Ca^{2+} regulation of the thin filaments: biochemical mechanism and physiological role

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The control of blood flow and blood pressure is primarily a question of the control of the contractility of the smooth muscle cells in blood vessel walls. The contractile machinery of smooth muscle cells is made up of filaments of the contractile proteins myosin (thick filaments) and actin (thin filaments), the interaction of which generates force and shortening at the expense of MgATP hydrolysis. The contractile proteins are associated with regulatory proteins which control the actin–myosin interaction in response to changes in the Ca^{2+} concentration in the surrounding cytoplasm.

One site of action of Ca^{2+} on the contractile apparatus: Ca^{2+}-dependent phosphorylation of myosin by a calmodulin-dependent myosin light chain kinase, leading to activation, has been demonstrated and studied extensively. The other site of Ca^{2+} action: Ca^{2+} regulation of the thin filaments, has now been identified in many smooth muscles and we have studied it in some detail in vascular smooth muscle (Marston & Smith, 1985).

We have developed a procedure to isolate native thin filaments from sheep aorta and many other smooth muscle types (Marston & Smith, 1984). Native thin filaments activate skeletal or phosphorylated smooth muscle myosin MgATPase in the presence of 10^{-5} M-Ca^{2+} in much the same way as pure actin or actin + tropomyosin, but this activation is Ca^{2+}-regulated, being 1/4–1/30th as much at Ca^{2+} concentrations of 10^{-8} M or less, whereas activation by pure actin or actin + tropomyosin does not change. This thin filament preparation therefore contains an intact Ca^{2+}-regulatory system and is a suitable material for our investigation of the mechanism of regulation (Smith & Marston, 1985).

Abbreviations used: HMM [SP], thiophorylated heavy meromyosin; AM, actomyosin; CBP, Ca^{2+}-binding protein.

Structure and function of the thin filament

The major functional components of the native thin filament are actin, tropomyosin, caldesmon and a calcium-binding protein which are present in molar ratios 28 actin:4 tropomyosin:1 caldesmon, determined by gel electrophoresis and densitometry. The calcium-binding protein component of thin filament preparations has not yet been positively identified, but is presumed to be present since it is required for Ca^{2+}-regulation (see below). Despite its low stoichiometry, caldesmon is the key component of the Ca^{2+}-regulatory system. This protein has a molecular mass of 120000 Da and is unique to smooth muscles and some non-muscle motile systems (Marston & Smith, 1985).

The structural location of caldesmon in the thin filament has been determined by electron microscope techniques. Single caldesmon molecules are very long (up to 150 nm) and are largely a random coil with a small compact head

