Cyclic GMP Causes Ca\(^{2+}\) Desensitization in Vascular Smooth Muscle by Activating the Myosin Light Chain Phosphatase*

(Received for publication, July 10, 1996, and in revised form, December 16, 1996)

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*This work was supported by National Institute of Health Grant HL51824. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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The primary mechanisms that regulate smooth muscle contraction and relaxation are, respectively, phosphorylation of the regulatory 20-kDa myosin light chain (MLC\(_{20}\))† at Ser-19 by MLC\(_{20}\) kinase (MLCK) and its dephosphorylation by MLC\(_{20}\) phosphatase (MLCP) (1, 2). Typically, intracellular Ca\(^{2+}\) levels modulate the MLCK to MLCP activity ratio and ultimately the degree of contractile force because MLCK activity depends on the amount of the Ca\(^{2+}\)-calmodulin complex, which itself hinges on cytosolic Ca\(^{2+}\) levels. In many cases, however, the sensitivity of force to Ca\(^{2+}\) can be changed by physiological modulation of the Ca\(^{2+}\) dependence of MLC\(_{20}\) phosphorylation.

Using permeabilized, arterial smooth muscle strips where membrane-associated pathways remain intact but intracellular Ca\(^{2+}\) stores are depleted, we investigated mechanism(s) for the Ca\(^{2+}\) desensitization of contractile force by cGMP. The nonhydrolyzable analog 8-bromo-cGMP, when applied to these strips with submaximal Ca\(^{2+}\) levels clamped, dramatically and reversibly reduced the steady state levels of phosphorylation at 20-kDa myosin light chain and contractile force, with a nanomolar concentration required to obtain 50% reduction. Supramaximal concentrations of 8-bromo-cGMP (10 \(\mu\)M), however, did not change the steady state relationship between phosphorylation and force. When light chain phosphatase activity was blocked at pCa 6.7, 10 \(\mu\)M 8-bromo-cGMP did not affect the rates of rise of light chain phosphorylation and contractile force. When light chain kinase activity was blocked, 10 \(\mu\)M 8-bromo-cGMP significantly accelerated light chain dephosphorylation and force relaxation from the maximal contraction steady state. The light chain phosphorylation time course of a pCa 6.0-induced contraction in the presence of 8-bromo-cGMP exhibited kinetics that are predictable from a mathematical model in which only light chain phosphatase activity is increased. The results of this study strongly suggest that cGMP indirectly activates light chain phosphatase, the first proposed mechanism for cGMP-induced Ca\(^{2+}\) desensitization in vasodilatation.

EXPERIMENTAL PROCEDURES

Tissue Preparation and Measurement—Smooth muscle strips (70 \(\mu\)m thick, 700–800 \(\mu\)m wide, and 3 mm long) were dissected from rabbit femoral arteries and carefully freed of connective tissue; the endothelia were removed by rubbing with a razor blade. The strips were then tied with silk monofilaments to the fine tips of two tungsten needles, one of which was connected to a force transducer (AM801, SensoNor), and mounted over a well filled with solution on a Teflon bubble plate to allow for moderately rapid (within a second) solution exchange and freezing (3). Experiments were, with a single exception (15 °C in Fig. 6), carried out at 20 °C.

Solutions—The standard relaxing solution (3) used for resting states of the permeabilized strips contained the following: 74.1 mM potassium methane sulfonate, 2 mM Mg\(^{2+}\), 4.5 mM MgATP, 1 mM EGTA, 10 mM creatine phosphate, 30 mM piperezine-N,N\(^{-}\)-bis(2-ethanesulfonic acid). At times, we used slightly modified relaxing solutions in which the concentration of EGTA was different. In the activating solution, 10 mM EGTA was used, and a calculated amount of calcium methanesulfonate was added to give the final desired concentration of free Ca\(^{2+}\) ions (14). In the rigor solutions, the solute composition was like that of the relaxing solutions except for the absence of MgATP and creatine phosphate. All solutions were neutralized to pH 7.1 with KOH at 20 °C, and an ionic strength of 0.2 was achieved by appropriately using more or less potassium methanesulfonate.

