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CALCIUM–FORCE COUPLING MECHANISMS DURING VASODILATOR-INDUCED RELAXATION OF FERRET AORTA

BY TIA T. DEFEO AND KATHLEEN G. MORGAN

From the Cardiovascular Division, The Charles A. Dana Research Institute, Harvard-Thorndike Laboratory, Beth Israel Hospital, Harvard Medical School, 330 Brookline Avenue, Boston, MA 02215, USA

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SUMMARY

1. The effects of three vasodilators, nifedipine, hydralazine and forskolin, were determined on isometric force and intracellular ionized calcium concentration ($[Ca^{2+}]_i$) as indicated by aequorin in ferret aorta. Three types of contraction were studied: the intrinsic tone induced by warming from 22 to 37 °C; the contraction to the phorbol ester 12-deoxyphorbol-13-isobutyrate-20-acetate (DPBA); and the contraction to potassium depolarization.

2. On warming there was no significant steady-state change in $[Ca^{2+}]$, even though $5{\cdot}7 \pm 0{\cdot}7$ mN of tone developed. During potassium depolarization, $[Ca^{2+}]_i$ rose to a sustained plateau while DPBA caused no significant rise in $[Ca^{2+}]_i$.

3. Nifedipine and hydralazine inhibited intrinsic tone while causing an associated decrease in $[Ca^{2+}]_i$; but in the presence of forskolin, a similar inhibition of tone was accompanied by no significant decrease in $[Ca^{2+}]_i$.

4. Nifedipine and hydralazine prolonged the characteristic lag phase before force development in response to DPBA but did not cause a significant change in contraction amplitude. In contrast, forskolin caused an essentially total inhibition of the contraction.

5. During potassium depolarization, all three vasodilators caused significant decreases in $[Ca^{2+}]_i$ coincident with decreases in steady-state force. Calcium–force curves were constructed by plotting the calibrated aequorin light signal against the resulting force. The control calcium–force curve was not shifted by nifedipine or hydralazine but was significantly shifted to the right by forskolin.

INTRODUCTION

Reports from this laboratory have previously described the use of the bioluminescent $[Ca^{2+}]$ indicator aequorin to monitor changes in intracellular calcium levels ($[Ca^{2+}]_i$) in vascular smooth muscle (Morgan & Morgan, 1982; Morgan & Morgan, 1984*a*). We have also shown that increases in contractile force are accompanied by increases in aequorin luminescence (a function of $[Ca^{2+}]_i$), but that the ratio of force to luminescence varies with the agonist used to induce contraction.

The mechanisms of action of the vasodilators are varied and not well understood.

We have previously reported the effects of several vasodilators on $[Ca^{2+}]_i$ in ferret portal vein (Morgan & Morgan, 1984*b*). It was shown that vasodilators can produce relaxation of the ferret portal vein not only by lowering cytoplasmic calcium levels, but also by causing an uncoupling of the calcium–force relationship. The latter mechanism was especially prominent in the presence of agents which increase cyclic AMP levels, such as forskolin. Because of uncertainties in the value of $[Mg^{2+}]$ at that time, the aequorin signals were not calibrated in these initial studies; therefore, complete calcium–force curves were not determined. Also, because of apparently contradictory results in other tissues in which other techniques were used (Somlyo, Haeusler & Somlyo, 1970; Meisheri & Van Breemen, 1982; Yamaguchi, Honeyman & Fay, 1982) these results in the ferret portal vein have been rather controversial.

In some respects, the ferret portal vein can be considered an unusual blood vessel because of the phasic nature of its contractile activity and its lack of ability to maintain tone. Therefore, we were anxious to extend these studies to a more tonic type of vascular smooth muscle. Hence, the purpose of the present study was to investigate the effects of three different types of vasodilators, hydralazine, nifedipine and forskolin, on $[Ca^{2+}]_i$ handling and to see if the quantitative $[Ca^{2+}]_i$–force relationship is shifted by these vasodilators.

