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

THOMAS M. LINCOLN,* TRUDY L. CORNWELL,* SUBASH S. RASHATWAR* and RANDOLPH M. JOHNSON†

**Department of Pharmacology, College of Medicine, University of South Alabama, Mobile, Alabama 36688, U.S.A. and †Department of Pharmacology, School of Medicine, University of Virginia, Charlottesville, Virginia 22908, U.S.A.*

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.

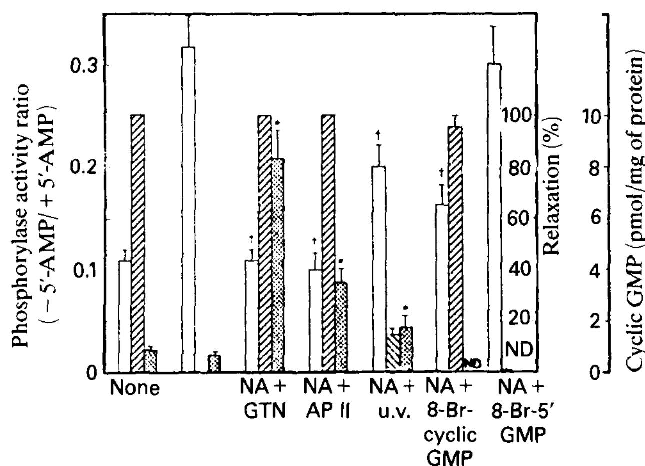

Fig. 1. *Effects of agents on phosphorylase a formation, cyclic GMP and relaxation in rat aortic smooth muscle*

Rat aortic strips were prepared for contractile studies as described previously (Lincoln, 1983). After 5 min with NA, strips were treated with either nitroglycerine (GTN) for 2 min, atriopeptin II (AP II) for 1 min, ultraviolet light (360 nm) for 20 s or were preincubated for 10 min with either 8-Br-cyclic GMP or 8-Br-5′-GMP before NA for 5 min. These tissues were compared with those untreated or NA-treated for 5 min. At the indicated times, aortas were removed from the incubation medium, frozen and the activity ratio of phosphorylase was determined in the presence and absence of 5′-AMP as described earlier (Johnson & Lincoln, 1985). Cyclic GMP was measured by radioimmunoassay. The data represent the results of four to eight experiments. † Denotes statistical significance ($P<0.05$) from NA-treated strips; * denotes statistical significance ($P<0.05$) from untreated and NA-treated strips; ND, not determined.

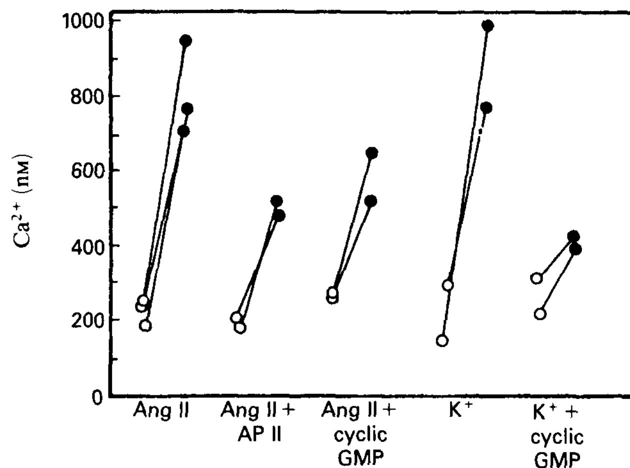

Fig. 2. *Effects of atriopeptin II (AP II) and 8-Br-cyclic GMP on Ca^{2+} levels in angiotensin II (Ang II)- and K^+-treated rat aortic smooth muscle cells*

Cells in passage 4–6 were loaded with fura-2 and fluorometric determinations of Ca^{2+} were performed as described previously (Rashatwar *et al.*, 1987). From left to right: 10 nM-Ang II at 30 s, 10 nM-Ang II at 30 s after treatment for 1 min with 100 nM-AP, 10 nM-Ang II at 30 s after treatment with 100 μM-8-Br-cyclic GMP for 15 min, 40 mM-KCl at 30 s, 40 mM-KCl at 30 s after treatment with 100 μM-8-Br-cyclic GMP for 15 min.