Cell Permeabilization—After measuring the force of contractions induced by high K\(^{+}\) (154 mM) and by phenylephrine (100 \(\mu\)M) in freshly dissected strips, these strips were incubated in the standard relaxing solution for several minutes. For plasma membrane permeabilization with \(\alpha\)-toxin, the strips were then treated for 30 min at 30 °C with 5000
units/ml of purified *Staphylococcus aureus* α-toxin (Life Technologies, Inc.) at pCa 6.7 buffered with 10 mM EGTA (15). For plasma membrane permeabilization with β-escin, the strips were instead treated with 40 μM β-escin in standard relaxing solution for 45 min at 5°C and then for 15 min at 30°C (15). The sarcoplasmic reticulum was also depleted of calcium by treating the strips with 10 μM A23187, a Ca$^{2+}$ ionophore, for 15–20 min at 30°C in standard relaxing solution. For the usage of Triton X-100 to heavily permeabilize strips, we simply used 0.1% in standard relaxing solution for 30 min at 4°C and for 15 min at 30°C.

Two-dimensional Gel Electrophoresis—For measuring MLC$_{20}$ phosphorylation, permeabilized preparations were rapidly frozen with liquid N$_2$-cooled, liquid chlorodifluoromethane at the desired conditions, with force monitored up to the time of freezing. The various phosphorylated states of MLC$_{20}$, unphosphorylated (U), monophosphorylated (P1), and diphosphorylated (P2), were separated by a two-dimensional isoelectric focusing SDS-polyacrylamide gel electrophoresis, blotted onto a nitrocellulose membrane, and stained with colloidal gold to produce separate spots for each phosphorylated state, and the amount at each spot was measured by its density (3). The percentage of MLC$_{20}$ phosphorylation was calculated by dividing (P1 + P2) by (U + P1 + P2).

**RESULTS**

**Ca$^{2+}$ Desensitization**—Fig. 1A graphically illustrates the marked effect of 8Br-cGMP to cause Ca$^{2+}$ desensitization of force in α-toxin-permeabilized rabbit femoral artery smooth muscle strips. This trace demonstrates how 10 μM 8Br-cGMP potently and reversibly elicits relaxation and how pretreatment with this compound inhibits development of force.

**FIG. 1.** Effectiveness of 8Br-cGMP on contractile force in permeabilized arterial smooth muscle. A, this trace of contractile force depicts a typical α-toxin-permeabilized strips where maximum force was obtained using a pCa 5.0 solution to demonstrate viability straight after permeabilization and then relaxed to the basal level with standard relaxing (pCa > 8) solution before introducing experimental conditions. B, Triton X-100 was used to demembranate the tissue, followed directly by onset of the desired experimental protocol (see "Results"). CaM; 3 μM calmodulin. MC; 10 μM MC-LR.

**FIG. 2.** Dose-response of 8Br-cGMP and 8Br-5GMP on contractile force. For at least 10 min, single strips were kept in standard relaxing solution and a given [8Br-xGMP], starting at 0 nM. Next, they were placed in a pCa 6.0 solution with the same pretreating [8Br-xGMP] until reaching a steady state level of force. 8Br-xGMP was then washed out with standard relaxing solution, and the process was repeated with increasingly higher 8Br-xGMP levels until 10 μM was reached. Steady state values were measured as relative force.

**FIG. 3.** 10 μM 8Br-cGMP on Ca$^{2+}$ sensitivity of phosphorylation (B) and force (A). A, relative force state was measured at each steady ± 10 μM 8Br-cGMP as Ca$^{2+}$ was increased stepwise. B, a similar protocol was followed except that individual strips were placed in a single, specific [Ca$^{2+}$] and then frozen at steady state. C, the summary between steady state phosphorylation and force ± 8Br-cGMP over the range of Ca$^{2+}$ levels in A and B was plotted. Only control values are fitted with a curve of $F = 0.56 - 0.69P + 0.046P^2 - 0.00029P^3$ ($R^2 = 0.998$), where $F$ is the force level, and $P$ is the phosphorylation level.
As a simple dose-response of the steady state, pCa 6.0-induced, submaximal (near 60%) contractile force to 8Br-cGMP shown in Fig. 2 depicts the high efficacy of this cyclic nucleotide to exert its desensitizing effect on force. The IC₅₀ was very low at 8.60.4 nM, whereas an 8Br-cGMP metabolite 8Br-5GMP had no significant effect on contraction, even in a micromolar range (Fig. 2).