METHODS

Twelve-week-old male ferrets were anaesthetized with chloroform. The chest cavity was opened and a portion of thoracic aorta removed to a physiological saline solution oxygenated with 95% O_2, 5% CO_2. The aorta was cut into circular strips (7 × 1·0–2·0 mm) and the endothelium removed by gently rubbing with a rubber policeman. The strip was placed in a specially designed light-collecting apparatus containing ellipsoidal mirrors. One end of the muscle was clamped and the other end attached to a Gould UC2 transducer. Aequorin was loaded into the strips by a method previously described (Morgan & Morgan, 1982). Briefly, the strips were incubated in four loading solutions for 30–90 min at 2 °C. 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, 0·5 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. $CaCl_2$ was added gradually to solution IV until a concentration of 2·5 mM was reached. The muscle was allowed to re-equilibrate overnight until basal light was absolutely stable. Light emitted by aequorin was detected with an EMI 9635QA photomultiplier tube specially selected for low dark current and is reported in nanoamperes of anode current. Calibration of aequorin light signals was performed in the manner of Allen & Blinks (1979). The muscle cells were lysed with Triton X-100 and maximal luminescence (L_{max}) was determined. Light levels during the experiment were then expressed as fractional luminescence (L/L_{max}) which was then converted to $[Ca^{2+}]_i$ by the use of an *in vitro* calibration curve assuming a value of 0·5 mM-$MgCl_2$ (Jiang & Morgan, 1987). As previously discussed (Bradley & Morgan, 1987) it is likely that the majority of the aequorin signal from these multicellular strips arises from smooth muscle cells since isolated individual cells give similar $[Ca^{2+}]_i$ signals to those from the strips (DeFeo & Morgan, 1986).

All experiments were performed at 37 °C in a modified Krebs solution containing the following (mM): NaCl, 120; KCl, 5·9; dextrose, 11·5; $NaHCO_3$, 25·0; $MgCl_2$, 1·2; $NaHPO_4$, 1·4; $CaCl_2$, 2·5. Solutions containing elevated potassium were obtained by equimolar replacement of NaCl with KCl. Aequorin was obtained from the laboratory of J. R. Blinks, Mayo Clinic (Rochester, MN, USA).

The following drugs were used: nifedipine (Pfizer), hydralazine (Ciba-Geigy), forskolin (Calbiochem), 12-deoxyphorbol-13-isobutyrate-20-acetate (LC Services Corp).

The results are reported as means ± standard error of the mean (S.E.M.). Statistical significance was accepted for comparisons at $P < 0·05$ using Student's *t* test.

RESULTS

Effects of vasodilators on intrinsic tone and basal $[Ca^{2+}]_i$

Ferret aorta is a tonic tissue which possesses intrinsic active tone at body temperature. It was therefore possible to examine the effect of the vasodilators on

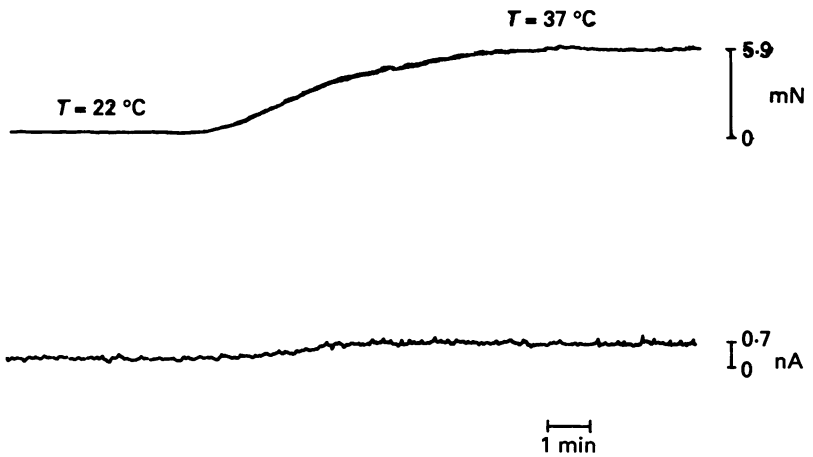

Fig. 1. Simultaneous recording of force (top) and light (bottom) when muscle is warmed from 22 to 37 °C. See text for details. Zero force is determined by the force at 4 °C with the muscle at the optimal length for force production. Zero light is the level of anode current (in nanoamperes) from the photomultiplier tube with the shutter closed.

