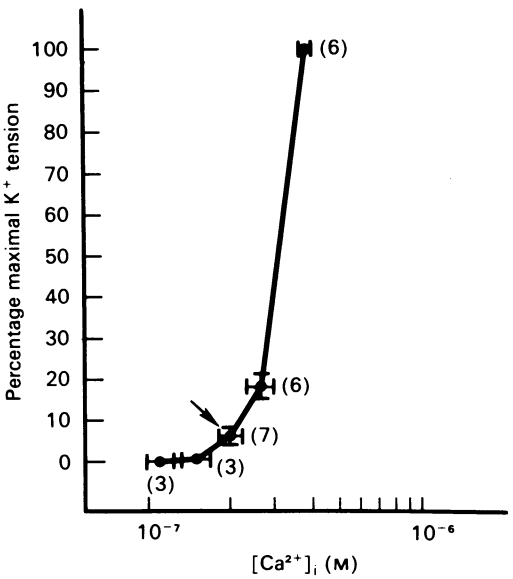

Fig. 1. Calcium–tension curve from the aequorin-loaded ferret portal vein at 35 °C. Numbers in parentheses refer to number of muscles. The point marked by the arrow represents the unstimulated muscle in normal physiological saline solution. See text for details.

the arrow represents the $[Ca^{2+}]_i$ in the unstimulated muscles in normal physiological saline solution containing 5·9 mM-K^+ and 2·5 mM-Ca^{2+}. Keeping Ca^{2+} constant, $[K^+]$ was increased from 5·9 to 19 and 66 mM. The curve begins to rise around 2×10^{-7} M-intracellular Ca^{2+} and reaches a peak at maximally effective concentrations of potassium (with respect to force generation) at $3{\cdot}8 \times 10^{-7}$ M. It is of interest that, if the assumed value of $[Mg^{2+}]_i$ is approximately correct, the calcium–force curve in this smooth muscle appears to be considerably left-shifted compared to aequorin and calcium-selective micro-electrode data from cardiac muscle (Allen & Kurihara, 1980; Lado *et al.* 1982). The resting value could be decreased by decreasing the extracellular calcium, giving, in some muscles, values as low as $8{\cdot}3 \times 10^{-8}$ M-intracellular Ca^{2+}. It appears that the 'unstimulated' muscle in normal Krebs solution is at or slightly above the $[Ca^{2+}]_i$ threshold for force development.

The mean calcium–tension curve determined with potassium depolarization for ferret aorta at 35 °C is shown in Fig. 2. The curve is practically superimposable with the curve for the ferret portal vein except that the unstimulated muscle in normal Krebs solution (arrow) appears to be moved further up the curve. The threshold portion of the curve could not be directly determined because the manoeuvre of removing extracellular calcium in aorta was not effective in lowering either basal tone

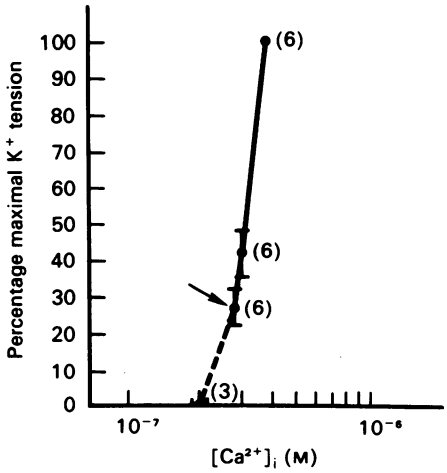

Fig. 2. Calcium–tension curve obtained in aequorin-loaded ferret aorta. The point marked by the arrow represents the unstimulated ferret aorta in normal physiological saline solution (2·5 mM-Ca^{2+} and 5·9 mM-K^+). Force was increased by increasing K^+ to 19 and 66 mM. See text for details.

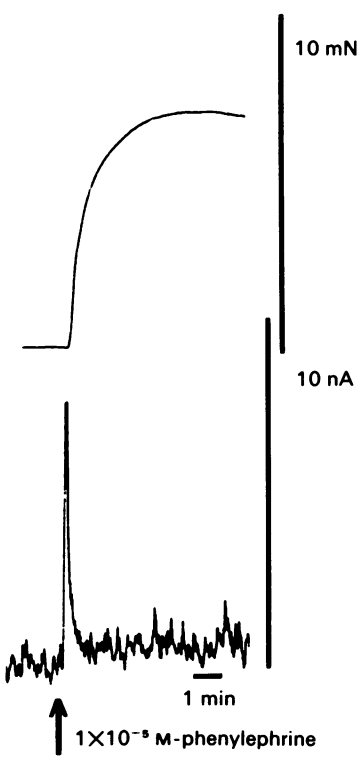

Fig. 3. Simultaneously recorded force (top trace) and light (bottom trace) in response to phenylephrine added at the arrow and kept in the bath for the duration of the trace.