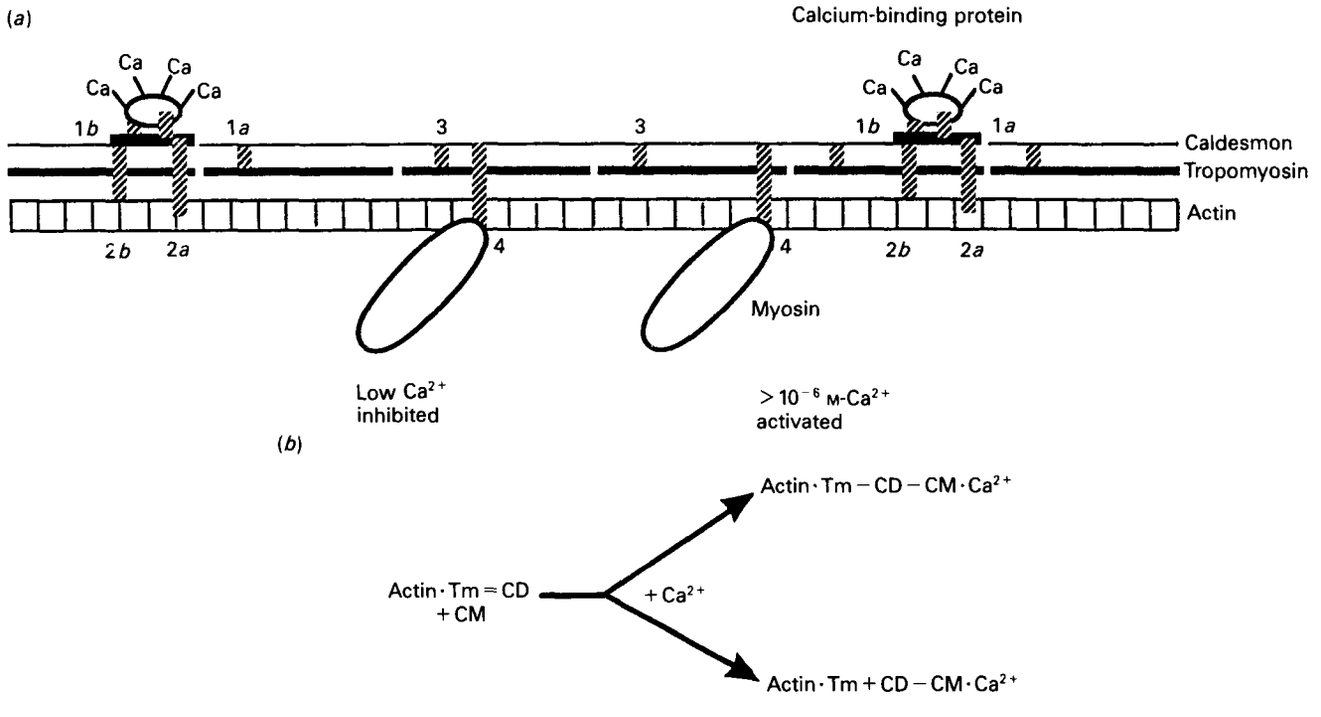

Fig. 1. *(a) A scheme for the structure of the aorta thin filament and (b) the mechanism of Ca^{2+} control of thin filament activity by calmodulin and caldesmon*

(*a*) The diagram represents one of the two actin strands which form a 38 nm pitch helix in the native thin filament. Key to interactions: 1*a*, CD–CBP inactivates 2*a* when Ca^{2+} is bound; 1*b*, CD–CBP binding contact, independent of Ca^{2+}; 2*a*, CD–actin binding with inhibition of up to 28 actins; 2*b*, CD–actin binding contact only; Ca^{2+} independent; 3, CD–tropomyosin, located in CD 'tail'; 4, CD–myosin binding contact only? Abbreviations: CD, caldesmon; CM, calmodulin; Tm, tropomyosin.

(Fürst *et al.*, 1986; Lynch *et al.*, 1987). Single thin filaments are indistinguishable from actin and actin–tropomyosin filaments when negatively stained so the caldesmon is not located as a compact molecule, which would be visible. Thin filaments are aggregated into parallel bundles by anti-caldesmon antibody; such bundles have no detectable periodicity, suggesting caldesmon is stretched along the whole filament surface (Marston *et al.*, 1988). In contrast, antibodies to the *C*-terminal 40000 M_r peptide (Szpacenko & Dabrowska, 1986) do show clear 40 nm periods (Lehman *et al.*, 1988).

A consensus model is suggested in Fig. 1(*a*). Caldesmon is a 150 nm long molecule occupying the grooves between the actin double helix, with a head piece located in register with tropomyosin and actin. This elongated structure explains caldesmon's stoichiometry and accounts for the ability of 1 caldesmon to influence the activity of 28 actins.