A more comprehensive and definitive example of the desensitization over a range of buffered Ca²⁺ levels is shown in Fig. 3. The 8Br-cGMP effect on steady state levels of contractile force (Fig. 3A) and MLC₂₀ phosphorylation (Fig. 3B) is a parallel shift to the right, with an average 2.5- and 2.9-fold increase in ED₅₀ of Ca²⁺ for force and phosphorylation, respectively. Of note, force is reduced from 62% to 8% at pCa 6.0, and phosphorylation is reduced from 62% to 30% at pCa 6.0, but the same concentration of the cyclic nucleotide affected neither maximal phosphorylation steady state nor maximal force steady state. With computer assistance, we extrapolated a line curve and its equation from the data values in the absence of 8Br-cGMP at the different Ca²⁺ levels of force and phosphorylation (Fig. 3C). The data points in the presence of the cyclic nucleotide lie virtually on top of the control line with no significant difference.

In the separate Triton X-100 experiments, 10 μM 8Br-cGMP was not capable of inducing significant relaxation of a submaximal steady state contraction at pCa 6.0 (Fig. 1). However, the addition of either calmodulin (Fig. 1, B1) or the selective phosphatase inhibitor microcystin-LR (MC-LR; Fig. 1, B2) did markedly enhance the level of force in the same preparations, thus demonstrating the integrity of contractile apparatus including MLCK and MLCP in the Triton X-100-permeabilized strips. Hence, it appears that cGMP is not able solely on its own to induce desensitization of contractile force to Ca²⁺ in the demembranated strips.

MLCK Activity Is Unaffected—After application of the membrane-impermeable (PP1- and PP2A-selective) phosphatase inhibitor MC-LR (16) at 10 μM in pCa 6.7 in β-escin-permeabilized strips, steady state levels of force and phosphorylation were attained that did not increase further when the Ca²⁺ concentration in the fibers was increased to a maximal pCa 4.5 (not shown). This demonstrates the ability of the chosen inhibitor MC-LR at 10 μM to abolish in situ MLCP activity. In pCa 6.7 solution containing 10 μM MC-LR, pretreatment with 8Br-cGMP did not affect the steady state levels of or the rate of rise for contraction (Fig. 4, A1) or phosphorylation (Fig. 4, A2). To verify the efficacy of this protocol, we used wortmannin (17) as a MLCK inhibitor that is known to have inhibitory effects on other signaling pathways. Strips were pretreated with 3 μM wortmannin instead of 8Br-cGMP, and the rate of rise did indeed decrease (Fig. 4, A1).

In another set of experiments, the structurally distinct, membrane-permeable (PP1- and PP2A-selective) phosphatase inhibitor calyculin A was used in combination with α-toxin-permeabilized strips. Neither the rate of rise for contraction (Fig. 4, B1) nor for phosphorylation (Fig. 4, B2) was affected by the presence of 10 μM 8Br-cGMP, similar to the results obtained using MC-LR in β-escin-permeabilized preparations.

MLCP Activity Increases—200 μM of the MLCK inhibitor ML-9 (18) together with a Ca²⁺-free (10 mM EGTA) solution potently decreased maximum levels of contractile force and...
Ca\textsuperscript{2+} for this protocol because of its relatively rapid inhibition from 53 to 39 s. ML-9, although its effect is weak, was chosen for each of the MLC\textsubscript{20} phosphorylation time course data points, as a single three predicted time courses of MLC\textsubscript{20} phosphorylation (Fig. 3). For MLCK, whereas wortmannin was not suitable for this protocol because of very slow kinetics of inhibition of even isolated MLCK by dephosphorylation, for MLCP, whereas it was not suitable for this protocol because of its relatively rapid inhibition of Ca\textsuperscript{2+}-activated contraction and its relatively high selectivity for MLCK, whereas wortmannin was not suitable for this protocol because of very slow kinetics of inhibition of even isolated MLCK (17).

