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CYCLIC AMP RELAXES SWINE ARTERIAL SMOOTH MUSCLE PREDOMINANTLY BY DECREASING CELL Ca^{2+} CONCENTRATION

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SUMMARY

1. Our objective was to evaluate the mechanism of cyclic AMP-dependent arterial smooth muscle relaxation. Cyclic AMP-dependent relaxation has been proposed to result from either (a) a decrease in intracellular [Ca^{2+}] or (b) a decrease in [Ca^{2+}] sensitivity of myosin light chain kinase by protein kinase A-dependent phosphorylation of myosin kinase.

2. We evaluated these proposed mechanisms by examining forskolin-induced changes in aequorin-estimated myoplasmic [Ca^{2+}], [cyclic AMP], myosin phosphorylation and stress generation in agonist-stimulated or KCl-depolarized swine common carotid media tissues.

3. Forskolin, an activator of adenylyl cyclase, increased [cyclic AMP] and reduced [Ca^{2+}], myosin phosphorylation and stress in tissues pre-contracted with phenylephrine or histamine. This relaxation was not associated with an alteration of the [Ca^{2+}] sensitivity of phosphorylation, nor the dependence of stress on phosphorylation.

4. Forskolin pre-treatment attenuated, but did not abolish, agonist-induced increases in [Ca^{2+}] and stress.

5. These results suggest that cyclic AMP-induced relaxation of the agonist-stimulated swine carotid media is primarily caused by cyclic AMP-mediated decreases in myoplasmic [Ca^{2+}].

INTRODUCTION

Ca^{2+}-dependent myosin light chain phosphorylation appears to be the predominant regulator of cross-bridge function in agonist-stimulated arterial smooth muscle (Kamm & Stull, 1985; Rembold & Murphy, 1988*a*; Hai & Murphy, 1988). Cross-bridge dephosphorylation may explain relaxation induced by removal of the stimulus (Gerthoffer, Trevethick & Murphy, 1984; Hai & Murphy, 1989). However, the role of cyclic adenosine 3′,5′-monophosphate (cyclic AMP) in modulating smooth

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muscle relaxation is less clear. β_2-Adrenergic stimulation or forskolin-dependent direct activation of adenylyl cyclase relax vascular smooth muscle, and the relaxation is associated with increases in the level of cyclic AMP (reviewed in Kramer & Hardman, 1980). However, the mechanism of cyclic AMP-induced smooth muscle relaxation is controversial.

de Lannerole, Nishikawa, Yost & Adelstein (1984) reported that myosin light chain kinase can be phosphorylated in smooth muscle tissue by cyclic AMP-dependent protein kinase (A kinase). This phosphorylation has been proposed to decrease the affinity of myosin kinase for the Ca_4^{2+}–calmodulin complex and could result in lower levels of myosin kinase phosphorylation for a given $[Ca^{2+}]$, thus decreasing the $[Ca^{2+}]$ sensitivity of the contractile apparatus (Adelstein, Conti & Hathaway, 1978). However, Miller, Silver & Stull (1983) found that the myosin kinase activity ratio was not altered during isoprenaline-induced relaxation, suggesting that the dependence of myosin kinase on Ca_4^{2+}–calmodulin was not significantly altered in isoprenaline-relaxed tissues. Subsequently, it has been found that cyclic AMP enhances the activity of cellular Ca^{2+} pumps (Kimura & Kobayashi, 1982), suggesting that cyclic AMP may relax smooth muscle by decreasing myoplasmic $[Ca^{2+}]$. It is not known whether cyclic AMP enhances Ca^{2+} extrusion into the extracellular space and/or increased Ca^{2+} sequestration into intracellular stores. Furakawa, Tawada & Shigekawa (1988) reported that the sarcoplasmic reticulum Ca^{2+} pump, but not the plasma membrane Ca^{2+} pump, is regulated by cyclic AMP. Thus, cyclic AMP elevation could result in a decrease in myoplasmic $[Ca^{2+}]$ without a change in the $[Ca^{2+}]$ sensitivity of myosin phosphorylation.

This study addresses two questions. (1) Does cyclic AMP relax smooth muscle predominantly by decreasing $[Ca^{2+}]$ without changes in the $[Ca^{2+}]$ sensitivity of phosphorylation? Or (2) is the predominant effect of cyclic AMP to decrease the $[Ca^{2+}]$ sensitivity of the contractile apparatus by A kinase-dependent phosphorylation of myosin kinase? These questions were evaluated by measuring aequorin-estimated myoplasmic $[Ca^{2+}]$, [cyclic AMP], phosphorylation of the 20 kDa myosin regulatory light chain and stress in the swine carotid media. Pre-contracted tissues were relaxed with small doses of forskolin that produced only modest relaxation. These lower forskolin concentrations (compared to other published studies) should approximate [cyclic AMP] changes characteristic of physiological relaxation without activation of mechanisms proposed to be operant at higher doses of forskolin and [cyclic AMP]. Modest changes in $[Ca^{2+}]$, myosin phosphorylation and [cyclic AMP] would be expected. Since there is a steep relationship between phosphorylation and stress (Rembold, 1990; Ratz, Hai & Murphy, 1989), even small decreases in $[Ca^{2+}]$ would be expected significantly to lower stress.