A mechanism for Ca^{2+} regulation of the smooth muscle thin filament

The regulatory mechanism has been determined by studies on the isolated protein components of the thin filaments (Smith *et al.*, 1987; Marston & Smith, 1985). Pure actin and actin + tropomyosin activate myosin MgATPase activity fully and show no Ca^{2+} sensitivity. The addition less than 0.1 caldesmon per actin strongly inhibits activation of MgATPase activity by actin–tropomyosin under all conditions and by actin at lower temperatures and ionic strengths (25°C, $I = 0.04$). Maximal inhibitions of > 80% with skeletal muscle myosin and > 95% with smooth muscle myosin are typical. The inhibition closely correlates with caldesmon binding to sites on actin–tropomyosin with a stoichiometry of 0.035 caldesmon/actin and affinity in the 10^7 M^{-1} range. Caldesmon also binds to actin, about five times weaker than to actin–tropomyosin, and this binding is often not accompanied by inhibition. Thus caldesmon interacts with actin at two sites, one which induces inhibition and one which does not, and also interacts with tropomyosin. Studies of proteolytic fragments indicate that the actin-binding sites are on the *C*-terminal head of caldesmon, while the tropomyosin sites are in the tail (Szpacenko & Dabrowska, 1986; Riseman & Bretscher, 1987). These data can be incorporated into the structural model as indicated in Fig. 1(*a*).

We have investigated how caldesmon inhibition modifies the enzymic mechanism of actomyosin ATPase by measuring ATPase activity and myosin·ADP·P_i–actin binding, using thiophosphorylated aorta heavy meromyosin (HMM[SP]). Caldesmon inhibits the MgATPase activity over 90% while leaving HMM[SP]·ADP·P_i–actin binding unaltered. Thus it is likely that caldesmon inhibition acts solely on the rate-limiting step [presumably product release from actomyosin (AM)·ADP·P_i]. This is the same step that is altered by troponin in skeletal muscles and by changes in myosin phosphorylation in smooth muscles (Sellers *et al.*, 1982, Chalovich & Eisenberg, 1982).

Caldesmon, tropomyosin and actin are not sufficient to reconstitute the smooth muscle thin filament, since the system is completely insensitive to Ca^{2+}-concentration changes. Ca^{2+} regulation requires an additional protein component. In many experiments *in vitro*, brain calmodulin has been used as a calcium-binding component. Ca^{2+}·calmodulin binds to caldesmon with an affinity around 10^6 M^{-1}, but calmodulin does not (Smith *et al.*, 1987; Marston *et al.*, 1988). Ca^{2+}·calmodulin can reverse caldesmon's inhibition of actin–tropomyosin, and thus produce a synthetic Ca^{2+}-regulated thin filament. This reversal of inhibition is accompanied by dissociation of the caldesmon-Ca^{2+}·calmodulin complex from actin–tropomyosin under some conditions, especially at 25°C and low KCl concentrations, but more usually we have observed that caldesmon and Ca^{2+}·calmodulin both remain bound on actin–tropomyosin while inhibition is reversed (Smith *et al.*, 1987).

Fig. 1(*b*) summarizes these interactions and how they act to produce Ca^{2+} control of the thin filament. The data certainly produce a self-consistent model mechanism, but there are a number of ways in which the synthetic system does not behave like native thin filaments. Explanation of such discrepancies is necessary before we can claim to know how native thin filaments are regulated.

The calcium-binding protein in native aorta thin filaments

It is likely that native thin filaments contain a Ca^{2+}-binding protein which is qualitatively different from the calmodulin which we have used in reconstituted systems. Thin filaments are prepared by repeated washes and extractions in Ca^{2+}-free buffers which would be expected to remove calmodulin from the preparation. Thin filaments show better Ca^{2+} sensitivity with smooth muscle myosin than with skeletal myosin, Ca^{2+} sensitivity is best at lower temperatures (15–25°C) and becomes small at 37°C. In contrast, systems reconstituted with calmodulin can give high Ca^{2+} sensitivity with skeletal myosin, but only at higher temperatures and [KCl] (37°C, > 70 mM-KCl); at 25°C, Ca^{2+}·calmodulin is very inefficient at reversing caldesmon inhibition.

We have obtained crude extracts from aorta containing Ca^{2+}-regulatory factors with properties which match the requirements of the thin filament calcium-binding protein (CBP) (Pritchard & Marston, 1988).

(a) If all the actin-binding proteins of the thin filament are stripped off by sedimenting the actin in dissociating conditions (0.8-M KCl) a mixture which includes tropomyosin and caldesmon is obtained. This mixture inhibits actin–tropomyosin, but only in the absence of Ca^{2+}, thus indicating the presence of a component which reverses caldesmon inhibition in Ca^{2+}. We are currently working on the purification of this CBP activity.