**Time Course Kinetics Is Predictably Altered**—In an unmodified pCa 6.0-induced contraction where MLCK and MLCP were not inhibited, 10 \textmu M 8Br-cGMP decreased the peak levels of contractile force (Fig. 6A) and MLC\textsubscript{20} phosphorylation (Fig. 6B) without affecting the initial rate of rise, thus decreasing the \( t_{1/2} \) as shown in the figure. According to a simple two-state MLC\textsubscript{20} model (phosphorylated or unphosphorylated), we generated three predicted time courses of MLC\textsubscript{20} phosphorylation (Fig. 6C). First, to simulate the steady state phosphorylation value obtained at pCa 6.0 (62% of total MLC\textsubscript{20}, see Fig. 3B), we chose an appropriate MLCK to MLCP activity ratio, 1.6 (control). To simulate possible 8Br-cGMP effects on the pCa 6.0-induced contraction, we either increased MLCP activity (MLCP \( \times 3.8 \)) or decreased MLCK activity (MLCK/3.8). The steady state levels were decreased to the same extent (30% of total MLC\textsubscript{20}) in both 8Br-cGMP simulations, but the actual experimental effect of 8Br-cGMP on initial time courses of both phosphorylation and contraction (Fig. 6, A and B) closely resembled the simulation with increased MLCP activity and deviated markedly from the simulation with decreased MLCK activity (Fig. 6C).

**DISCUSSION**

This study illustrates how nanomolar concentrations of 8Br-cGMP but not 8Br-5′GMP strongly and reversibly reduces the ability of Ca\textsuperscript{2+} to cause the vascular smooth muscle contractile machinery to generate force *in situ*. Moreover, it analyzes this effect of 8Br-cGMP to identify which of the contractile or regulatory apparatuses might be altered sufficiently to account for the Ca\textsuperscript{2+} desensitizing effect.

The cGMP-induced desensitization of contractile force to Ca\textsuperscript{2+} must involve a Ca\textsuperscript{2+}-independent reduction in the MLC\textsubscript{20} phosphorylation by cGMP at a given Ca\textsuperscript{2+} level and/or a change to some MLC\textsubscript{20} phosphorylation-independent regulatory mechanism(s). An example of the latter could be a cGMP-induced increase in inhibition of actomyosin ATPase by the thin filament-associated proteins caldesmon and calponin (5, 6, 19). However, because 8Br-cGMP does not appear to change the steady state relationship between phosphorylation and force (Fig. 3C), it is reasonable to exclude a significant involvement of such phosphorylation-independent mechanisms. In this tissue, it appears that the Ca\textsuperscript{2+} desensitization of force by 8Br-cGMP is mainly mediated through Ca\textsuperscript{2+}-independent decreases in phosphorylation at MLC\textsubscript{20}. However, we cannot rule out the possibility of more complicated mechanisms of Ca\textsuperscript{2+} desensitization of force by cGMP during the nonsteady state phases of contraction and relaxation.

