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Involvement of the protein kinase C system in calcium-force relationships in ferret aorta

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1 Intracellular Ca^{2+}-force relationships were investigated in ferret aortic smooth muscle by the simultaneous measurement of aequorin luminescence and isometric force. Complete calcium-force curves were constructed by plotting calibrated aequorin luminescence versus force, while intracellular $[Ca^{2+}]$ was made to change by increasing degrees of K^+ depolarization or decreasing extracellular $[Ca^{2+}]$.

2 The steady state calcium-force curve in response to K^+ depolarization exhibited maximal force generation at an intracellular $[Ca^{2+}]$ of approximately 4×10^{-7} M. Further increases in intracellular $[Ca^{2+}]$ did not yield additional increments in force.

3 Protein kinase C activation with the phorbol ester, 12-deoxyphorbol-13-isobutyrate 20 acetate (DPBA) produced contractions accompanied by no detectable increases in aequorin luminescence. DPBA significantly shifted the control $[Ca^{2+}]$-force relationship leftward to lower intracellular $[Ca^{2+}]$ with an increase in the magnitude of maximal generated force.

4 In aorta maximally precontracted by K^+ depolarization, the addition of DPBA resulted in a significant increase in force in the absence of further increases in intracellular $[Ca^{2+}]$. Conversely, in muscles maximally precontracted with DPBA, responses to K^+ depolarization resulted in subsequent increases in force in the presence of simultaneous sustained increases in intracellular $[Ca^{2+}]$.

5 The relatively specific protein kinase C antagonist H-7 caused a significant decrease in intrinsic myogenic tone in the absence of any statistically significant decrease in intracellular $[Ca^{2+}]$.

6 These results suggest that protein kinase C may be an important regulator of vascular smooth muscle contractility by: (1) providing a mechanism by which the apparent $[Ca^{2+}]$ sensitivity of the contractile apparatus during agonist-induced contractions is increased, and (2) maintaining intrinsic myogenic tone by a mechanism the $[Ca^{2+}]$ requirement of which is satisfied by the resting $[Ca^{2+}]_i$.

Introduction

Tumour-promoting phorbol esters, which presumably activate protein kinase C, have been shown to cause sustained contractions of vascular smooth muscle (Rasmussen *et al.*, 1984; Danthuluri & Deth, 1984; Baraban *et al.*, 1985) in the absence of increasing levels of $[Ca^{2+}]_i$ (Jiang & Morgan, 1987). Protein kinase C activation has also been shown to modulate the phosphorylation of heavy meromyosin (Nishikawa *et al.*, 1984). However, this protein kinase C-mediated myosin phosphorylation occurred at sites different from those phosphorylated by myosin light chain kinase, suggesting a mechanism of myosin regulation in addition to the Ca^{2+}-calmodulin dependent pathway. This phosphorylation resulted in an inhibition of actomyosin ATPase activity. Therefore, the mechanism of protein kinase C-mediated contraction remains unknown.

In the present study we have determined the $[Ca^{2+}]_i$-force relationships in ferret aortic smooth muscle in the presence and absence of protein kinase C activation and have investigated the degree to which protein kinase C activation contributes to α-adrenoceptor-mediated contractions as well as to the

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phenomenon of additivity of agonists (Somlyo & Somlyo, 1968).

Methods

Adult male ferrets were anaesthetized with an overdose of chloroform by procedures approved by the Institutional Committee on Animal Research. Strips of ferret aorta were prepared in a manner similar to that previously described (DeFeo & Morgan, 1985). Experiments were performed 37°C in a modified Krebs solution of the following composition (mM): NaCl 120, KCl 5.9, $MgCl_2$ 1.2, $CaCl_2$ 2.5, $NaHCO_3$ 25, NaH_2PO_4 1.2 and dextrose, 11.5; aerated with 95% O_2:5% CO_2. Solutions containing elevated K^+ were obtained by isotonic replacement of NaCl by KCl and therefore resulted in solutions which were somewhat Na-deficient. 'Isometric' contractions were recorded simultaneously with aequorin signals from circular strips, 1–2 mm wide, 5–7 mm long and approximately 200 μm thick. Stress was determined by dividing the force of contraction by the cross sectional area. The cross sectional area of the strips was calculated by weight by the method of Herlihy & Murphy (1973), assuming that area = (mass/(density × length) and that density is 1.05 g ml^{-1}.

The viability of each strip was assessed at room temperature with a maximally effective dose of phenylephrine before the bioluminescent protein, aequorin, was loaded into the smooth muscle cells. Aequorin was loaded into the vascular smooth muscle cells by a chemical loading procedure previously described (Morgan & Morgan, 1982; Bradley & Morgan, 1987). Briefly, this procedure consists of incubating the muscle in a series of EGTA- and ATP-containing solutions at 2°C. As previously shown (Morgan & Morgan, 1984; Jiang & Morgan, 1987), this procedure does not alter the responsiveness of aortic smooth muscle when compared before and after the aequorin loading. Aequorin luminescence was monitored using a light-tight enclosure for the muscle bath and photomultiplier tube (Bradley & Morgan, 1987). All experiments were performed at 37°C.