(b) If all the proteins containing a Ca^{2+}-dependent hydrophobic patch are isolated from crude aorta homogenates by chromatography on phenyl-Sepharose (Mathew *et al.*, 1986), we obtain a CBP activity which can reverse caldesmon inhibition at low doses and under conditions (such as 25°C, 50 mM-KCl) where thin filaments are optimally Ca^{2+} sensitive, but calmodulin cannot reverse caldesmon inhibition. This preparation contains S-100, calmodulin and 'calcimedins' and we are currently engaged in identifying which of the components is the thin-filament regulator.

Although the thin filament CBP is clearly functionally distinct from calmodulin, until it is isolated we do not know whether it is structurally quite different or very similar.

Caldesmon function in the native aorta thin filaments

The belief that caldesmon is the key regulatory protein of the native thin filament is based on the fact that smooth muscle thin filaments uniquely contain caldesmon and that caldesmon isolated from thin filaments is a regulator (Fig. 1). Direct evidence for this proposition has come from experiments using an anti-caldesmon polyclonal antibody. The antibody antagonizes caldesmon inhibition in reconstituted systems. It also antagonizes Ca^{2+} regulation in thin filaments (with skeletal myosin) by preventing the inhibition of ATPase activity when [Ca^{2+}] is lowered to 10^{-8} M.

It is, however, not yet clear whether caldesmon in native thin filaments behaves exactly the same as the reconstituted system. One puzzling observation is the dependence of Ca^{2+} sensitivity on conditions. In general, thin filament Ca^{2+} sensitivity is best under conditions which are optimal for caldesmon binding, but thin filaments regularly show much higher

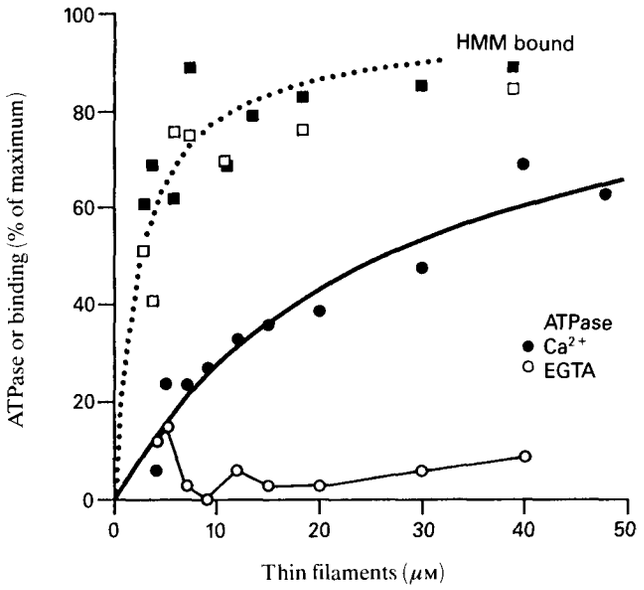

Fig. 2. *Comparison of thin filament activation of HMM[SP] MgATPase and binding in the presence and absence of* Ca^{2+}

Conditions: 25°C, pH 7.1, 5 mM-Pipes (dipotassium salt), 2.5 mM-$MgCl_2$, 1 mM-dithiothreitol, 3 μM-HMM[SP], 0–100 μM-aorta thin filaments, 0.1 mM-$CaCl_2$ or 1 mM-EGTA.