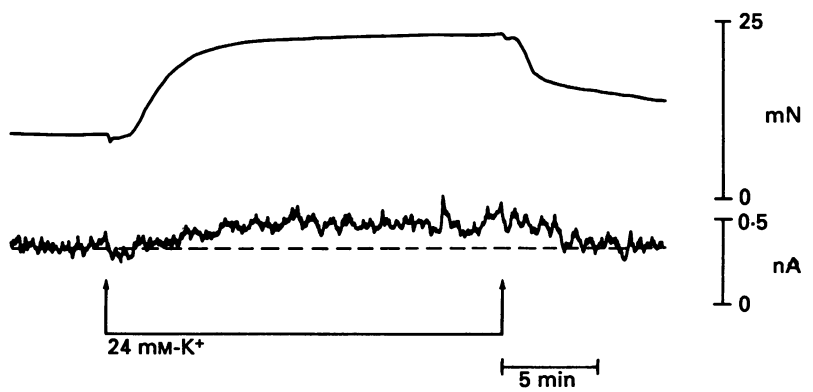

Fig. 2. Effect of potassium on tone (top) and basal $[Ca^{2+}]_i$ (bottom) at 37 °C. Zero force and light defined as in Fig. 1.

basal tone and $[Ca^{2+}]$ without having to precontract the tissue to facilitate the observation of vasodilatation.

Ferret aorta generates $5{\cdot}7 \pm 0{\cdot}7$ mN ($0{\cdot}17 \times 10^5 \pm 0{\cdot}02 \times 10^5$ N/m^2) of force in response to increasing the temperature from 22 to 37 °C ($n = 18$). We have previously reported that there is no statistically significant increase in $[Ca^{2+}]_i$ in the ferret aorta over this temperature range (DeFeo & Morgan, 1985). An example of the effect of warming from 22 to 37 °C is shown in Fig. 1. Notice that both force and light increase but when steady-state fractional luminescence is determined at the two

temperatures, using the appropriate calibration curve and rate constant for consumption of aequorin at each temperature (Allen & Blinks, 1979; Jiang & Morgan, 1987), it is found that absolute $[Ca^{2+}]_i$ remains the same (DeFeo & Morgan, 1985). The mean resting $[Ca^{2+}]_i$ for ferret aorta at 37 °C was $2{\cdot}55 \times 10^{-7} \pm 0{\cdot}09 \times 10^{-7}$ M ($n = 17$).

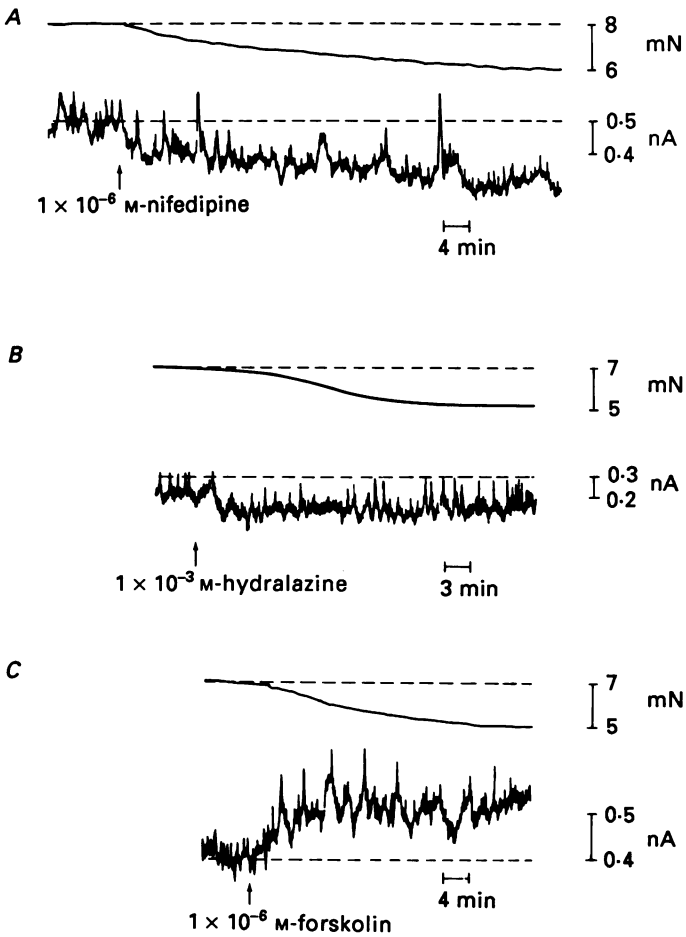

Fig. 3. Effect of vasodilators on intrinsic tone (top) and basal $[Ca^{2+}]_i$ (bottom) at 37 °C. Dashed line marks position of basal force and light. Zero force and light defined as in Fig. 1. *A*, effect of nifedipine. *B*, effect of hydralazine. *C*, effect of forskolin.