or intracellular [Ca^{2+}] in a reasonable period of time. The point on the curve connected by the dashed line was obtained at 7 °C. By cooling the preparation below 10 °C it was possible to lower both intracellular calcium and basal tone to subthreshold values; however, an interpretation of calcium threshold being between 2·0 and $2{\cdot}9 \times 10^{-7}$ M has to be made cautiously since the change in temperature could change the position of the calcium threshold for contraction. The possible problem of changes in aequorin sensitivity with temperature have been circumvented by using a calibration curve (see Methods) obtained at the temperature of the measurement.

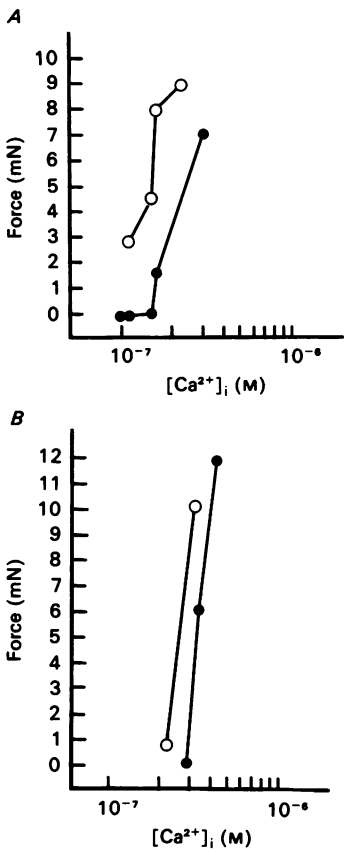

Fig. 4. Calcium–force curves in the presence of either potassium (filled circles) or phenylephrine (open circles) while $[Ca^{2+}]_i$ was made to change. *A* is from a ferret portal vein, *B* from a ferret aorta. In each case all of the data points are from the same individual muscle. In both cases zero force was arbitrarily defined as the force present in the resting muscle in normal physiological saline solution.

Effect of α-stimulation on calcium–force relationships

We have previously shown that the aequorin signal in response to α-stimulation in the ferret portal vein consists of an initial spike peaking during the period of force development followed by a low plateau-like elevation of light during the period of maintenance of the tonic contraction (Morgan & Morgan, 1984*a*). A similar signal is seen in ferret aorta (Fig. 3).

Calcium–force relationships were also investigated by altering extracellular calcium during the steady-state response to a maximally effective concentration of phenylephrine (10^{-5} M). Interestingly, although the manoeuvre of changing extracellular calcium in the ferret aorta was ineffective in altering resting tone or resting $[Ca^{2+}]_i$, this manoeuvre was effective in altering both tone and $[Ca^{2+}]_i$ in the ferret aorta in

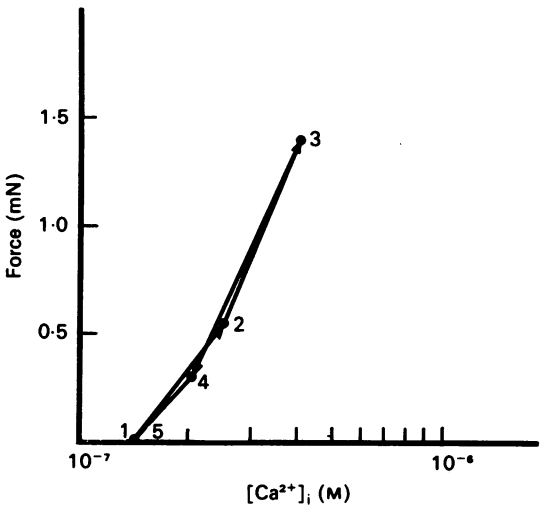

Fig. 5. Calcium–force curve from an aequorin-loaded ferret portal vein at 35 °C. The level of force in the unstimulated muscle in normal physiological saline solution was arbitrarily designated as zero force. The numbered points indicate the order in which the data were obtained. $[Ca^{2+}]_i$ and force were increased and decreased by using different levels of potassium depolarization.