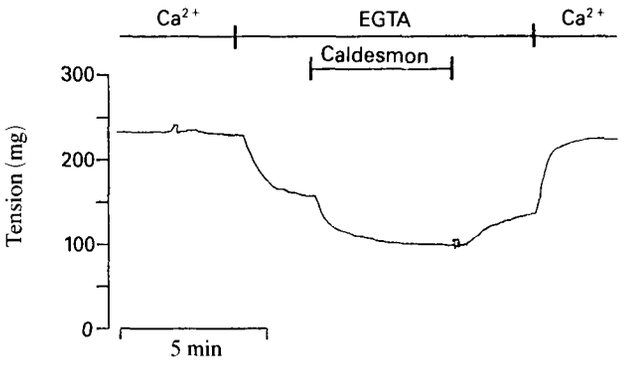

Fig. 3. *Effect of caldesmon on desensitized rabbit muscle fibres*

Glycerinated rabbit psoas fibres (bundle of six, 0.5 cm long) were mounted on a tension-measuring apparatus in a flow cell. Treatment in desensitizing solution for 30 min at 39°C resulted in 75% of maximal Ca^{2+}-activated tension being produced in the incubating solution in the absence of Ca^{2+}. Caldesmon (8 μM) added to fibres generating 170 mg tension in the absence of Ca^{2+} produced a rapid and reversible loss of tension. Desensitizing solution: 50 mM-KCl, 20 mM-Tris, pH 8.0. Incubating solution: 5 mM-Pipes, 5 mM-EGTA or 5 mM-EGTA+4 mM-$CaCl_2$, 5 mM-$MgCl_2$, 1.5 mM-NaN_3, 4 mM-MgATP, pH 7.1, 25°C.

Ca^{2+} sensitivity when activating smooth muscle myosin, compared with skeletal myosin: this feature is not encountered in reconstituted systems.

When we determined thin-filament activation of aorta HMM[SP] we found the MgATPase to be highly Ca^{2+} sensitive (Fig. 2). At 10^{-5} M-Ca^{2+}, the maximal activation was 30 min^{-1} and the K_m was 26 μM, similar to actin-tropomyosin under the same conditions. At 10^{-8} M-Ca^{2+} the activation was less than 1/10th of this and did not follow a Michaelis-Menten form of curve. The binding of HMM[SP]·ADP·P_i to the thin filaments was measured simultaneously by the sedimentation method (Sellers *et al.*, 1982). Binding reached a maximum at 5 μM and the dissociation constant was estimated as $K_d = 1.8$ μM, and was independent of Ca^{2+} concentration (Fig. 2).

Examination of the known enzymic mechanism of actomyosin ATPase (Taylor, 1979) shows that it is not possible for K_d to be significantly less than K_m. This extra tight binding is thus clearly not related to the ATPase mechanism or Ca^{2+} regulation; we must suppose that there is a 'second site' for myosin-thin filament binding. Such 'extra tight' binding has been reported in some reconstituted systems (we have observed it in actin-tropomyosin-caldesmon) (Lash *et al.*, 1986) and is thus due to caldesmon or caldesmon + tropomyosin. It has been suggested (Hemric & Chalovitch, 1988) that the 'extra tight' binding occurs only with smooth and not skeletal myosin: consequently it could be related to the variability of Ca^{2+} sensitivity with myosin type. These observations suggest an added dimension to thin filaments in smooth muscles, which deserves intensive investigation.

The role of thin filament regulation in the control of contractility

There are now several lines of evidence that indicate caldesmon can control muscle contractility as well as ATPase activities.

(a) The contraction of actomyosin gels is inhibited by caldesmon (Nomura & Sobue, 1987).

(b) The movement of myosin along the actin 'cables' of *Nitella* is inhibited by caldesmon (Sellers & Shirinsky, 1987).

(c) Caldesmon will decrease tension in glycerinated skeletal muscle fibres, which have been treated to inactivate their intrinsic troponin regulatory system (Taggart & Marston, 1988). A maximum tension decrease of up to 50% was observed with half-maximal effect at 2–5 μM-caldesmon (Fig. 3).

Currently, direct evidence for a regulatory role of thin filaments and caldesmon *in vivo* is lacking. Such evidence as there is for a second regulatory system other than myosin phosphorylation is negative. Myosin phosphorylation does not account for some maintained 'tonic' contractures and it has been proposed that a second system takes over from myosin, forming a 'latch' in which tension is maintained with low ATP consumption, low phosphorylation levels and low Ca^{2+} concentrations (Murphy, 1982; Kamm & Stull, 1985).