Figure 2 illustrates a typical response to potassium depolarization. Note that both force and light exhibit a slow increase to a sustained plateau level.

Figure 3*A* illustrates the effect of 1×10^{-6} M-nifedipine, a calcium channel blocker, on the intrinsic tone and basal $[Ca^{2+}]_i$ at 37 °C. Note that upon the addition of the drug, light decreased concomitantly with tone. The change in $[Ca^{2+}]_i$ was small but statistically significant. In nine experiments the mean decreases in tone and $[Ca^{2+}]_i$ produced by nifedipine were $1{\cdot}6 \pm 0{\cdot}4$ mN and 52 ± 8 nM respectively.

Unlike nifedipine, the physiological basis for vasodilator actions of hydralazine has been largely unexplored. The effect of hydralazine on intrinsic tone and basal $[Ca^{2+}]_i$ at 37 °C is illustrated in Fig. 3*B*. As with nifedipine, there are parallel decreases in

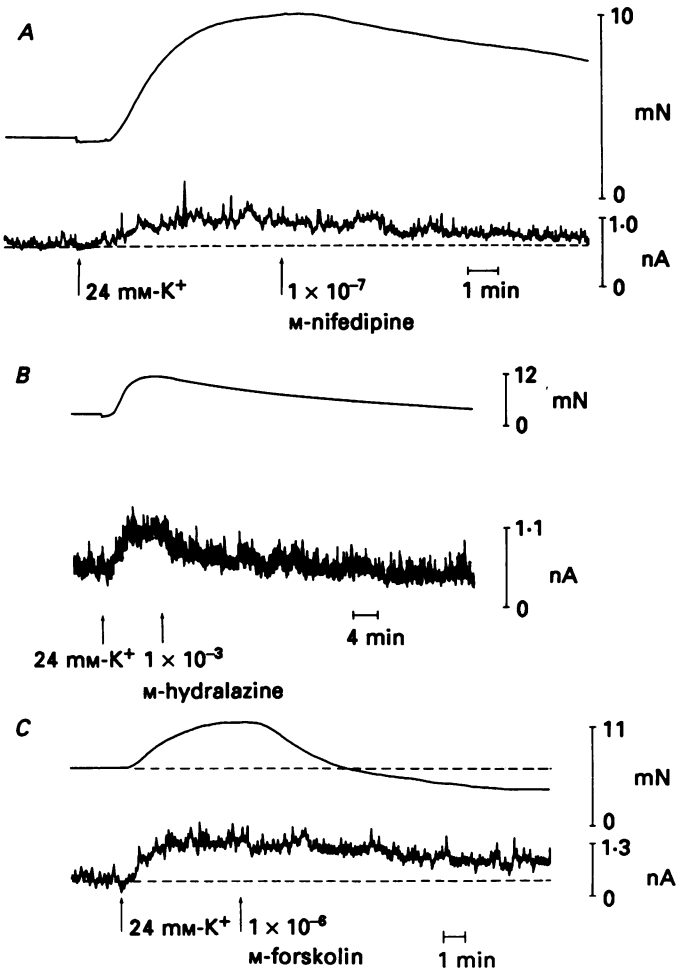

Fig. 4. Effect of vasodilators on K^+-induced increase in force (top) and light (bottom). Dashed line marks position of resting light. Zero force and light as in Fig. 1. *A*, effect of nifedipine. *B*, effect of hydralazine. *C*, effect of forskolin.

force and light. The mean decreases in tone and light were $3{\cdot}1 \pm 0{\cdot}6$ mN and 45 ± 8 nM respectively ($n = 6$).

We also investigated the actions of forskolin, as a prototype of the group of vasodilators which are known to increase cyclic AMP levels. The effect of forskolin on intrinsic tone and basal $[Ca^{2+}]_i$ at 37 °C was also investigated and is illustrated in Fig. 3*C*. Although a relaxation of $1{\cdot}8 \pm 0{\cdot}3$ mN of force was seen, light did not decrease in any experiment and, instead, was found to be 57 ± 7 nM higher than control resting calcium ($n = 9$).