the presence of phenylephrine, suggesting that phenylephrine increases the permeability of the membrane or increases the turnover of intracellular stores. A typical steady-state calcium–force relationship in the presence of phenylephrine in the ferret portal vein at 35 °C is shown in Fig. 4*A* and compared to a calcium–force relationship using potassium depolarization in the same muscle. The corresponding relationships for the ferret aorta at 35 °C are shown in Fig. 4*B*. The phenylephrine curve was shifted to the left in both muscles compared to the respective curves obtained for potassium depolarization. Although there was some variability in the exact position of the curves in different muscles, in six out of six ferret aorta and five out of five ferret portal vein preparations the phenylephrine curve was consistently positioned to the left of the potassium curve. Inspection of Fig. 4 shows that, in fact, when the $[Ca^{2+}]_i$ in the phenylephrine-stimulated muscle was made to lower to 'unstimulated' values, that a significant amount of tone remained, compared to the control.

Hysteresis in calcium–force relationships

It has been suggested that there is a counter-clockwise hysteresis in the calcium–tension curve for skinned smooth muscle (Chatterjee & Murphy, 1983). One might suggest that the left-ward shift in the calcium–force curve for phenylephrine is due to a similar hysteresis in the calcium–force relationship, since all of the values for

phenylephrine shown in the preceding Figures are steady-state values following exposure to the high $[Ca^{2+}]_i$ of the initial phenylephrine-induced spike. The phenylephrine values, but not potassium values had a history of being exposed to this high spike of calcium transiently so the difference between the two curves might be due to this sort of hysteresis. Since it is difficult to quantitate spike-like aequorin signals

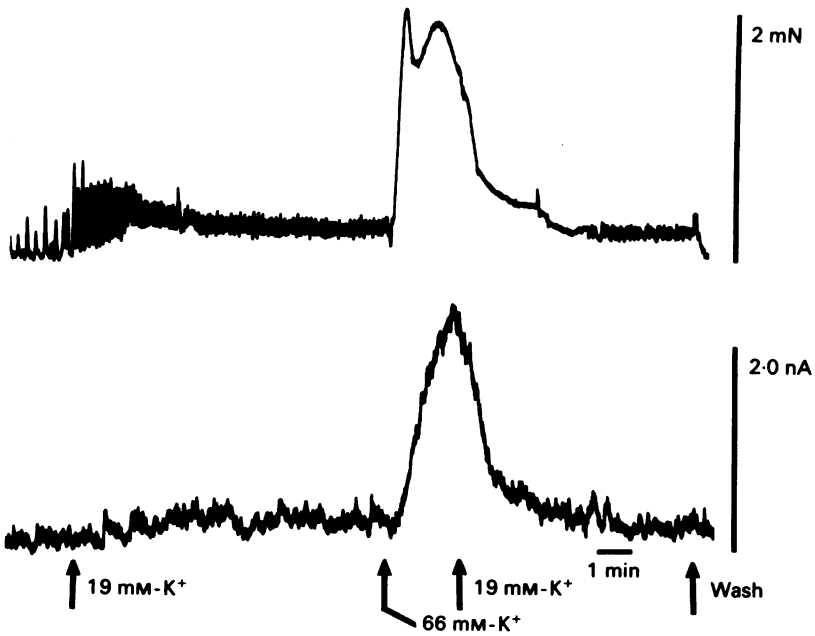

Fig. 6. Force (top) and light (bottom) from a ferret portal vein at 35 °C.

(Blinks *et al.* 1982) we did not attempt to describe a calcium–force curve for the transient situation. Instead, we have determined calcium–force curves using steady-state potassium depolarization both before and after exposure to a high potassium concentration (i.e. the ascending and descending portions of a hysteresis loop). We were unable to demonstrate any hysteresis in thirteen out of thirteen ferret portal vein strips at 22 °C or seven out of seven ferret portal vein strips at 35 °C. An example of the lack of hysteresis in this preparation is plotted in Fig. 5. The numbers indicate the order in which the points were obtained. Fig. 6 shows raw data from an experiment where the early and late exposure to a low concentration of K^+ gave similar $[Ca^{2+}]_i$ so that the forces can be directly compared without quantitation. Note that, in order to avoid significant consumption of aequorin, the higher $[K^+]$ was washed out as soon as the level of tonic force (after the initial 'phasic component') reached a plateau. It is of interest that although individual $[Ca^{2+}]$ responses to potassium depolarization were highly reproducible when the preparation was washed with normal physiological saline between exposures there was more variability in the $[Ca^{2+}]_i$ response to a certain level of $[K^+]$ after exposure to high $[K^+]$. This suggests some sort of history dependence of the voltage-sensitive channels, but we did not pursue this point further.