So far, the thin filament regulation system has been proposed as being the second system merely on the basis that it is there and a system is needed to account for the muscle's behaviour. This is an experimentally difficult area where direct evidence will not be easy to obtain. However, the finding of the 'extra tight' second-site binding effect in caldesmon-regulated thin filaments (Fig. 2) suggests a way of holding tension which does not involve the conventional ATPase cycle. This is an intriguing speculative mechanism for latch.

The work reported here has been supported by the British Heart Foundation and the M.R.C.

Chalovich, J. M. & Eisenberg, E. (1982) *J. Biol. Chem.* **257**, 2432–2437

Fürst, D. O., Cross, R. A., DeMey, R. A. & Small, J. V. (1986) *EMBO J.* **5**, 251–257

Hemric, M. E. & Chalovitch, J. M. (1988) *J. Biol. Chem.* **263**, 1878–1885

Kamm, K. E. & Stull, J. T. (1985) *Annu. Rev. Pharmacol.* **25**, 593–620

Lash, J. A., Sellers, J. R. & Hathaway, D. R. (1986) *J. Biol. Chem.* **261**, 16155-16160
Lehman, W., Moody, C. & Craig, R. (1988) *Biophys. J.* **53**, 182a
Lynch, W. P., Riseman, V. M. & Bretscher, A. (1987) *J. Biol. Chem.* **262**, 7429-7437
Marston, S. B. & Smith, C. W. J. (1984) *J. Muscle Res.* **5**, 559-575
Marston, S. B. & Smith, C. W. J. (1985) *J. Muscle Res.* **6**, 669-708
Marston, S. B., Lehman, W., Moody, C. & Pritchard, K. (1988) in *Calcium and Calcium binding Proteins* (Gerday, C., Gilles, R. & Bolis, L., eds.), pp. 69-81, Springer Verlag, Heidelberg
Mathew, J. K., Krolak, J. M. & Dedman, J. R. (1986) *J. Cell. Biochem.* **32**, 223-234
Murphy, R. A. (1982) *Hypertension* **4** (Suppl. II), 3-7
Nomura, M. & Sobue, K. (1987) *Biochem. Biophys. Res. Commun.* **144**, 936-943
Pritchard, K. & Marston, S. (1988) *Biochem. Soc. Trans.* **16**, 355-356
Riseman, V. M. & Bretscher, A. (1988) *J. Cell. Biol.* **103**, 536a
Sellers, J. R. & Shirinsky, V. P. (1987) *J. Cell. Biol.* **105**, 194a
Sellers, J. R., Eisenberg, E. & Adelstein, R. S. (1982) *J. Biol. Chem.* **257**, 13880-13882
Smith, C. W. J. & Marston, S. B. (1985) *FEBS Lett.* **184**, 115-119
Smith, C. W. J., Pritchard, K. & Marston, S. (1987) *J. Biol. Chem.* **262**, 116-122
Szpacenko, A. & Dabrowska, R. (1986) *FEBS Lett.* **202**, 182-186
Taggart, M. & Marston, S. (1988) *Biochem. Soc. Trans.* **16**, 360-361
Taylor, E. W. (1979) *CRC Crit. Rev. Biochem.* **6**, 103-164

Received 27 January 1988

Mechanism of cyclic-GMP-dependent relaxation in vascular smooth muscle

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It has become evident that cyclic GMP is one of the major factors controlling vascular smooth muscle tone. It is well established that several vasodilator drugs, particularly the nitrogen oxide vasodilator drugs such as nitroglycerine and nitroprusside, stimulate increases in smooth muscle cyclic GMP content (Ignarro & Kadowitz, 1985; Waldman & Murad, 1987). Furthermore, analogues of cyclic GMP produce relaxation at micromolar concentrations. More recently, several physiologically important regulators of vascular smooth muscle tone, notably the endothelial-derived relaxing factor (EDRF) and atrial natriuretic factor (ANF), have been found to stimulate cyclic GMP formation (Holtzman, 1983; Winquist *et al.*, 1984).