Aequorin luminescence was calibrated in terms of absolute $[Ca^{2+}]_i$ by the method of fractional luminescence (Allen & Blinks, 1979) as adapted to vascular muscle by DeFeo & Morgan (1985). A time constant of 0.4 s was used in the calculation of L_{max}. The ratio of L/L_{max} was then converted to $[Ca^{2+}]_i$ by the use of a standard *in vitro* calibration curve constructed at a free $[Mg^{2+}] = 0.5$ mM (Jiang & Morgan, 1987). The choice of 0.5 mM $[Mg^{2+}]$ was based on NMR measurements of $[Mg^{2+}]$ in bladder and uterine smooth muscle (Kushmerick *et al.*, 1986). For a discussion of the effect of errors in the estimation of $[Mg^{2+}]_i$ on the calculation of $[Ca^{2+}]_i$ from aequorin luminescence in ferret aorta, see DeFeo & Morgan, 1985.

The aequorin used in this study was obtained from Professor J.R. Blinks of the Mayo Clinic. The following drugs were used as aqueous solutions: 1-(5-isoquinolinesulphonyl)-2-methylpiperazine dihydro-chloride (H-7) (Seikagaku Kogyo), N-(6-aminohexyl)-5-chloro-1-naphthalenesulphonamide hydrochloride (W-7) (Seikagaku Kogyo), phenylephrine (Sigma). 12-Deoxyphorbol-13-isobutyrate 20-acetate (DPBA) (LC Services) was dissolved in dimethylsulphoxide.

Data are reported as mean values with standard errors of the mean. Statistical significance was accepted for comparisons at $P < 0.05$ using Student's t test.

Results

Resting $[Ca^{2+}]_i$

The quantitation of $[Ca^{2+}]_i$ from aequorin signals requires a knowledge of the intracellular Mg^{2+} concentration ($[Mg^{2+}]_i$) (Blinks *et al.*, 1978), which is still controversial in smooth muscle. A value of $[Mg^{2+}]_i = 0.5$ mM was assumed in these studies. This assumption is based on n.m.r. measurements of $[Mg^{2+}]_i$ in smooth muscle cells (Kushmerick *et al.*,

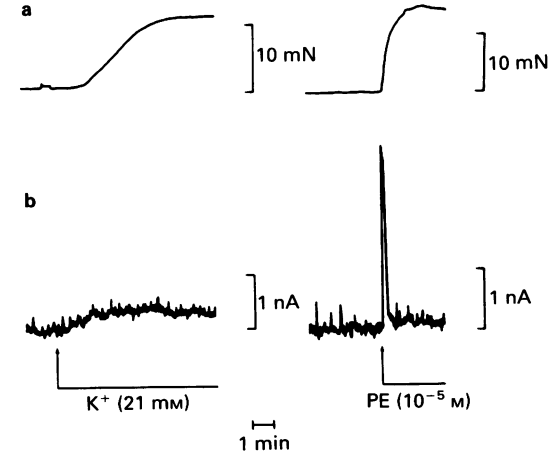

Figure 1 Simultaneously recorded tension in mN (102 mg = 1 mN) (a) and light in nA (b) in ferret aorta at 37°C. Left pair of traces, 21 mM K^+. Right pair of traces, 10^{-5} M phenylephrine (PE).

1986). Using this value for $[Mg^{2+}]_i$, resting $[Ca^{2+}]_i$ was $2.60 \pm 0.09 \times 10^{-7}$ M ($n = 33$ preparations, 33 animals) in ferret aortic smooth muscle cells at 37°C.

Concentration-response relationships

Morgan & Morgan (1984) have previously shown that different contractile stimuli produce very different temporal changes in $[Ca^{2+}]_i$. However, the concentration-dependence of these responses was not previously determined in ferret aorta. Figure 1 illustrates typical light and tension responses of the ferret aorta at 37°C to the addition of 21 mM K^+ and 10^{-5} M phenylephrine. In the presence of 10^{-5} M phenylephrine light rises rapidly but the increase is transient, quickly declining toward resting levels while isometric tension continues to increase to a steady state level. When the complete concentration-response relationships were determined (Figure 2) it was found that 10^{-5} M phenylephrine is a maximally effective concentration. The mean 50% effective concentration (EC_{50}) for phenylephrine was $4.4 \pm 0.5 \times 10^{-8}$ M ($n = 13$ preparations, 9 animals). The aequorin response to 21 mM K^+ (a near EC_{50} concentration) stimulation rises gradually with force and both responses plateau at a steady state level. The maximally effective concentration of K^+ was approximately 51 mM (Figure 2) and the mean EC_{50} was 19.6 ± 1 mM ($n = 17$ preparations, 7 animals). Paired analysis of the amplitude of the maximal contractions to the two stimuli ($n = 15$ preparations, 7 animals) revealed that maximal phenylephrine responses (mean stress, 0.755 ± 0.03 N m$^{-2} \times 10^5$) were significantly larger (9%) than maximal K^+ stimulated responses (mean stress, 0.695 ± 0.03 N m$^{-2} \times 10^5$).