With that in mind, we set out to establish whether cGMP causes inactivation of MLCK, activation of MLCP, or some combination thereof. It is well known that *in vitro* phosphorylation of MLCK at its site A by protein kinase A, protein kinase C, and Ca\textsuperscript{2+}-calmodulin-dependent kinase II inactivates MLCK by decreasing the affinity of MLCK for Ca\textsuperscript{2+}-calmodulin (20). Hence phosphorylation at site A by any of the above mentioned kinases would cause a lower than normal MLC\textsubscript{20} phosphorylation at a given Ca\textsuperscript{2+} level. Not surprisingly, it had been proposed that the cGMP-activated kinase (PKG) phosphorylates MLCK and accounts for the Ca\textsuperscript{2+} desensitizing effect of cGMP. Direct phosphorylation of MLCK by PKG does indeed occur *in vitro* but not at site A; furthermore, this phosphorylation does not modify the activity of MLCK (21, 22). Thus from the biochemical studies, it seems that cGMP is not capable of affecting MLCK activity through its protein kinase PKG. Still, the biochemical research on isolated MLCK could not provide any useful information to evaluate whether an indirect inhibition of MLCK by cGMP occurred. Our experiment, however, involving virtually complete elimination of MLCP activity (Fig. 4, A and B) lends physiological support to the idea that MLCK is unaffected by cGMP. 8Br-cGMP did not change the activity of MLCK, as evidenced by its ineffectiveness on the phosphorylation rate and rate of development of force. This result was confirmed in two different types of permeabilized preparations using two structurally different selective MLCP inhibitors. We realize that it is conceivable, although unlikely, that our chosen two phosphatase inhibitors MC-LR and calyculin A somehow interfere with the putative ability of PKG to inhibit MLCK, thus hiding such an effect.

At this point it now seemed by default that cGMP was exerting Ca\textsuperscript{2+} desensitization through activation of MLCP. The experiment summarized in Fig. 5 indeed indicates such a mechanism; 8Br-cGMP significantly increased the rates of dephosphorylation and relaxation. The \( t_{1/2} \) for dephosphorylation was 1.4 times greater in the absence of 8Br-cGMP and the \( t_{1/2} \) for...
phosphorylated MLC20, respectively, and the rate of rise could be more precisely analyzed. The fraction of the phosphorylation was lowered slightly in order to slow down the time course so that the two-state model for the reaction of MLC20 phosphorylation is assumed, where:

\[
M \xrightarrow{k_1} P_M \quad \text{(Eq. 1)}
\]

The time course of force development was similar to that of MLC20 phosphorylation based on a single phosphorylation site for MLCK. Although in vitro MLCK can phosphorylate 2 mol/mol of MLC20 (Ser-19 and Thr-18), the rate of phosphorylation at the latter site is much lower than at the former site and their dephosphorylations by MLCP occur at similar rate (26), suggesting that only a very small portion of MLC20 is diphosphorylated under physiological conditions. In fact, the extent of phosphorylation is 1 mol/mol or lower in intact smooth muscle (27) and 1.1–1.2 mol/mol in the permeabilized muscle maximally stimulated by Ca\(^{2+}\) ions alone or with GTPγS (15). Additional Thr-18 phosphorylation increases the actin-activated myosin ATPase (26) but does not increase the rate of movement of actin filaments in the in vitro motility assay (28). Therefore, only one phosphorylation site at MLC20 is enough and appropriate to simulate the time course of MLC20 phosphorylation in our conditions (23, 24).

The time course of force development was similar to that of MLC phosphorylation (Fig. 4), but the relaxation occurred much more slowly than the dephosphorylation (Fig. 5). The relaxation and dephosphorylation rates were somewhat affected by diffusion of EGTA and ML-9, but MLC phosphorylation at 1 min in the Ca\(^{2+}\)-free (EGTA 10 mM), ML-9 solution is comparable to how 8Br-cGMP changed the phosphorylation kinetics of actual pCa 6.0-induced contractions. Thus we conclude from experimental results that cGMP creates marked Ca\(^{2+}\) desensitization of contractile force in vascular smooth muscle by activating MLCP. That finding would also imply that MLCP activity in situ under normal physiological conditions is not maximal. It has been interestingly suggested that PKG in pituitary tumor cells stimulates Ca\(^{2+}\) and voltage-activated potassium channels through activation of an okadaic acid-sensitive protein phosphatase (25). We suggest that activation of protein phosphatase by cGMP may be a general function in biological systems.