Effects of vasodilators on potassium depolarization

Figure 4*A* illustrates the effect of nifedipine on a potassium-induced contraction. Krebs solution containing 24 mM-potassium was added at the first arrow and kept in the bath for the duration of the trace. Nifedipine (1×10^{-7} M) was added (at the second arrow) as soon as steady-state force and luminescence were achieved. As can be seen, light and force fell concomitantly in response to nifedipine.

Figure 4*B* illustrates the effect of hydralazine on a potassium-induced contraction and the accompanying light signal. Roughly parallel decreases in force and light were observed in response to the addition of hydralazine.

Figure 4*C* shows the effect of forskolin on the potassium contraction and accompanying light signal. In contrast to the effect of forskolin on basal $[Ca^{2+}]_i$ a fall in $[Ca^{2+}]_i$ was consistently observed to parallel the relaxation of potassium contraction; however, in comparison to the effects of nifedipine, a much larger decrease in force appeared to be associated with a smaller decrease in luminescence.

Effects of vasodilators on the calcium–force relationship

The apparent discrepancy in the magnitudes of the changes in $[Ca^{2+}]_i$ and force in the presence of forskolin prompted an analysis of the $[Ca^{2+}]_i$–force relationship in the presence of the vasodilators. The control $[Ca^{2+}]_i$–force curve was generated by causing graded degrees of potassium depolarization of the aorta and plotting the calibrated aequorin light signal against the force developed at 37 °C in each case. The points below baseline were obtained by removing extracellular Ca^{2+} in the presence of depolarization with 24 mM-potassium and also cooling the muscle to 7 °C. Although cooling, by itself, does not significantly lower $[Ca^{2+}]$, it is necessary for the complete removal of active tone. The signals were calibrated in terms of absolute $[Ca^{2+}]_i$ by the method of Allen & Blinks (1979). Figure 5*A* illustrates the effect of nifedipine on the $[Ca^{2+}]_i$ force curve. The aorta was contracted with 24 mM-potassium Krebs solution. Increasing concentrations (10^{-8} to 10^{-5} M) of nifedipine were added to the bath to generate the nifedipine points. As can be seen, the four nifedipine points do not significantly deviate from the control curve.

A similar effect was observed with hydralazine (Fig. 5*B*). Because of concerns about non-specific effects, concentrations above 10^{-3} M were not investigated. It can be seen from Fig. 5*B* that the three hydralazine points (10^{-5} to 10^{-3} M) are essentially superimposable on the control curve.

A different effect was seen with the adenylate cyclase stimulator, forskolin. Figure 5*C* shows that the forskolin curve was shifted significantly to the right compared to the control curve. In this case, a constant concentration of forskolin was used in the presence of three different levels of potassium. Thus, in addition to the decrease in $[Ca^{2+}]_i$ caused by forskolin during the potassium contraction (Fig. 4*C*), it also has a second effect to decrease the amount of force generated at any one $[Ca^{2+}]_i$.

Effects of vasodilators on phorbol ester contractions

It has previously been shown that the phorbol ester 12-deoxyphorbol-13-isobutyrate-20-acetate (DPBA), a protein kinase C activator (Baraban, Gould, Peroutka & Snyder, 1985), produces a significant increase in tone in the ferret aorta

without causing an increase in $[Ca^{2+}]_i$ (Jiang & Morgan, 1987) indicating that resting calcium levels are sufficient to produce phorbol ester-induced contractions in this tissue. Therefore, we were curious to see if agents with known effects on the control