Control $[Ca^{2+}]_i$-force relationships

$[Ca^{2+}]_i$-force curves were generated in intact aequorin-loaded cells by causing graded degrees of K^+ depolarization of the muscle. Maintaining $[Ca^{2+}]$ constant in the Krebs solution at 2.5 mM, $[K^+]$ was increased from 5.9 mM to 81 mM. The K^+-stimulated force response increased in a concentration-dependent fashion achieving a maximum at 51 mM K^+. Luminescence increased in parallel with force up to 51 mM K^+ and further increased in the presence of 81 mM K^+ in the absence of a further increase in generated force. A mean $[Ca^{2+}]_i$-force curve is shown in Figure 3. Values for $[Ca^{2+}]_i$ and force below resting levels were obtained by depletion of extracellular calcium with EGTA (4 mM) in the presence of 24 mM K^+.

In order to obtain an index of the steepness of the $[Ca^{2+}]_i$-force relationship the data were fitted to the Hill equation: relative force = $100\%/(1 + 10^{N(pCa - pK)})$ where $pCa = -\log_{10}[Ca^{2+}]$, pK = the pCa at the half maximal force level of the Hill fit, and N = the Hill coefficient, a measure of the slope of the curve at the pK. The mean pK was 6.50 ± 0.03 which is $3.15 \pm 0.2 \times 10^{-7}$ M [Ca] with $N = 12.2 \pm 2.2$.

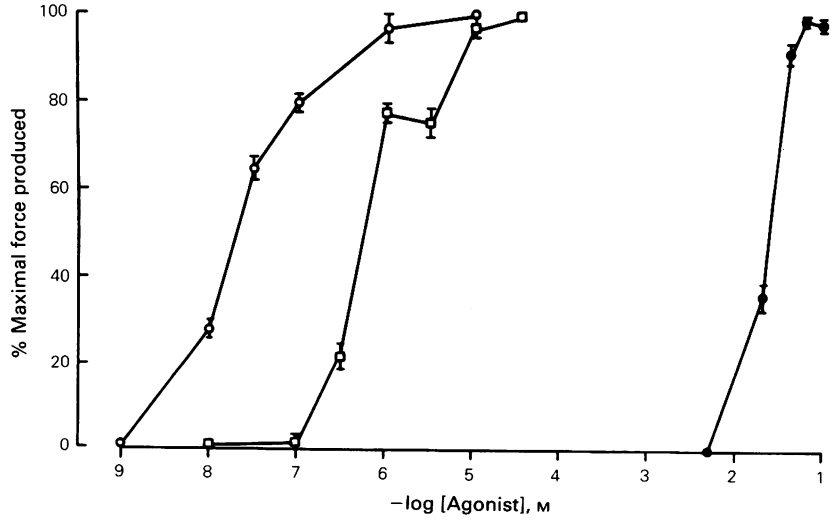

Figure 2 Mean concentration-force relationships for phenylephrine (○), DPBA (□), and K^+ depolarization (●) in ferret aorta at 37°C. The mean EC_{50} values given in the text were determined from the mean of the EC_{50} values determined for each individual curve.

Dependence of phenylephrine contraction on presence of Ca^{2+} transient

The transient light responses associated with phenylephrine-induced contractions were most pronounced upon the addition of a single high concentration ($>10^{-6}$ M) of the agonist and were generally not demonstratable upon cumulative addition of the agonist. This was in spite of the fact that essentially identical force was obtained with a maximal concentration of phenylephrine applied alone or in a cumulative protocol. In 8 preparations from 6 animals, in a cumulative protocol, 10^{-5} M phenylephrine caused no detectable Ca transient but produced 98 ± 2.3% of the contractile force to a challenge of 10^{-5} M phenylephrine. These results suggest that the calcium spike may be merely coincidental with and may not be directly related to the generation and maintenance of the contraction.

Additivity of responses

As has been previously reported for portal vein smooth muscle (Johansson & Somlyo, 1980; Morgan & Morgan, 1984), we have found that the addition of phenylephrine (10^{-5} M) to ferret aortic smooth muscle which was maximally pre-contracted with respect to K^+ stimulation (81 mM), produced a further sustained increase in force. This subsequent increase in force (0.19 ± 0.01 N m^{-2} × 10^5 or 23% of the K^+ generated precontraction; cf. Table 1) occurred in the presence of only a transient increase in the $[Ca^{2+}]_i$ level over that due to the initial K^+ stimulation. The steady state $[Ca^{2+}]$ was not significantly changed (Table 1).

A further sustained contraction (0.14 ± 0.02 N m^{-2} × 10^5 or 25% of the force generated by the precontraction; Table 1) was also generated when 81 mM K^+ was added on top of a maximally effective concentration of phenylephrine (10^{-5} M), with a concurrent sustained increase in $[Ca^{2+}]_i$ above the $[Ca^{2+}]_i$ observed during the phenylephrine-induced steady state contraction. The increase in $[Ca^{2+}]_i$ induced by 81 mM K^+ under these conditions was not significantly different from that observed under control conditions.