A preliminary abstract of this study has been published (McDaniel, Rembold & Murphy, 1988).

METHODS

Swine common carotid arteries were obtained from a slaughterhouse and transported at 0 °C in physiological salt solution (PSS). Arteries were used the day of harvest or stored at 4 °C in PSS which was changed daily. Dissection of medial strips, mounting and determination of the optimum length for stress development at 37 °C were performed as previously described (Driska, Aksoy &

Murphy, 1981). The intimal surface was mechanically rubbed to remove the endothelium. PSS contained (mM): NaCl, 140; KCl, 5; 3-(*N*-morpholino)propanesulphonic acid (MOPS), 2; $CaCl_2$, 1·6; $MgCl_2$, 1·2; Na_2HPO_4, 1·2; D-glucose, 5·6; pH adjusted to 7·4 at 37 °C. Agonist stimulation was performed by injecting an appropriate volume of 10 mM stock histamine or phenylephrine into the tissue bath. Stock solutions of agonists were prepared daily. Depolarization was accomplished by replacement of the bathing solution with PSS in which KCl was stoichiometrically substituted for NaCl. Tissues that did not produce a stress greater than $1{\cdot}0 \times 10^5$ N/m^2 after 109 mM-KCl depolarization or 10 μM-histamine stimulation were discarded (Rembold & Murphy, 1988*a*).

Identical protocols were used to measure [Ca^{2+}] and stress in one set of tissues and [cyclic AMP], myosin phosphorylation and stress in a second set of tissues. The destructive phosphorylation and cyclic AMP assays precluded paired controls. The [Ca^{2+}] and stress data are presented as means and standard error of four separate tissues with four or more separate and different arteries as control. Some of the control 10 μM-histamine (eight of the ten control contractions averaged in Fig. 3) and 109 mM-KCl (four of the six control contractions averaged in Fig. 8) results have been published previously (Rembold & Murphy, 1988*a*, 1990). The phosphorylation and [cyclic AMP] data represent results from paired samples from at least four different arteries for each time point and basal condition.

Aequorin (batch 2, obtained from Dr John Blinks, Mayo Medical School, Rochester, MN, USA) was loaded as previously described (Morgan & Morgan, 1982; Rembold & Murphy, 1988*a*). This procedure involves incubation of free-floating tissues in a series of Ca^{2+}-free solutions at 2 °C. Solution 1 chelates extracellular Ca^{2+} with high EGTA (bis(β-aminoethylether)*N*,*N*,*N*′,*N*′-tetraacetic acid), solution 2 contains aequorin, solution 3 contains higher [Mg^{2+}] which may help to 'reseal' the membrane, and solution 4 is Ca^{2+}-free PSS with high $MgCl_2$. The tissues were mounted isometrically, stretched to a length that produced a stress of approximately $0{\cdot}5 \times 10^5$ N/m^2, and warmed to 22 °C. Extracellular $CaCl_2$ was slowly increased to 1·6 mM over 40 min, and the tissues were equilibrated overnight at 37 °C. The next morning, the tissues were stretched to within 5% of their optimal length for stress development.

Simultaneous aequorin-emitted light and stress measurements were made and the light and force signals were collected by a personal computer. Stress was calculated as force per cross-sectional area estimated from measured length, weight and a density of 1·050 g/cm^3. Aequorin light signals are presented as log L/L_{max}, where L is the photon count (in counts per second) and L_{max} is a measure of the total undischarged aequorin present in the tissue (Allen & Blinks, 1979). L_{max} was calculated at each time point to correct for aequorin consumption. Aequorin light emission was calibrated in a series of Ca^{2+}/EGTA buffers with [Mg^{2+}] = 0·5 mM at 37 °C (Rembold & Murphy, 1988*b*). Resting [Ca^{2+}] was 92 ± 5 nM in the thirty-six preparations shown in Figs 2, 3, 5 and 7. Changes in log L/L_{max} and active stress were compared using Student's unpaired t test and significance was defined as $P < 0{\cdot}05$.

Phosphorylation was estimated in tissues treated identically to those used to measured [Ca^{2+}] except these tissues were not loaded with aequorin. Tissues were frozen by immersion in a dry ice–acetone slurry (20 g/20 ml) at −78 °C. The frozen tissues were slowly warmed, first in a −20 °C freezer for 1 h and then 1 h at room temperature, air-dried for 15 min, cut in half, and both halves weighed. One portion was processed for determination of phosphorylation and the other for the cyclic AMP assay. Phosphorylation of the smooth muscle-specific 20 kDa myosin light chain was determined by the two-dimensional isoelectric focusing and sodium dodecyl sulphate (SDS) polyacrylamide electrophoresis (Driska, Aksoy & Murphy, 1981; Aksoy, Mras, Kamm & Murphy, 1983). Phosphorylation is reported as mol inorganic phosphate (P_i)/mol total smooth muscle-specific light chain (MLC). Force was measured isometrically with Grass FT.03 (Quincy, MA, USA) force transducers in the tissues that were assayed for phosphorylation and [cyclic AMP].