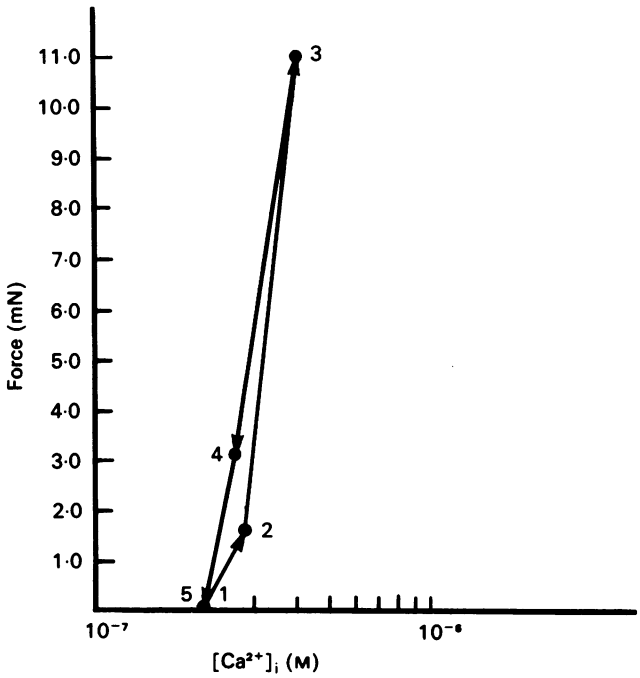

Fig. 7. Calcium–force curve from the ferret aorta at 35 °C. Data points were obtained in the order indicated by the numbers. Levels of calcium and force above base line were obtained by graded potassium depolarization. The level of force present in the normal Krebs solution was arbitrarily assigned the value of zero.

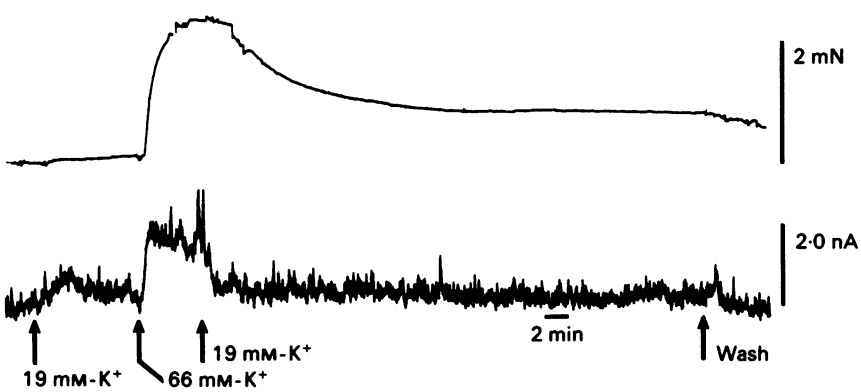

Fig. 8. Force (top trace) and light (bottom trace) from an aequorin-loaded ferret aorta at 35 °C.

Surprisingly, when the experiments of the same protocol were performed on ferret aorta at 35 °C, in seven out of eight preparations the calcium–force curve demonstrated a hysteresis. In one out of eight experiments there was no evidence for hysteresis but the exposure to the high concentration of K^+ was exceptionally short. Fig. 7 shows a calcium–force plot of such an experiment and Fig. 8 shows raw data in a case where $[Ca^{2+}]_i$ before and after exposure to high K^+ were comparable.

In all cases the background light after returning to normal potassium was identical to the control background light (or else the preparation was rejected) arguing against any complicating effect due to aequorin consumption. The possibility that noradrenaline was being released from nerve terminals in the aorta but not in the portal vein which might explain hysteresis in the former tissue was made unlikely by the observation that hysteresis was not prevented in the ferret aorta by pre-treatment with 3×10^{-6} M-phentolamine.