To investigate whether the increase in maintained force associated with the addition of phenylephrine in the presence of maximal K^+ stimulation could be attributed to a protein kinase C-mediated pathway, we determined the degree to which the effects of the active phorbol ester DPBA, a putative protein kinase C agonist, could mimic the actions of phenylephrine. At a maximally effective concentration of DPBA (10^{-5} M; Figure 2) the mean generated contraction was larger than either maximal K^+ or phenylephrine contractions (mean stress, 0.91 ± 0.07 N m^{-2} × 10^5).

The addition of DPBA (10^{-5} M) to a muscle maximally precontracted with K^+ resulted in a further slowly developing sustained contraction in the absence of any significant change in $[Ca^{2+}]_i$ (Table 1). The mean increase in force caused by the addition of a maximally effective concentration of DPBA on top of maximum K^+ was 0.21 ± 0.03 N m^{-2} × 10^5 (23% of the precontraction; cf. Table 1). This increase in force was not significantly different from the increase in force observed upon addition of phenylephrine to a maximally K^+-contracted tissue

Table 1 Effects of stimulant combinations on isometric force generation in ferret aorta

Initial stimulus	*Secondary stimulus*	*Additional force (as a % of the precontraction)*	*Steady state $[Ca^{2+}]_i$*	
			Precontraction	*Secondary contraction*
$[K^+]_{max}$	$[PE]_{max}$	23 ± 2 (15,8)	421 ± 28 nM (11,11)	389 ± 88 (4,4)
	$[DPBA]_{max}$	23 ± 2 (15,8)		420 ± 51 (3,3)
$[PE]_{max}$	$[K^+]_{max}$	25 ± 3 (16,10) *	304 ± 43 (6,5)	405 ± 32 (6,5)
	$[DPBA]_{max}$	11 ± 4 (15,8)		307 ± 21 (4,3)
$[DPBA]_{max}$	$[K^+]_{max}$	30 ± 4 (17,12) *	263 ± 12 nM (13,12)	416 ± 24 nM (13,12)
	$[PE]_{max}$	7 ± 4 (12,7)		282 ± 32 (7,7)

PE = phenylephrine; DPBA = 12-deoxyphorbol-13-isobutyrate 20 acetate.
$[K^+]_{max}$ = 81 mM; $[PE]_{max}$ = 10^{-5} M; $[DPBA]_{max}$ = 10^{-5} M. *$P < 0.01$ between values above and below asterisk.
The number of preparations used, followed by the number of animals used are given in parentheses.

(see Table 1). However, the increase in force observed upon addition of DPBA to tissues maximally precontracted with phenylephrine was far less, $0.08 \pm 0.02\,N\,m^{-2} \times 10^5$ or 11% of the precontraction (Table 1).

DPBA (10^{-5} M) contracts ferret aortic smooth muscle in the absence of any significant rise in $[Ca^{2+}]_i$, as previously reported (Jiang & Morgan, 1987) and confirmed in this study. Addition of 81 mM K^+ to a maximally DPBA-precontracted muscle resulted in a further significant increase in force ($0.22 \pm 0.02\,N\,m^{-2} \times 10^5$; or 30% of the precontraction; cf. Table 1) concurrent with a sustained increase in $[Ca^{2+}]_i$. The steady state $[Ca^{2+}]_i$ level reached during this subsequent addition of 81 mM K^+ was not significantly different from that observed in the presence of 81 mM K^+ alone. Moreover, the magnitude of the combined force generated by maximal concentrations of K^+ and DPBA ($1.05 \pm 0.07\,N\,m^{-2} \times 10^5$) was not significantly different from the combined force generated by maximal concentrations of K^+ and phenylephrine ($1.02 \pm 0.05\,N\,m^{-2} \times 10^5$).

Addition of 10^{-5} M phenylephrine to a maximally DPBA-contracted muscle strip caused a small increase in force which reached significance in a paired t test. The transient spike in light normally associated with phenylephrine-induced responses was also abolished in the presence of 10^{-5} M DPBA.

The relative differences in forces generated by the different combination protocols employed are summarized in Table 1. These results, taken together, demonstrate an additivity of responses between K^+ and phenylephrine contractions and between K^+ and DPBA contractions but little or no additivity between DPBA and phenylephrine contractions.

$[Ca^{2+}]_i$-force relationship and C kinase activation

It has been suggested that phorbol esters, via protein kinase C activation, contract vascular smooth muscle, in part, by increasing the apparent sensitivity of the contractile apparatus to calcium (Miller *et al.*, 1986; Jiang & Morgan, 1987). However, the actual effect of protein kinase C activation on the $[Ca^{2+}]_i$-force relationship has not been demonstrated. To evaluate further the calcium-dependence of DPBA-induced contractions of ferret aorta, mean calcium-force curves were constructed in the presence and absence of DPBA (10^{-5} M). As mentioned previously, these curves were generated by causing graded degrees of K^+ depolarization in the presence and absence of the phorbol ester. In each case, steady state active force was plotted against steady state $[Ca^{2+}]_i$. As shown in Figure 3, under control conditions (absence of phorbol ester) active force increased with $[Ca^{2+}]_i$ from a sub-resting level of 200 nM