[Cyclic AMP] was determined in the same tissues used to estimate phosphorylation. The dehydrated tissues (after freezing and rewarming in acetone) were homogenized in ice-cold 100 mM-HCl with Belco glass homogenizers (Vineland, NJ, USA) for 3 min. The homogenate was centrifuged at 14000 *g* for 20 min and the resulting supernatant was subjected to radio-immunoassay for cyclic AMP content on Gammaflo (Squibb) (Brooker, Terasake & Price, 1976). Data are expressed as pmol cyclic AMP/mg tissue wet weight.

We compared tissues frozen in acetone–dry ice as above with paired tissues frozen in liquid nitrogen and immediately homogenized in ice-cold 100 mM-HCl after treatment with 0, 0·1, 0·3 and 1·0 μM-forskolin. There was no difference from the line of identity when the values obtained by each

method were plotted with respect to each other. Thus, there was no significant loss of cyclic AMP due to acetone extraction or warming.

The acetone used in the freezing procedure was evaporated to dryness. The remaining residue, resuspended in 100 mM-HCl, contained an estimated [cyclic AMP] of $0{\cdot}494 \pm 0{\cdot}026$ pmol cyclic AMP/ml ($n = 16$). This value did not significantly differ from the [cyclic AMP] estimated in fresh

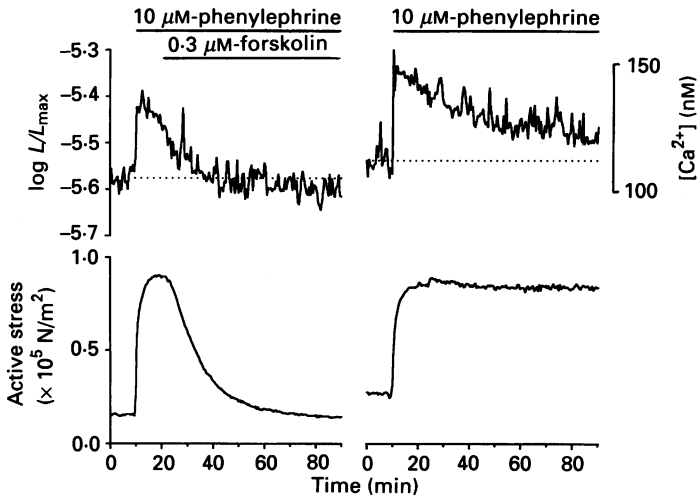

Fig. 1. Raw data depicting the change in aequorin-estimated $[Ca^{2+}]$ (expressed as $\log L/L_{max}$) and stress upon stimulation with 10 μM-phenylephrine at 10 min and relaxed with 0·3 μM-forskolin at 20 min (the left panel). The right panel is the control 10 μM-phenylephrine stimulation at 10 min without forskolin treatment. The L/L_{max} and stress data are plotted at 30 s intervals. At the far right is a $[Ca^{2+}]$ calibration obtained with an assumed $[Mg^{2+}]_i$ of 0·5 mM.

acetone after evaporation ($0{\cdot}433 \pm 0{\cdot}031$) or in fresh 100 mM-HCl ($0{\cdot}463 \pm 0{\cdot}048$, $n = 5$). The efficiency of cyclic AMP extraction was evaluated by addition of 5 pmol/ml of cyclic AMP to acetone alone or tissue homogenates stimulated with 109 mM-KCl or 1 μM-forskolin. The recovery of the added cyclic AMP was 95, 87 and 89% respectively. We conclude that the extraction procedures did not involve a substantial loss of cyclic AMP.

RESULTS

In the swine carotid artery, phenylephrine (an α_1-adrenergic agonist) produced approximately half the stress that is elicited by histamine of KCl depolarization. Stimulation with 10 μM-phenylephrine produced small increases in the aequorin-estimated myoplasmic $[Ca^{2+}]$ that peaked within the first 5 min of stimulation (Fig. 1, right panel). $[Ca^{2+}]$ decreased to intermediate levels and was maintained above resting values for at least 2·5 h (the longest protocol) with stress maintenance near peak values. Addition of 0·3 μM-forskolin (an activator of adenylyl cyclase) to a second tissue after 10 min of phenylephrine stimulation induced a slow but near-complete relaxation that was associated with a decrease in $[Ca^{2+}]$ to resting values (Fig. 1, left panel).

Mean data revealed the same results as the raw data (Fig. 2). $[Ca^{2+}]$ and phosphorylation peaked in the first 5 min of 10 μM-phenylephrine stimulation and

then decreased to intermediate levels with stress maintenance and no change in [cyclic AMP] in tissues not treated with forskolin (dashed lines, labelled Control). The addition of 0·3 μM-forskolin was associated with significant increases in [cyclic AMP] and decreases in [Ca^{2+}], myosin phosphorylation and stress (continuous lines,