Table 1. *Effects of various protein kinases and calmodulin on Ca^{2+}-ATPase activity in rat aortic smooth muscle cell microsomal fractions*

Microsomal fractions from cultured rat aortic smooth muscle cells were prepared by the methods described previously (Rashatwar *et al.*, 1987) and Ca^{2+}-ATPase was assayed. Units are defined as the amount of enzyme required to transfer 1 nmol of phosphate from ATP to histone substrate in 1 min. The results represent the average of two separate experiments.

Additions	Ca^{2+}-ATPase (nmol/min per mg of protein)
None	6.09
Cyclic-dependent protein kinase (3.6 u.)	6.08
Cyclic-GMP-dependent protein kinase (0.7 u.)	19.98
Protein kinase C (2.5 u.)	12.60
Calmodulin alone	11.88
Cyclic-GMP kinase + calmodulin	27.67

To define the mechanism of action of cyclic GMP, we have taken into account the ability of the nucleotide to inhibit increases in Ca^{2+} produced by two distinct pathways: agonists, such as NA and angiotensin, which stimulate phosphatidylinositol breakdown and the formation of inositol 1,4,5-triphosphate (InsP_3), and depolarization which increases intracellular Ca^{2+} via influx through the cell membrane without affecting InsP_3 levels (Rashatwar *et al.*, 1987). Our approach, therefore, was to examine the effect of cyclic-GMP-dependent protein kinase on potential Ca^{2+} removal processes in cultured smooth muscle cells, and specifically, the effect of cyclic-GMP-dependent protein kinase on Ca^{2+}- stimulated ATPase activity. As shown in Table 1, cyclic-GMP-dependent protein kinase, but not cyclic-AMP-dependent protein kinase or protein kinase C, activated membrane-associated Ca^{2+}-ATPase activity over 2-fold. Calmodulin, a known activator of Ca^{2+}-ATPase from plasma membranes, produced a similar 2-fold activation of ATPase activity in the same preparations. The effect of cyclic-GMP-dependent protein kinase was additive with that of calmodulin. It was apparent that kinase and calmodulin either affected different enzymes, or that the same ATPase was stimulated by both proteins in a dual fashion. However, our attempts to use cyclic-GMP-dependent protein kinase to activate a partially purified Ca^{2+}-ATPase which had been detergent extracted from bovine aorta have been unsuccessful. Indeed, we have recently separated the Ca^{2+}–calmodulin-activated ATPase from substrate proteins for the cyclic-GMP-dependent protein kinase by affinity chromatography (our unpublished work). Therefore, the mechanism by which cyclic-GMP-dependent protein kinase activates Ca^{2+}-ATPase remains obscure. It is likely that other membrane proteins are phosphorylated by cyclic-GMP-dependent protein kinase which in some as yet undefined manner results in the activation of Ca^{2+}-ATPase.

In conclusion, our laboratory has shown that cyclic GMP formation is associated with a reduction of free intracellular Ca^{2+} in rat aorta and cultured rat aortic smooth muscle cells. The reduction in Ca^{2+} could mediate the relaxation produced by cyclic GMP in contracted rat aortic strips. The mechanism by which this occurs must explain the effect of cyclic GMP to inhibit Ca^{2+} increases produced either by agonist-induced InsP_3 release or those effects due to activation of voltage-sensitive Ca^{2+} channels. One effect of cyclic GMP which has been observed is the activation of membrane-associated Ca^{2+}-ATPase(s) which would presumably pump Ca^{2+} from the cytoplasm (Popescu *et al.*, 1985; Rashatwar *et al.*, 1987). However, direct phosphorylation of the Ca^{2+}-

ATPase from the plasma membrane has not been observed. Further work will be required to understand the exact role of cyclic-GMP-dependent protein kinase in regulating Ca^{2+}-ATPase activity.

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Myosin light chain and heavy chain phosphorylation in smooth muscle: potential regulatory roles for calcium, phospholipids and cyclic nucleotides

DAVID R. HATHAWAY, LEONARD P. ADAM, ROBERT C. TURNER and JOSEPH A. LASH
Department of Medicine and the Krannert Institute of Cardiology, Indiana University School of Medicine, Indianapolis, IN 46223, U.S.A.