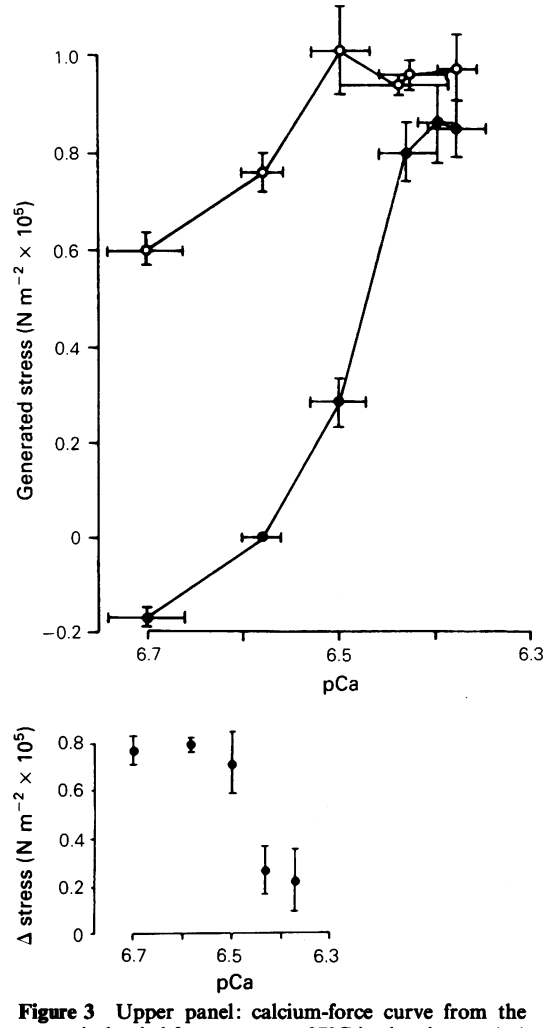

Figure 3 Upper panel: calcium-force curve from the aequorin-loaded ferret aorta at 37°C in the absence (●) and presence of 10^{-5} M DPBA (○). Zero force is arbitrarily defined as the resting level of force at body temperature in Krebs solution. Lower panel: Net DPBA-mediated calcium-force relationship. See text for details.

(achieved by depletion of extracellular calcium with 4 mM EGTA and 24 mM K^+) to approximately 390 nM after which further increases in $[Ca^{2+}]_i$ did not result in further significant increases in force. When $[Ca^{2+}]_i$ was lowered from resting levels by this protocol, active resting force decreased and was recorded as negative generated force. DPBA (10^{-5} M) was found to cause a leftward shift in the calcium-force relationship along with an increase in the magnitude of the maximal generated force relative to the control condition. In the presence of the phorbol ester, maximal force was reached at lower $[Ca^{2+}]_i$

(320 nM) with further increases in $[Ca^{2+}]_i$ not generating further force.

It is apparent from Figure 3 that, if there are two force generating systems in this tissue, their contributions overlap (and consequently add) in the range of pCa's between 6.7 and 6.5. If the 'control' curve is obtained solely by depolarization-induced increases and no other pharmacological or receptor mediated actions, it would be unlikely that there would be significant Ca^{2+}-dependent activation of protein kinase C under these conditions. Therefore, if this 'control' curve is subtracted from the curve obtained in the presence of the phorbol ester, the true $[Ca^{2+}]_i$-force relationship utilized by the phorbol ester should become evident. In fact, the 'net' DPBA-induced $[Ca^{2+}]_i$ force curve (Figure 3, lower panel) shows that there does not appear to be a $[Ca^{2+}]_i$-dependence of DPBA-mediated force production over a range of $[Ca^{2+}]_i$ from 200 nM (sub-resting) to 320 nM. At higher $[Ca^{2+}]_i$ less force was obtained, probably, as a result of a ceiling on the total amount of force the muscle could generate, preventing complete additivity of the two stimuli.

Effects of inhibition of C kinase and myosin light chain kinase

Recently, Hidaka & Hagiwara (1987) described a group of novel naphthalenesulphonamide enzyme inhibitors with relative selectivity for either protein kinase C or calmodulin. The isoquinoline derivative, H-7, was reported to be a direct inhibitor of protein kinase C with a 2–20 fold selectivity over cyclic nucleotide-dependent protein kinases and myosin light chain kinase, respectively. Another structurally related compound, W-7, has been described as a direct calmodulin inhibitor (Kanamori *et al.*, 1981) which inhibits protein kinase C only indirectly at higher concentrations (Hidaka & Hagiwara, 1987), allowing for the possible discrimination of protein kinase C and Ca^{2+}-calmodulin (myosin light chain kinase)-mediated responses (Wright & Hoffman, 1987).