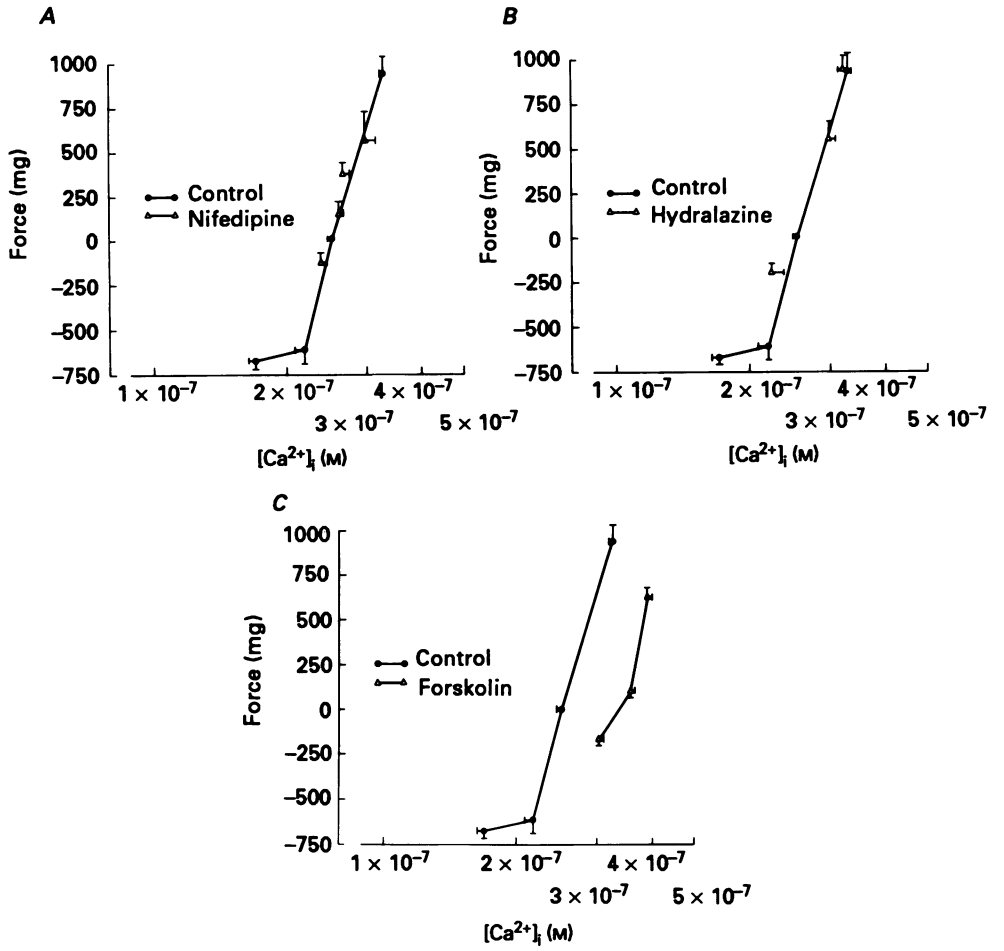

Fig. 5. $[Ca^{2+}]_i$–force relationships. Zero force is defined as the baseline level at 37 °C. Points plotted on the *X*-axis are the log of $[Ca^{2+}]_i$. Points shown are means ± S.E.M. Where the S.E.M. was smaller than the size of the symbol it was drawn touching the symbol. *A*, in the presence and absence of nifedipine. *B*, in the presence and absence of hydralazine. *C*, in the presence and absence of forskolin. See text for details.

$[Ca^{2+}]_i$–force relationship could antagonize the DPBA-induced contraction. Aortic strips were pre-treated with each vasodilator and paired controls were used to establish latency (lag time from application of DPBA until the onset of force), time to peak contraction and magnitude of peak tension. Table 1 summarizes the effects of the vasodilators on phorbol ester-induced contractions in ferret aorta. Both nifedipine and hydralazine significantly increased the latency with no effect on peak force development compared to their paired controls. Forskolin, in contrast,

TABLE 1. Effects of vasodilators on phorbol ester-induced contractions in ferret aorta

	Nifedipine (1×10^{-6} M) $n = 8$	Hydralazine (1×10^{-3} M) $n = 8$	Forskolin (1×10^{-6} M) $n = 6$
Latency (min):			
Control	3·80 ± 0·5	4·80 ± 1·4	3·6 ± 0·5
With drug	6·3 ± 0·9* ($P < 0·05$)	8·5 ± 0·5* ($P < 0·05$)	No response
Time to peak (min):			
Control	34·4 ± 1·8	33·0 ± 4·1	40·0 ± 3·6
With drug	43·1 ± 2·8* ($P < 0·05$)	75·4 ± 3·1* ($P < 0·05$)	No response
Peak tension (mN):			
Control	7·6 ± 1·2	11·8 ± 1·5	8·5 ± 1·9
With drug	8·7 ± 1·2 ($P > 0·05$)	12·4 ± 1·8 $P > 0·05$)	No response

* Statistically significant differences from control.