Because of its pivotal role in the regulation of vascular function and blood flow, there has been a renewed interest in the manner in which cyclic GMP acts in cells to produce physiologically meaningful responses. In smooth muscle, the major receptor protein for cyclic GMP is the cyclic-GMP-dependent protein kinase. This protein has been purified, characterized and sequenced, yet its physiological role is still unknown (Lincoln & Corbin, 1983). Obviously, the identification of proteins which are phosphorylated in the cell in response to cyclic GMP elevations would contribute to our understanding of the mechanism of action of cyclic GMP in smooth muscle. However, no such proteins have been identified and characterized to date. Therefore, to define the role of cyclic GMP and the cyclic-GMP-dependent protein kinase in smooth muscle, it would appear necessary to first identify the site of action of cyclic GMP.

One area where our understanding of the regulation of smooth muscle function is considerably advanced is in the biochemistry of smooth muscle contraction. It is generally accepted that phosphorylation of the 20000 Da light chain of smooth muscle myosin (MLC) by the Ca^{2+}-calmodulin-dependent activation of myosin light chain kinase (MLCK) leads to active cross-bridge cycling and contraction (Aksoy *et al.*, 1983). Since activation of MLCK requires increases in free cytosolic Ca^{2+} levels, it is conceivable that cyclic GMP leads to decreases in either cytosolic Ca^{2+} levels or to MLC dephosphorylation, or both. Since there is no evidence that cyclic-GMP-dependent protein kinase causes the inactivation of MLCK by the direct phosphorylation of the enzyme (Nishikawa *et al.*, 1984), most investigators have focused on the role of cyclic GMP in controlling intracellular Ca^{2+} levels.

Our laboratory, using activation of the Ca^{2+}-sensitive enzymes phosphorylase *b* kinase and MLCK to monitor changes in intracellular Ca^{2+} levels in rat aorta, provided compelling evidence that nitrogen oxide vasodilators and analogues of cyclic GMP lower the levels of free intracellular Ca^{2+} in vascular smooth muscle (Johnson & Lincoln, 1985; Lincoln *et al.*, 1985). As shown in Fig. 1, the formation of phosphorylase *a* as a result of the Ca^{2+}-dependent stimulation of rat aortic phosphorylase kinase due to noradrenaline (NA) was inhibited by agents which elevate cyclic GMP in smooth muscle (nitroglycerine, ANF, or ultraviolet radiation). A similar result was obtained when depolarization (i.e. KCl) was used to elevate Ca^{2+} instead of NA (not shown). This finding was consistent with the effects of cyclic-GMP-elevating agents or analogues to relax rat aortic strips contracted with either NA or KCl (Lincoln, 1983).

Although phosphorylase *a* formation appeared to be a useful indicator of free Ca^{2+} concentrations in smooth muscle, there were disadvantages using this approach. First, quantitative information on the amount of Ca^{2+} mobilized inside the cell could not be obtained since Ca^{2+}-sensitive enzyme activation reflected only relative changes in Ca^{2+}. Secondly, other factors such as cyclic AMP, 5'-AMP and dephosphorylation of phosphorylase *a* may be affected by the experimental manipulations. Thus the direct measurement of Ca^{2+} was necessary to confirm the effects of cyclic GMP on decreasing Ca^{2+} levels. For these reasons, we have used cultured vascular smooth muscle cells from rat aorta to study changes in Ca^{2+} using the fluorescent probe fura-2. As shown in Fig. 2, both agonist treatment (i.e. angiotensin II) and depolarization using KCl, increased Ca^{2+} concentrations in cultured rat aortic cells. In addition, 8-bromo-cyclic GMP and ANF inhibited the increase in intracellular Ca^{2+} produced by either angiotensin or KCl. Thus, the data obtained using fura-2-loaded cultured cells complemented the information obtained using phosphorylase *a* formation in the rat aorta with respect to the effects of cyclic GMP on Ca^{2+} levels.

Abbreviations used: ANF, atrial natriuretic factor; MLC, myosin light chain; MLCK, myosin light chain kinase; NA, noradrenaline; $InsP_3$, inositol 1,4,5-trisphosphate.