As shown in Figure 4, 30 μM H-7 caused a significant decrease in resting active tone of the ferret aorta ($0.10 \pm 0.01\,N\,m^{-2} \times 10^5$ or 20%; $n = 15$ preparations, 11 animals) in the absence of any significant decreases in $[Ca^{2+}]_i$ at 37°C; this compares with a control active resting tone of $0.48 \pm 0.03\,N\,m^{-2} \times 10^5$ ($n = 38$ preparations, 29 animals). The magnitude of resting tone was measured as the difference between the force on the transducer after stretching the muscle to the optimal length (L_M) for force production and cooling to 2°C and the force with the muscle at L_M but warmed to 37°C. Lowering of $[Ca^{2+}]_i$ to sub-resting levels in the presence of H-7, achieved by depletion of extracellular calcium

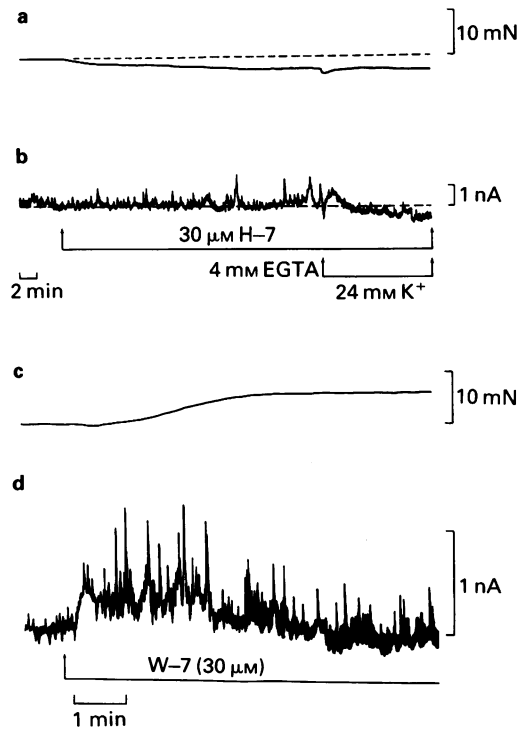

Figure 4 Upper pair of traces: force (a) and light (b) in response to the addition H-7. The dashed lines indicate baseline levels. Lower pair of traces: force (c) and light (d) in response to the addition of W-7.

with EGTA under mildly depolarizing conditions (in presence of 24 mM K^+), produced a detectable decrease in luminescence (Figure 4) but failed to cause a further decrease in resting tone. This ruled out the possibility that the lack of a fall in luminescence during H-7 exposure was a result of being below the range of $[Ca^{2+}]_i$ to which aequorin is sensitive.

In contrast, 30 μM W-7 caused a small but significant rise ($0.09 \pm 0.02\,N\,m^{-2} \times 10^5$ or 18%; $n = 10$ preparations, 8 animals) in resting active tone (Figure 4). In the presence of this agent, $[Ca^{2+}]_i$ did not appear to rise in a sustained manner but rather, frequent oscillations of rapid transient light spikes were observed in aequorin-loaded muscle strips. Additionally, these changes in $[Ca^{2+}]_i$ did not temporally parallel observed changes in resting tone.

To evaluate further the role of protein kinase C in K^+ and phenylephrine-mediated contractile responses, the effects of both compounds, H-7 and W-7, were compared and contrasted. Since both agents also affected baseline tone (see above) the determination of the net effects of the inhibitor on agonist-mediated contractions is reported after com-

Table 2 Effect of protein kinase C inhibition (+30 μM H-7) and calmodulin inhibition (+30 μM W-7) on agonist-stimulated isometric force

Stimulus	*Generated stress* ($N m^{-2} \times 10^5$)	*% inhibition*
K^+ (21 mM)	0.299 ± 0.01 (16,9)	
PE (10 nM)	0.370 ± 0.02 (16,9)	
DPBA (0.7 μM)	0.373 ± 0.04 (15,8)	
K^+ + H-7	0.262 ± 0.04 (8,4)*	12
PE + H-7	0.302 ± 0.07 (8,4)*	18
DPBA + H-7	0.181 ± 0.05 (8,3)*	51
K^+ + W-7	0.323 ± 0.06 (8,5)*	−8
PE + W-7	0.268 ± 0.08 (8,5)*	28
DPBA + W-7	0.247 ± 0.06 (8,5)*	34

Forces generated are given after compensating for the effects of H-7 or W-7 (see text for details). The number of preparations used, followed by the number of animals used are in parentheses.
* Indicates significant ($P < 0.05$) change from corresponding control stress in the absence of inhibitors.
PE = phenylephrine; DPBA = 12-deoxyphorbol-13-isobutyrate 20 acetate; H-7 = 1-(5-isoquinolinesulphonyl)-2-methylpiperazine dihydrochloride; W-7 = N-(6-amino-hexyl)-5-chloro-1-naphthalene sulphonamide hydrochloride.

pensating for the effects on resting active tone in the absence of an agonist. This compensation was made by adding an amount equal to the expected mean decrease in basal tone ($0.10 N m^{-2} \times 10^5$) onto the observed generated force in the presence of H-7 and subtracting the expected mean rise in basal tone ($0.09 N m^{-2} \times 10^5$) in the presence of W-7. H-7 at an approximately mean inhibitory concentration of 30 μM (Hidaka *et al.*, 1984), inhibited EC_{50} K^+-phenylephrine- and DPBA-induced contractions. DPBA contractions were most sensitive to inhibition by H-7 (51%) whereas phenylephrine responses were affected much less (18%) and K^+ were affected least of all (12%; Table 2).