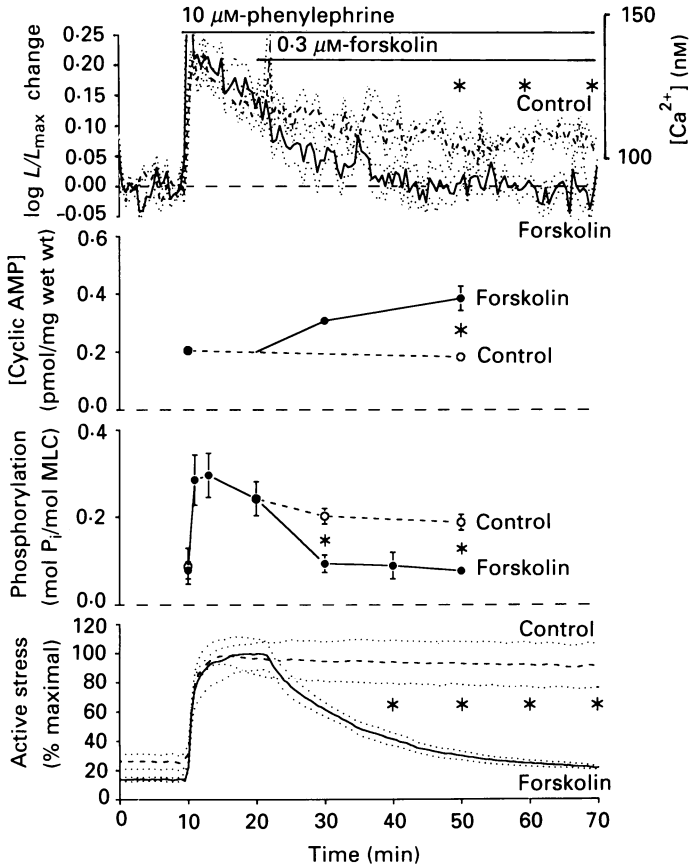

Fig. 2. The change in aequorin-estimated [Ca^{2+}] (expressed as log L/L_{max}), [cyclic AMP], myosin phosphorylation and stress upon stimulation with 10 μM-phenylephrine at 10 min and relaxed with 0·3 μM-forskolin at 20 min (labelled as Forskolin). The L/L_{max} and stress data are plotted as 30 s averages and shown as mean (continuous line) $\pm$ S.E.M. (dotted lines) with $n = 4$. The [cyclic AMP] and phosphorylation data are shown as filled circles ($n \geqslant 4$). Data from tissues contracted with phenylephrine but not exposed to forskolin are shown as open circles ($n \geqslant 4$) or dashed lines (mean data $\pm$ S.E.M., $n = 4$, labelled as Control). $*P < 0{\cdot}05$. at the right is a Ca^{2+} scale with an assumed $[Mg^{2+}]_i$ of 0·5 mM.

labelled Forskolin). Smaller, but similar changes in all measurements and half-maximal relaxations were found with addition of 0·1 μM-forskolin to tissues pre-contracted with 10 μM-phenylephrine (data not shown).

Ten micromolar histamine produced large [Ca^{2+}] and phosphorylation transients associated with rapid stress development. Sustained histamine stimulation induced

a persistent contraction with steady-state elevations in [Ca^{2+}] and phosphorylation with no significant change in [cyclic AMP] (Fig. 3, dashed line labelled Control). Forskolin (0·3 μM) addition to the histamine-pre-contracted tissues significantly increased [cyclic AMP] and significantly decreased [Ca^{2+}], phosphorylation and stress (Fig. 3, continuous line labelled Forskolin).

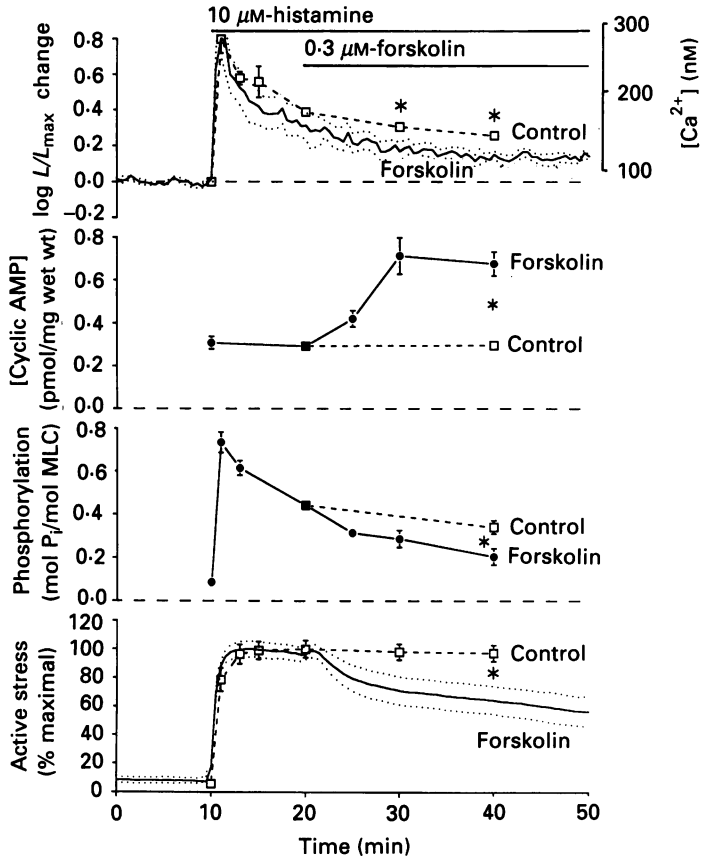

Fig. 3. The change in aequorin-estimated [Ca^{2+}] (expressed as the change in log L/L_{max}), [cyclic AMP], myosin phosphorylation and stress upon stimulation with 10 μM-histamine at 10 min followed by 0·3 μM-forskolin at 20 min (labelled Forskolin: mean (continuous line) $\pm$1 S.E.M. (dotted lines), $n = 4$, and filled circles, $n \geq 4$). The control values from tissues contracted with histamine, but not treated with forskolin, are shown as open squares and dashed lines ($n = 10$). *$P < 0{\cdot}05$. Calcium scale at right as previously defined.