Regulation of myosin light chain kinase by the catalytic subunit of cyclic-AMP-dependent protein kinase

The early phase of contraction in vascular smooth muscle is characterized by rapid phosphorylation of the 20000 Da light chain subunits of myosin. Light chain phosphorylation correlates directly with the shortening velocity of the muscle as well as the intracellular Ca^{2+} transient measured by photodye methods. The enzyme which catalyses this phosphorylation, myosin light chain kinase (MLCK), has been purified to homogeneity from bovine aortic smooth muscle and has a molecular mass of 160000 Da [1]. Vascular MLCK activity is absolutely dependent upon Ca^{2+} and calmodulin and, in saturating Ca^{2+}, is activated by calmodulin in a 1:1 stoichiometry. Whereas Ca^{2+} and calmodulin serve to activate MLCK, it has been proposed that the catalytic subunit of cyclic-AMP-dependent protein kinase (PK_A) can effect the opposite [2]. Phosphorylation of vascular MLCK by PK_A results in the incorporation of up to 2 mol of phosphate/mol of enzyme *in vitro*, depending upon incubation conditions [3]. Tryptic peptide mapping of MLCK phosphorylated by PK_A in the absence of Ca^{2+}-calmodulin yields two major phosphorylated peptides by thin layer electrophoresis (peptides 1 and 2). When saturating Ca^{2+} and calmodulin are included in the assay, only peptide 1 is phosphorylated by PK_A. The effect of phosphorylation of MLCK by PK_A depends upon the specific site(s) phosphorylated and, hence, incubation conditions. When phosphorylated on both peptides 1 and 2, approximately 20-fold more calmodulin is required for full activation of MLCK in saturating Ca^{2+}. When peptide 1 alone is phosphorylated (i.e. incubation of MLCK and PK_A in the presence of Ca^{2+} and calmodulin), there is no measurable effect on the calmodulin requirement for maximal activity or upon the K_m values for myosin light chains or ATP [2].

Abbreviations used: MLCK, myosin light chain kinase; PK_A, catalytic subunit of cyclic-AMP-dependent protein kinase.

In its native environment, all structural domains of MLCK may not be freely accessible to other kinases. Chemical skinning of smooth muscle removes neither MLCK nor all calmodulin [4]. Thus, under more physiological conditions, MLCK and calmodulin must lie in rather close proximity or apposition to one another. To test the suitability of MLCK as a substrate for PK_A under more native conditions, the catalytic subunit of cyclic-AMP-dependent protein kinase (50 μg/ml) was added to the bath of glycerinated porcine carotid vascular smooth muscle in the absence or presence of Ca^{2+} (100 μM) and calmodulin (10^{-6} M). The results are summarized in Fig. 1. In the absence of Ca^{2+} and calmodulin, 1.09 mol of phosphate could be incorporated into MLCK following a 30 min incubation at 25°C. Tryptic peptide mapping of the phosphoryalted enzyme *in situ* revealed that nearly 80% of the phosphate was incorporated into peptide 1 with the remainder in peptide 2. Under identical conditions, except for the inclusion of Ca^{2+} and calmodulin in the bathing solution, phosphate was almost exclusively incorporated into peptide 1. Under these conditions, the stoichiometry of MLCK phosphorylation was reduced to approximately 0.7 mol of PO_4/mol of MLCK. These results suggested that under all conditions, peptide 2 is a relatively poor substrate for PK_A. Moreover, preliminary studies with intact vascular smooth muscle loaded with $^{32}PO_4$ confirm that very little phosphate can be incorporated into peptide 2 of MLCK in response to pharmacological agents that increase cyclic AMP. Thus, regulation of MLCK activity as proposed from studies *in vitro* (i.e. regulation by PK_A of the binding affinity of calmodulin for MLCK) seems unlikely in intact smooth muscle [7, 8].

Phosphorylation of the heavy chain of vascular smooth muscle: potential role for protein kinase C and Ca^{2+}-calmodulin kinase II in modulating contractility

Myosin heavy chain phosphorylation has been reported for invertebrate and several non-muscle myosins. Although effects of myosin heavy chain phosphorylation on the enzymic or structural properties of myosin have been rather scanty, modulation of filament formation or actin-activated Mg^{2+}-ATPase activity has been described [9, 10]. Fig. 2 illustrates the capacity for purified protein kinase C and Ca^{2+}-calmodulin kinase II to phosphorylate the heavy chain