In contrast, the calmodulin inhibitor, W-7, exhibited a very different profile against these responses. At a mean inhibitory concentration (30 μM), W-7 inhibited both DPBA- (34%) and phenylephrine-mediated responses (28%) whereas K^+-stimulated contractions were slightly but significantly potentiated (8%; Table 2). These responses are not consistent with the expected inhibition of force as a result of inhibition of calcium-calmodulin-mediated myosin light chain phosphorylation, but were found to be consistent with the observed changes in $[Ca^{2+}]_i$ (see below).

In a separate series of studies, the effects of W-7 on K^+ and phenylephrine contractions were determined in aequorin-loaded preparations. The potentiation of K^+ contractile responses by W-7 occurred in the presence of further small transient increases in $[Ca^{2+}]_i$ in addition to that produced by K^+ stimulation (Figure 5). The inhibition of phenylephrine-generated force by 30 μM W-7 was accompanied by complete abolition of the characteristic $[Ca^{2+}]_i$ spike response associated with maximal phenylephrine (10^{-5} M) stimulation.

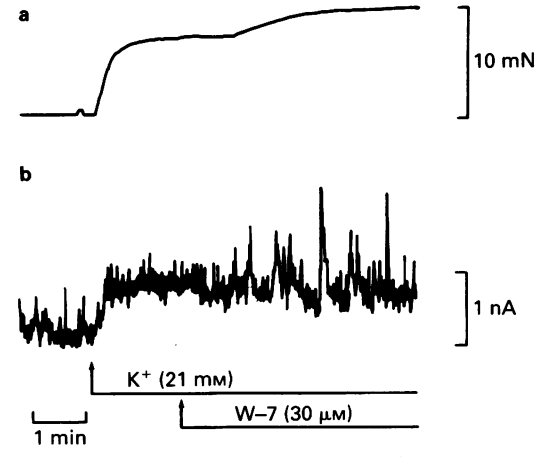

Figure 5 Force (a) and light (b) from ferret aorta at 37°C.

Discussion

The main finding of the present study is that there appear to exist at least two mechanisms mediating intrinsic tone maintenance in intact aortic smooth muscle of the ferret. One, requires a higher $[Ca^{2+}]_i$, and can be distinguished by a characteristic $[Ca^{2+}]_i$-force relationship with a p*K* of 6.50. This p*K* value is similar to that reported by Sobieszek (1977) for calcium-dependent myosin phosphorylation in myofibrils by myosin light chain kinase. Thus this first mechanism may utilize the Ca^{2+}-calmodulin-dependent activation of myosin phosphorylation by myosin light chain kinase for force production. The second mechanism appears to have a lower $[Ca^{2+}]_i$ requirement that may in fact be below resting $[Ca^{2+}]_i$. Such a pathway was demonstrated in the presence of phorbol esters, and may also be involved in the generation of intrinsic tone and the phenylephrine-induced contraction.

One of the more interesting findings of this study is that we were able to demonstrate that additional force could be generated under conditions where a calcium-dependent force production pathway was apparently at its maximal capacity (further increases in $[Ca^{2+}]_i$ could not produce further force). This was observed during maximal K^+ contractions subsequently challenged with either phenylephrine or a

phorbol ester. These results indicate that the additivity occurs at a step in the excitation-contraction coupling sequence beyond the calcium-dependent step and point to the operation of a second force generating pathway in addition to the first calcium-dependent pathway.

In this tissue, the addition of phenylephrine to a ferret aorta precontracted with a high level of KCl, produced both a further contraction and a transient increase in $[Ca^{2+}]_i$. These observations are somewhat different from those reported in ferret portal vein from the same laboratory (Morgan & Morgan, 1984) in that the phenylephrine transient calcium signal was absent when added to a 96 mM K^+ response in portal vein. This probably reflects tissue specific differences in calcium mobilization processes. Even though a transient $[Ca^{2+}]_i$ increase was observed with the addition of phenylephrine (but not phorbol ester) in maximally K^+-stimulated tissues, the phenylephrine-induced calcium spike was not thought to cause the further sustained force since it was observed in the presence of an apparent saturation of the $[Ca^{2+}]_i$-dependent force production pathway (by the K^+ precontraction). Similarly, we have shown in cumulative phenylephrine concentration-response experiments that this transient calcium increase is probably only coincidental with and is not necessary for phenylephrine-mediated force production.

The generation and maintenance of phorbol ester-mediated vascular tone in the absence of detectable elevations in $[Ca^{2+}]_i$ (Jiang & Morgan, 1987) or in the presence of low levels of myosin light chain phosphorylation (Chatterjee & Tejada, 1986; Jiang & Morgan, 1988) have suggested a role for endogenous phospholipid hydrolysis products (diacylglycerols) in contractile signal transduction. Phorbol ester-induced contractions in intact (Jiang & Morgan, 1987) and skinned vascular smooth muscle (Chatterjee & Tejada, 1986; Miller *et al.*, 1986) were maintained at a $[Ca^{2+}]_i$ which could not support other contractions (K^+ stimulated) requiring myosin light chain phosphorylation. These findings indicate that protein kinase C activation can result in an apparently increased sensitivity of the contractile apparatus to calcium.