Clearly, both [Ca^{2+}] and phosphorylation decreased during the cyclic AMP-dependent relaxation of agonist-stimulated tissues (Figs 2 and 3). If forskolin-induced relaxation was caused by cyclic AMP-dependent phosphorylation of myosin light chain kinase, then the [Ca^{2+}] sensitivity of phosphorylation should be altered in forskolin-treated tissues. The [Ca^{2+}] and phosphorylation data from forskolin-treated tissues (Figs 2 and 3) were compared to data generated during agonist stimulation. No shift in the [Ca^{2+}] sensitivity of phosphorylation was evident (Fig.

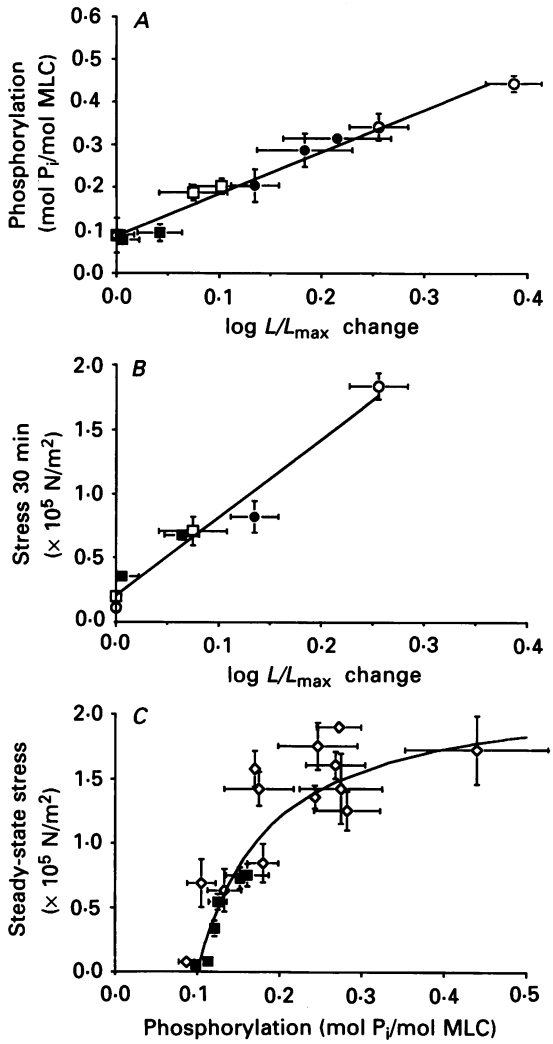

Fig. 4. *A*, the dependence of myosin phosphorylation on changes in myoplasmic [Ca^{2+}] (log L/L_{max} change). The filled symbols represent data from Figs 2 and 3 obtained with forskolin-induced relaxation of agonist-stimulated tissues. The histamine plus forskolin data points (●) were obtained at 15, 20 and 30 min after the histamine addition. The phenylephrine plus forskolin data points (■) were obtained at 20, 30 and 40 min after phenylephrine addition. The open symbols represent control data from Figs 2 and 3 obtained 10 and 30 min after histamine addition (○) and 20 and 40 min after phenylephrine addition (□). Linear regression revealed that $r^2 = 0{\cdot}97$. *B*, the dependence of stress on changes in myoplasmic [Ca^{2+}] (log L/L_{max} change). The filled symbols represent data from Figs 2 and 3 obtained 20 min after forskolin-induced relaxation of agonist-stimulated tissues (i.e. 30 min after addition of the agonist). The open symbols represent control data from Figs 2 and 3 obtained 30 min after agonist addition. Circles are histamine and squares phenylephrine data. Linear regression revealed that $r^2 = 0{\cdot}96$. *C*, the dependence of steady-state stress on phosphorylation. The open symbols represent data obtained during contractions induced by various types of contractile agonists (data from Rembold & Murphy, 1988*b*). The filled symbols represent data from tissues

4*A*). Furthermore, there was no shift in the dependence of stress (measured 30 min after stimulation) on [Ca^{2+}] (Fig. 4*B*). These data, in combination with the result that [Ca^{2+}] significantly decreased during forskolin-induced relaxed, suggest that the primary mechanism of cyclic AMP-induced relaxation in this tissue was a decrease in myoplasmic [Ca^{2+}]. If protein kinase A-dependent myosin kinase phosphorylation was the predominant regulator of cyclic AMP-dependent relaxation, we would have expected a decrease in the [Ca^{2+}] sensitivity of phosphorylation (i.e. a shift of this curve to the right). The phosphorylation dependence of steady-state stress was also unaffected by forskolin (Fig. 4*C*), suggesting that small increases in [cyclic AMP], which induced substantial relaxation, did not affect the response of the contractile apparatus to cross-bridge phosphorylation.