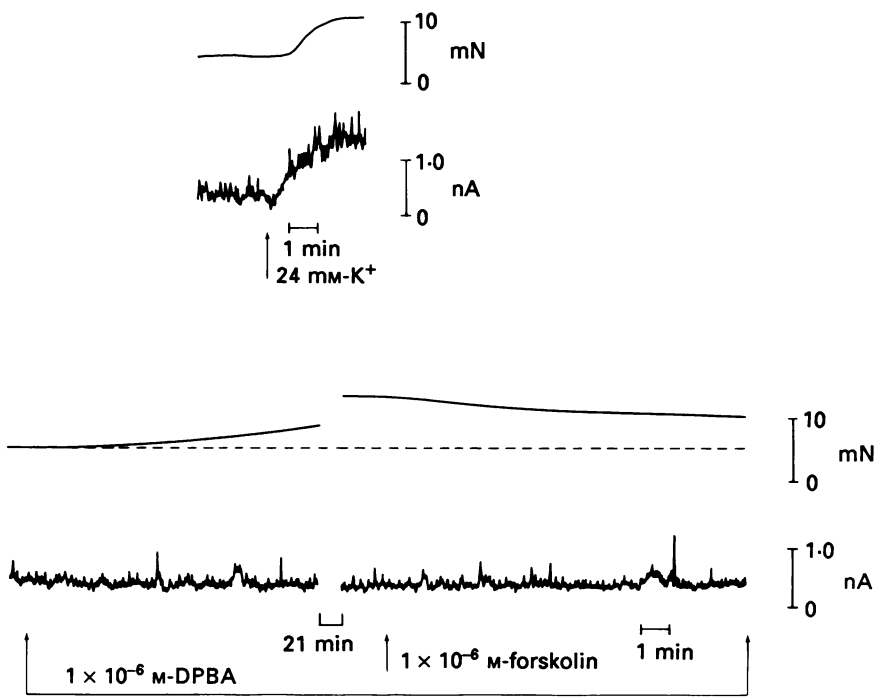

Fig. 6. Two pairs of traces from the same muscle. In each case force is on the top, light is on the bottom. Zero force and light defined as in Fig. 1. The response to 24 mM-K^+ (top pair of traces) is included to demonstrate adequate aequorin loading of the muscle. The lower pair of traces show the response of the tissue to DPBA and forskolin and were interrupted for 21 min.

completely abolished the DPBA-induced contraction in five out of six preparations and thus an average value for latency and time to peak contraction could not be determined.

Since forskolin pre-treatment caused such a dramatic inhibition of the DPBA contraction, we were curious to see if it could antagonize a steady-state DPBA-induced contraction and to determine the effect on $[Ca^{2+}]_i$ at that time. Figure 6 illustrates the effect of forskolin on the DPBA response. DPBA (1×10^{-6} M) was added at the arrow and, after a lag phase, caused a contraction in the absence of any significant rise in luminescence. Once steady-state force was reached, 1×10^{-6} M-forskolin was added. While force decreased significantly, $[Ca^{2+}]_i$ did not, consistent with the previous observation that forskolin does not significantly decrease basal $[Ca^{2+}]_i$ in this tissue. The fact that an adequate amount of aequorin was loaded into these preparations was demonstrated by challenging each preparation with a low concentration of potassium. In each case a significant $[Ca^{2+}]_i$ signal was observed during a potassium contraction of equal or lesser magnitude than that caused by DPBA (Fig. 6). Also, past studies (Morgan & Morgan, 1984*a*), as well as present studies on hydralazine and nifedipine, have indicated that a decrease in $[Ca^{2+}]_i$ below baseline in vascular smooth muscle can be detected by aequorin.

DISCUSSION

The main conclusion of this study is that different calcium–force relationships exist in the presence of the vasodilators nifedipine, hydralazine and forskolin in ferret aorta. We have found that forskolin (an agent known to increase cyclic AMP levels) causes a shift in the $[Ca^{2+}]_i$–force relationship. Nifedipine and hydralazine cause parallel decreases in force and $[Ca^{2+}]_i$. Neither of these vasodilators significantly shifts the $[Ca^{2+}]_i$–force curve away from control and, therefore, the vasodilatation due to nifedipine and hydralazine appears to be due solely to the decrease in $[Ca^{2+}]_i$.