The $[Ca^{2+}]_i$-force relationships reported in this study are consistent with the hypothesis that protein kinase C activation, in the presence of agonists which apparently release diacylglycerol, may cause the apparent increase in the sensitivity of the contractile apparatus to calcium observed in the presence of other agonists (Bradley & Morgan, 1987). In the presence of DPBA, the $[Ca^{2+}]_i$-force relationship was shifted significantly leftward (to lower $[Ca^{2+}]_i$) relative to the control curve. The presence of an increased level of maximal force in comparison to the control curve, however, suggests the existence of more than a simple change in sensitivity. Additionally, there is an indication that a large part of this force producing mechanism may be calcium independent. This became apparent when the 'control' $[Ca^{2+}]_i$-force curve was subtracted from the curve determined in the presence of protein kinase C activation (inset, Figure 3). With this manipulation two distinct force generating mechanisms are apparent, with one being activated by calcium over the same general range as myosin light chain kinase and the other appearing to be calcium-independent over the physiological range of pCa's in this tissue. A similar calcium independence has been suggested for some agonist-induced platelet function (Rink & Hallam, 1984). These observations may in part be explained by reports of protein kinase C-mediated phosphorylation of other proteins such as the actin binding protein, caldesmon (Umekawa & Hidaka, 1985).

The Hill coefficient for the 'control' $[Ca^{2+}]_i$-force relationship in the presence of K-depolarization is 12. This value is relatively high compared to values reported for skinned skeletal muscle preparations (Brandt *et al.*, 1982) but in intact (aequorin loaded) preparatons a value as high as 9.1 has been reported (Yue *et al.*, 1986). Since there were relatively few points in the critical middle portion of the curve, the results should be interpreted with caution but they do suggest a relatively high degree of cooperativity and complexity in the $[Ca^{2+}]_i$-force relationship in the intact muscle strip. This relationship agrees in general with the previously determined $[Ca^{2+}]_i$-force relationship in ferret aorta (DeFeo & Morgan, 1985), to the extent that the p*K*'s appear similar, but the complete $[Ca^{2+}]_i$-force curve was not previously determined.

Interestingly, the relatively specific C kinase inhibitor H-7 caused a significant (20%) decrease in intrinsic myogenic tone in the absence of any statistically significant decrease in $[Ca^{2+}]_i$, in spite of the fact that further detectable decreases in $[Ca^{2+}]_i$ were observed on addition of EGTA in the absence of further decreases in active force. This finding is consistent with recent studies by Laher & Bevan (1987) indicating an important direct role of protein kinase C in the modulation of vascular myogenic tone and providing evidence that this pathway may be critical to the expression of subsequent agonist-stimulated vascular contractility. Conversely, the calmodulin inhibitor W-7 caused a significant rise in intrinsic myogenic tone with the appearance of frequent transient oscillations in $[Ca^{2+}]_i$. Thus the expected inhibition of the calcium-calmodulin-dependent enzyme myosin light chain kinase was not the dominant effect of this agent. The mechanism of these oscillations is not currently understood. This

may relate to the disruption of the functioning of various calmodulin-dependent calcium pumps on the sarcoplasmic reticulum or sarcolemma (Yamaguchi, 1987). The temporal dissociation of force generation from the observed increase in $[Ca^{2+}]_i$ may also reflect an alteration in calcium-force relationships at the level of the contractile proteins.

Attempts to distinguish the force maintaining mechanisms of phenylephrine- and K^+-induced contractions with these antagonists of protein kinase C (H-7) and calmodulin (W-7) yielded somewhat confusing results. Whereas DPBA-induced contractions were significantly more sensitive to inhibition by H-7 than W-7, a significant amount (34%) of inhibition by the calmodulin inhibitor was observed. This result can most probably be attributed to the lack of total specificity for calmodulin of this inhibitor, a structural congener of H-7 (Hidaka & Hagiwara, 1987). Alternatively, protein kinase C may interact with calmodulin in exerting its cellular actions. However, in contrast to previous experiments suggesting phenylephrine responses are mediated through protein kinase C, submaximal phenylephrine responses were found to be slightly more sensitive to inhibition by W-7 than H-7. It is difficult to say whether this suggests a co-involvement of the Ca^{2+}-calmodulin pathway in α-adrenoceptor-mediated contractions in this tissue or reflects a lack of specificity of the inhibitor.

In conclusion, we have shown that protein kinase C activation can not only cause a state of increased apparent calcium sensitivity of vascular smooth muscle contractile apparatus but may also regulate intrinsic myogenic tone in a calcium-requiring but not necessarily calcium-dependent manner. The α-adrenoceptor agonist phenylephrine may contract ferret aorta by multiple mechanisms involving both protein kinase C- and calmodulin-dependent pathways. It is possible that other Ca-dependent mechanisms may also play a role, such as caldesmon (Marston, 1982), or Ca-dependent regulation of phosphatases (Ingebristen & Cohen, 1983). With respect to the latter possibility, however, it appears that there is little Ca-dependence of the main phosphatases present in smooth muscle (DiSalvo *et al.*, 1985).

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