Takuwa, Takuwa & Rassmussen (1988) reported that isoprenaline, which is known to elevate cyclic AMP by β_2-adrenergic receptor activation, increased aequorin-estimated myoplasmic [Ca^{2+}] without increasing force in the resting bovine trachealis. Furthermore, stimulation of these isoprenaline-treated tissues with contractile agonists was associated with a decrease of [Ca^{2+}] to resting values. The swine carotid artery does not show consistent relaxation in response to isoprenaline, probably because β_2-receptors are inadequately expressed. Addition of 1·0 μM-forskolin did not affect aequorin-estimated myoplasmic [Ca^{2+}] in unstimulated tissues (Fig. 5). Ten micromolar forskolin slightly elevated [Ca^{2+}] with a decrease in tone. The forskolin pre-treatment attenuated, but did not abolish, the [Ca^{2+}] and force response to 10 μM-histamine (when compared to 10 μM-histamine without forskolin, Fig. 3). In contrast, the response to 109 mM-KCl depolarization was not affected. The results suggest that large increases in [cyclic AMP] can slightly increase [Ca^{2+}] and partially interfere with agonist-induced contractions.

Depolarization induces a decrease in the [Ca^{2+}] sensitivity of phosphorylation when compared to the [Ca^{2+}] sensitivity induced by agonist stimulation (Rembold & Murphy, 1988*a*). We hypothesized that a higher [cyclic AMP] would be required to relax depolarized tissues. The forskolin dose required to relax depolarized tissues was nearly 100 times larger than the dose required to relax 10 μM-phenylephrine-induced contractions (Fig. 6). The [cyclic AMP] associated with relaxation of depolarized tissues was nearly 25 times higher than the concentration required for phenylephrine-stimulated tissues. Small changes in [cyclic AMP] in phenylephrine-stimulated tissues were associated with almost complete relaxation while very large elevations of [cyclic AMP] in depolarized tissues were associated with only modest relaxation. Abe & Karaki (1989) report similar insensitivity to forskolin in depolarized rat aortic tissue.

Depolarization (109 mM-KCl) induced large increases in [Ca^{2+}], phosphorylation and stress with no change in [cyclic AMP] (Fig. 7). Addition of 30 μM-forskolin (100 times the dose used with agonist contractions in Figs 2 and 3) after 10 min of contraction induced a slow relaxation that was associated with very large increases

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contracted with phenylephrine, treated with various doses of forskolin added 10 min after phenylephrine addition, and frozen 120 min later. Forskolin typically induced oscillations in force in prolonged histamine contractions such that steady-state relationships could not be obtained.

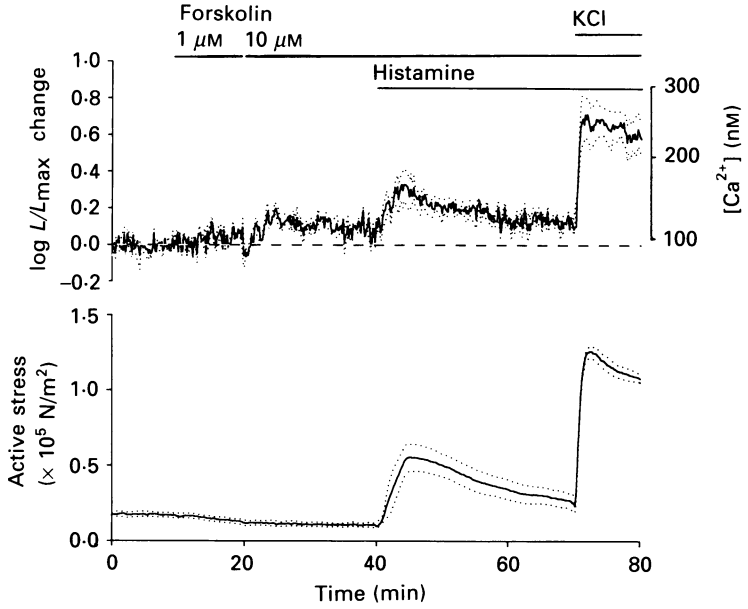

Fig. 5. The change in aequorin-estimated [Ca^{2+}] (expressed as log L/L_{max}) and stress upon exposure of tissues to 1 μM-forskolin at 10 min and 10 μM-forskolin at 20 min. The tissues were then contracted with 10 μM-histamine at 40 min and the solution changed to 109 mM-KCl with 10 μM-histamine and 10 μM-forskolin at 70 min. Continuous and dotted lines are mean and S.E.M. as in Fig. 2 with $n = 4$.

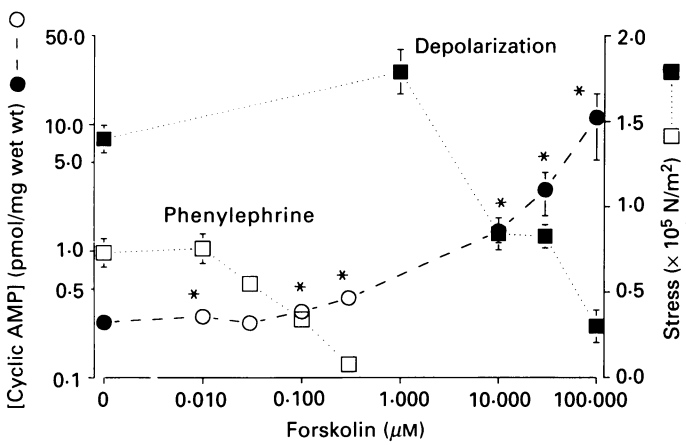

Fig. 6. The forskolin dose dependence of steady-state stress (□ and ■) and [cyclic AMP] (○ and ●) in tissues pre-contracted with 10 μM-phenylephrine (open symbols) or 109 mM-KCl depolarization (filled symbols). Tissues were contracted with phenylephrine or KCl, treated with various doses of forskolin 10 min later and frozen 120 min after contraction ($n \geqslant 4$). *$P < 0{\cdot}05$ for elevations in [cyclic AMP] over control values.

in [cyclic AMP] and significant decreases in phosphorylation (Fig. 7). However, no change in aequorin-estimated myoplasmic [Ca^{2+}] was detected.