DISCUSSION

The use of aequorin to quantitate $[Ca^{2+}]_i$ requires a knowledge of $[Mg^{2+}]_i$. Although the Mg^{2+} concentration is still controversial, Dillon *et al.* (1982) have reported that $[Mg^{2+}]_i$ is lower in smooth muscle than in striated muscle based on nuclear magnetic resonance measurements. The use of a value of 0·5 mM (Dillon *et al.* 1982; M. J. Kushermick, personal communication) for $[Mg^{2+}]_i$ in defining the calibration curve for aequorin also places the resting calcium values in closer agreement with the preliminary reports of $[Ca^{2+}]_i$ using other methods than would a value of 3–4 mM such as has been reported for cardiac and skeletal muscle $[Mg^{2+}]_i$ (Hess, Metzger & Weingart, 1982; Lopez, Alamo, Caputo, Vergera & DiPolo, 1984). Assuming the value of the 0·5 mM for $[Mg^{2+}]_i$, a value of approximately 2×10^{-7} M-intracellular Ca^{2+} is obtained for the Ca^{2+} threshold for contraction in the ferret portal vein and aorta at 35 °C. This value is in fair agreement with values of Ca^{2+} thresholds estimated from skinned smooth muscle preparations (Peterson, 1982; Arner, 1982; Ruegg & Paul, 1982; Itoh *et al.* 1983). One interesting feature of the control calcium–tension curve in smooth muscle is that the slope of the curve is much steeper than has been generally reported for chemically skinned smooth muscle preparations. It is generally accepted that the chemically skinned smooth muscle preparations lose calmodulin and possibly other regulatory factors. This is one possible explanation for the difference in slope of intact aequorin-loaded fibres from the chemically skinned fibres, but certainly other interpretations are possible. Since aequorin emphasizes heterogeneities in cytoplasmic $[Ca^{2+}]_i$ (Blinks *et al.* 1982), transient calcium–force relationships might be modified by the properties of aequorin but this is less likely to be a factor in steady-state relationships such as we report here. It should be pointed out, however, that the extreme steepness of the $[Ca^{2+}]_i$–force curve suggests a high degree of co-operativity of the system. It is of interest that the calcium dependence of myosin light chain kinase phosphorylation and also of myosin ATPase activation has been reported to be similarly more steep than has been reported for the calcium–force relationship in chemically skinned smooth muscle fibres (Sobieszek, 1977).

One of the more interesting findings of this study is that we were able to demonstrate a hysteresis of the calcium–force relationship of the ferret aorta but not in ferret portal vein. Ferret portal vein is a relatively fast contracting 'phasic' muscle which does not maintain tonic contractions well. The aorta, in contrast, is a very slowly contracting smooth muscle which maintains tone better and does not generally display any phasic contractions. A hysteresis in the calcium–force relationship of chemically skinned smooth muscle has been described so far only in hog carotid arteries, another vascular smooth muscle which is of the tonic type (Chatterjee &

Murphy, 1983). It appears then, that some smooth muscles and not others may be capable of displaying a hysteresis in the calcium–force relationship and that the hysteresis may be related to tone maintenance.

In spite of the lack of the hysteresis in ferret portal vein we were still able to demonstrate a shift in the calcium–force curve in the presence of the α-agonist phenylephrine indicating an increase in the sensitivity of the contractile apparatus to $[Ca^{2+}]_i$. Although this high sensitivity state was preceded by transient exposure to high $[Ca^{2+}]_i$ (cf. the spike of light in Fig. 3), it appears that prior exposure to high $[Ca^{2+}]_i$ alone is not sufficient to change the $[Ca^{2+}]_i$ sensitivity in ferret portal vein. The fact that prostaglandin $F_{2\alpha}$ also appears to produce a high $[Ca^{2+}]_i$ sensitivity state in the absence of any detectable transient light spike also argues against a hysteresis-like mechanism explaining the high sensitivity state (Bradley & Morgan, 1985). It appears that some second messenger, other than the $[Ca^{2+}]_i$ spike, must be causing the increased sensitivity of the contractile apparatus to $[Ca^{2+}]$ in the presence of phenylephrine. Others have suggested that α-stimulation causes the release of diacylglycerol and subsequent activation of protein kinase C which can increase the sensitivity of many systems to Ca^{2+} (Nishizuka, 1984; Rasmussen & Barrett, 1984; Berridge & Irvine, 1984; Williamson, Cooper, Joseph & Thomas, 1985). Our data are consistent with a phenylephrine-induced activation of protein kinase C.

One might raise the question of the 'purpose', if any, of the spike in the phenylephrine light response. The spike may represent a focal signal in response to the release of Ca^{2+} from stores and may not be an accurate reflexion of average cytoplasmic $[Ca^{2+}]$. Alternatively, we have noticed that the rate of tension development in the presence of phenylephrine is much faster than in the presence of prostaglandin $F_{2\alpha}$, even when tonic contractions of similar amplitude are compared (A. B. Bradley & K. G. Morgan unpublished data). Although a number of interpretations of this finding are possible, a phenylephrine-induced spike of $[Ca^{2+}]_i$ could allow greater myosin light chain phosphorylation and consequently a greater initial rate of cross-bridge cycling.

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