The results with forskolin in ferret aorta are consistent with previous results in ferret portal vein in that we have previously reported that isoprenaline, papaverine and forskolin, which would all be expected to increase cyclic AMP levels, are capable of relaxing ferret portal vein in the absence of a decrease in $[Ca^{2+}]_i$ (Morgan & Morgan, 1984*b*). These results are also consistent with a recent report by Taylor & Stull (1988) that forskolin shifts the $[Ca^{2+}]_i$–phosphorylation relationship to the right in tracheal smooth muscle. In contrast, others (e.g. Somlyo *et al.* 1970; Meisheri & Van Breemen, 1982; Yamaguchi *et al.* 1982) have suggested that increases in cyclic AMP levels in various types of smooth muscle should result in decreases in $[Ca^{2+}]_i$. In ferret stomach, using the same methods as those used in the present study, we have found that isoprenaline does cause a decrease in $[Ca^{2+}]_i$ (Morgan & Jiang, 1987). Therefore, it appears that increases in cyclic AMP can have multiple effects on intracellular Ca^{2+} mobilization and $[Ca^{2+}]_i$–force coupling mechanisms which combine to produce the net relaxation observed. In the present study we have found that forskolin both decreases potassium-induced increases in $[Ca^{2+}]_i$ as well as altering the Ca^{2+} requirement of force production.

The mechanism of action of hydralazine has not previously been extensively investigated. Jacobs (1984) suggested that it may decrease myosin light chain

phosphorylation in bovine carotid arteries. This suggestion is consistent with our observation of a decrease in $[Ca^{2+}]_i$, since myosin light kinase is a $[Ca^{2+}]$-dependent enzyme (Kamm & Stull, 1985).

Tumour-promoting phorbol esters are similar in structure to diacylglycerol and increase the affinity of protein kinase C for Ca^{2+} resulting in full activation at physiological Ca^{2+} concentrations (Nishizuka, 1986; Jiang & Morgan, 1987). In ferret aorta, nifedipine and hydralazine decrease basal $[Ca^{2+}]_i$ and increase the latency and time to peak contraction of phorbol ester-induced responses, but peak tension is not affected. This finding suggests that these two vasodilators leave the activation mechanism intact and may only affect the time required for protein kinase C to become membrane-bound. The increase in latency may be related to the observed decrease in basal $[Ca^{2+}]_i$ caused by these agents in this tissue.

In contrast, forskolin completely prevents the phorbol ester-induced contraction when the muscle is pre-treated with forskolin. It is also capable of producing relaxation of the phorbol-induced contraction when added at steady state in spite of the fact that no concomitant decrease in $[Ca^{2+}]_i$ was seen. This observation suggests that the inhibitory effect could be due to a direct effect on protein kinase C. It could prevent the enzyme from becoming membrane-bound, or, if the enzyme is already bound, enhance its dissociation from the plasma membrane. Nishizuka (1986) has reported that cyclic AMP can inhibit the formation of diacylglycerol and the consequent activation of protein kinase C but we are unaware of any report that cyclic AMP-activated kinase can directly interact with protein kinase C. Alternatively, the forskolin-induced increases in cyclic AMP could antagonize the effects of protein kinase C at the level of the final effector protein phosphorylation.

It was also found that the relaxation of intrinsic tone by forskolin occurs in the absence of a detectable decrease in basal $[Ca^{2+}]_i$, and that forskolin induced a shift of the $[Ca^{2+}]_i$–force curve in this tissue. It is possible that these effects are also a consequence of an antagonism of intrinsic protein kinase C-mediated activity in this tissue. This interpretation is consistent with the report of Ruzycky & Morgan (1988) that the protein kinase C inhibitor H-7 (Hidaka, Inagaki, Kawamoto & Sasaki, 1984) inhibits intrinsic tone in ferret aorta in the absence of a detectable change in basal $[Ca^{2+}]_i$ as well as the fact that phorbol esters cause a leftward shift in the $[Ca^{2+}]_i$–force curve in this tissue (Ruzycky & Morgan, 1988).

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