DISCUSSION

The mechanism of cyclic AMP-dependent relaxation of smooth muscle is controversial. Myosin light chain kinase can be phosphorylated during cyclic AMP-

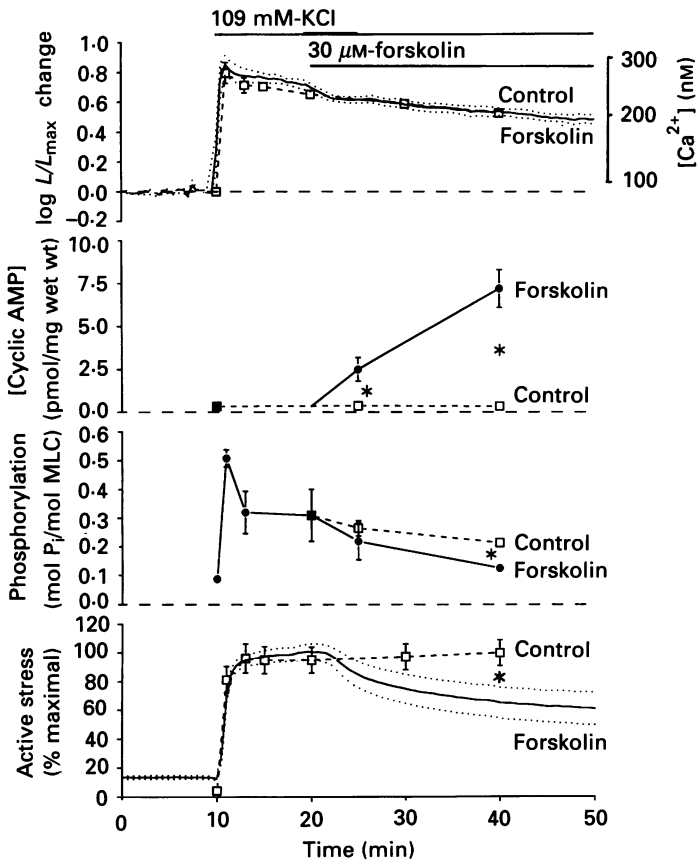

Fig. 7. The change in aequorin-estimated [Ca^{2+}] (expressed as log L/L_{max}), [cyclic AMP], myosin phosphorylation and stress upon depolarization with 109 mM-KCl at 10 min and relaxation with 30 μM-forskolin at 20 min (continuous and dotted lines as in Fig. 1, $n = 4$; filled circles $n \geqslant 4$, labelled as Forskolin). The mean change of log L/L_{max}, [cyclic AMP], myosin phosphorylation and active stress observed in tissues depolarized with 109 mM-KCl, but not treated with forskolin, is shown as open squares and dashed lines ($n \geqslant 4$, labelled as Control; $^*P < 0{\cdot}05$).

dependent relaxation (Adelstein *et al.* 1978; Conti & Adelstein, 1981; de Lanerolle *et al.* 1984) Since phosphorylated myosin kinase has a lower affinity for Ca_4^{2+}–calmodulin *in vitro*, the phosphorylated myosin kinase would be less active and therefore phosphorylate less myosin producing relaxation. Alternatively, recent studies have shown that cyclic AMP increases the activity of cellular Ca^{2+} pumps

(Kimura *et al.* 1982; Furakawa *et al.* 1988). Some studies (Kimura *et al.* 1982; Parker, Ito, Kuriyama & Miledi, 1987; Furakawa *et al.* 1988; Gunst & Bandyopadhyay, 1989), but not all (Morgan & Morgan, 1984; Takuwa *et al.* 1988), have found that forskolin or β-adrenergic stimulation of smooth muscle is associated with decreases in myoplasmic [Ca^{2+}], which would also decrease myosin kinase activity and produce relaxation. The goal of this study was to evaluate which of these mechanisms has more physiological significance in this tissue. In contrast to most previous studies, this goal was evaluated by (1) examining the response to the forskolin dose producing modest relaxation and (2) making measurements of the major systems thought to be changing during the cyclic AMP-dependent relaxation. We evaluated doses of forskolin that did not induce maximal or rapid relaxation. Therefore, large changes in measured values were not observed. However, this approach produced significant decreases in [Ca^{2+}] and permitted evaluation of the effect of cyclic AMP on the relationship between [Ca^{2+}] and phosphorylation in the range of values thought to closely represent physiological conditions.