In our theoretical model, we made predictions regarding MLC20 phosphorylation based on a single phosphorylation site for MLCK. Although in vitro MLCK can phosphorylate 2 mol/mol of MLC20 (Ser-19 and Thr-18), the rate of phosphorylation at the latter site is much lower than at the former site and their dephosphorylations by MLCP occur at similar rate (26), suggesting that only a very small portion of MLC20 is diphosphorylated under physiological conditions. In fact, the extent of phosphorylation is 1 mol/mol or lower in intact smooth muscle (27) and 1.1–1.2 mol/mol in the permeabilized muscle maximally stimulated by Ca\(^{2+}\) ions alone or with GTPγS (15). Additional Thr-18 phosphorylation increases the actin-activated myosin ATPase (26) but does not increase the rate of movement of actin filaments in the in vitro motility assay (28). Therefore, only one phosphorylation site at MLC20 is enough and appropriate to simulate the time course of MLC20 phosphorylation in our conditions (23, 24).

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8Br-cGMP, even though force levels in both conditions were reduced only by 3–4% (Fig. 5). These observations are consistent with previous results (29) and can be explained by a slow onset detachment of dephosphorylated cross-bridges (30). Recently, Khromov et al. (31) precisely analyzed the biphasic time course of relaxation in permeabilized arterial smooth muscle by photolysis of a caged Ca$^{2+}$ chelator. They suggested that the initial plateau phase of relaxation followed by an exponential decay may be due to continued cycling of remaining phosphorylated cross-bridges and cooperative cycling of phosphorylated cross-bridges. It is therefore the length of the plateau phase of relaxation in tonic smooth muscle that may depend inversely upon the MLC dephosphorylation rate. The second exponential decay phase was suggested to be representative of the detachment rate of dephosphorylated cross-bridges, which is independent of the dephosphorylation rate. The time course of relaxation initiated by EGTA and ML-9 in this study was also biphasic (Fig. 5A). 8Br-cGMP reduced the duration of the plateau phase of relaxation but did not significantly affect the subsequent exponential decay. Using the work of Khromov et al. (31) to interpret our results shown in Fig. 5, one again concludes that 8Br-cGMP increases the rate of MLC dephosphorylation but does not affect detachment rate of dephosphorylated cross-bridge.

The loss of 8Br-cGMP’s desensitizing effect in heavily permeabilized, demembranated samples implies that some soluble cytosolic target(s), such as PKG and/or diffusible cofactor(s), mediates that effect in intact and selectively permeabilized fibers, confirming previous work of Pfizter et al. (10). This idea is in agreement with other studies involving cGMP because no known action of cGMP in smooth muscle relaxation has been shown to function independently of PKG (9). One can then reasonably assume from the low nanomolar IC$_{50}$ of 8Br-cGMP shown in this study that the unknown anticipatory target involved in coupling GMP to the activation of MLCP is likely PKG. However, because cGMP has also been shown to activate cAMP-dependent protein kinase (32), the possibility that high micromolar concentrations of 8Br-cGMP stimulate both PKG and protein kinase A signaling pathways cannot be excluded.

The Ca$^{2+}$ level in smooth muscle is the primary determinant in the phosphorylation of MLC$_{20}$ and hence in the contraction. The known mechanisms in this tissue for handling cytoplasmic Ca$^{2+}$ and for modulating Ca$^{2+}$ sensitivity of the contraction are synergistic and cooperative pathways. Excitatory agonists produce contraction by elevating Ca$^{2+}$ levels and increasing Ca$^{2+}$ sensitivity (33, 34), whereas vasodilating substances cause relaxation by reducing Ca$^{2+}$ levels and decreasing Ca$^{2+}$ sensitivity (35, 36). Furthermore, the excitatory agonists cause Ca$^{2+}$ sensitization through inhibition of MLCP (23, 37), whereas we have shown in this study that cGMP cause Ca$^{2+}$ desensitization through activation of MLCP. Thus, nitrovasodilators and the endothelium via production of NO appear to antagonize excitatory agonists in almost every reported mechanism, including a new apparent activation of MLCP versus agonist-induced inhibition of MLCP.

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