Phosphorylation of the regulatory light chain of myosin appears to determine stress in the swine carotid arterial media. A unique relationship between aequorin-estimated myoplasmic [Ca^{2+}] and myosin phosphorylation was found with agonist simulation of swine carotid media (Rembold & Murphy, 1988*a*). Forskolin-induced relaxation did not affect either the relationship between [Ca^{2+}] and phosphorylation (Fig. 4*A*) or the dependence of stress on [Ca^{2+}] (Fig. 4*B*). As the forskolin-induced relaxations were associated with significant decreases in myoplasmic [Ca^{2+}], we conclude that the primary physiological mechanism for cyclic AMP-induced relaxation of agonist-stimulated swine carotid artery is by decreasing myoplasmic [Ca^{2+}]. Phosphorylation changed secondary to the change in [Ca^{2+}] and not from the action of cyclic AMP-dependent protein kinase directly on myosin kinase.

Depolarization of swine arterial smooth muscle is associated with a decrease in the [Ca^{2+}] sensitivity of phosphorylation (i.e. the [Ca^{2+}] sensitivity of phosphorylation is shifted to the right). Cyclic AMP levels were not affected by histamine, phenylephrine or KCl depolarization (Figs 2, 3 and 7). This result suggests that cyclic AMP-mediated phosphorylation of myosin kinase cannot explain the decrease in the [Ca^{2+}] sensitivity of phosphorylation observed with depolarization (Rembold & Murphy, 1988*a*; Rembold, 1990). In contrast to cyclic AMP-induced relaxation in agonist-stimulated tissue, much larger doses of forskolin were required to relax depolarized tissues. These relaxations were associated with decreases in phosphorylation without detectable changes in aequorin-estimated myoplasmic [Ca^{2+}]. This result can be interpreted in several ways. (1) When [Ca^{2+}] is high, the capacity of cyclic AMP-dependent Ca^{2+} pumps may be overwhelmed. Therefore, relaxation of KCl-depolarized tissues may only occur by protein kinase A-dependent phosphorylation of myosin kinase. However, (2) recent reports suggest that high doses of forskolin (50 μM) may produce effects besides elevating [cyclic AMP] such as altering K^+ channel activity (Hoshi, Garber & Aldreich, 1988; Wagoner & Pallotta, 1988). Finally, (3) it is possible that the aequorin light signal in depolarized tissues may overestimate myofibrillar [Ca^{2+}] (Rembold & Murphy, 1988*a*; Rembold, 1989).

The mechanism whereby increases in [cyclic AMP] activate the cellular Ca^{2+} pumps is also controversial. Recent reports suggest that smooth muscle relaxation is

better correlated with activation of cyclic GMP-dependent protein kinase (G kinase) than A kinase (Lincoln, Cornwell & Taylor, 1990). G kinase is only relatively specific for cyclic GMP over cyclic AMP (Francis, Noblett, Todd, Wells & Corbin, 1988). In smooth muscle resting [cyclic AMP] is tenfold higher than [cyclic GMP]; therefore, increases in [cyclic AMP] may also activate cyclic GMP-dependent protein kinase. Lincoln *et al.* (1990) found that forskolin decreased [Ca^{2+}] in smooth muscle cell lines that expressed G kinase; however, forskolin increased [Ca^{2+}] in cell lines deficient in G kinase. If G kinase was reintroduced into the cell lines deficient in G kinase, then forskolin decreased [Ca^{2+}], suggesting that the forskolin-dependent decrease in [Ca^{2+}] was produced only through activation of G kinase. It is known that A kinase can phosphorylate Ca^{2+} channels and increase Ca^{2+} flux (reviewed in Brown, Yatani, Imoto, Codina, Mattera & Birnbaumer, 1989). This effect may explain the small increases in [Ca^{2+}] observed with high doses of forskolin in the swine carotid (Fig. 6). If the bovine trachealis is relatively deficient in G kinase, then this hypothesis would explain the large isoprenaline-induced [Ca^{2+}] increases observed by Takuwa *et al.* (1988).

Pre-treatment of swine carotid with large doses of forskolin attenuated the response to histamine stimulation (Fig. 5). This effect may be caused by a decrease in the [Ca^{2+}] sensitivity of phosphorylation as was observed with relaxation of KCl-depolarized tissues (Fig. 7). However, Hall & Hill (1988) found that β-adrenergic stimulation inhibited histamine-stimulated inositol phosphate metabolism in bovine tracheal smooth muscle. Potentially, 10 μM-forskolin may decrease histamine-stimulated intracellular Ca^{2+} release by decreasing 1,4,5-inositol trisphosphate production. However, Tawada, Furukawa & Shigekawa (1988) report that cyclic AMP enhances the peak calcium release with ATP stimulation of cultured rat aortic cells. The dissimilarities in the data presented by Takuwa *et al.* (1988) and ours may represent differences in G kinase content, method of increasing cyclic AMP, species or tissue origin.

In summary, cyclic AMP predominantly relaxes agonist-stimulated swine arterial smooth muscle by decreasing myoplasmic [Ca^{2+}]. Other effects of cyclic AMP elevation may be important with high forskolin doses, other methods of elevating cyclic AMP or in other tissues. If protein kinase A or G have other actions that induce relaxation, they appear to be dependent on very large increases in [cyclic AMP] as may be needed to relax depolarized smooth muscle. Such effects may have minimal physiological significance (Kamm & Stull, 1985).

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