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Effects of Nitroprusside, Glyceryl Trinitrate, and 8-Bromo Cyclic GMP on Phosphorylase *a* Formation and Myosin Light Chain Phosphorylation in Rat Aorta

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SUMMARY

The effects of nitroprusside (NP), glyceryl trinitrate (GTN), and the 8-bromo analog of cyclic GMP (8-Br-cGMP) on norepinephrine (NE)-stimulated phosphorylase *a* formation and myosin light chain (MLC) phosphorylation were examined in the rat aorta. NE produced a time-dependent increase in tension, phosphorylase *a* formation, and MLC phosphorylation. The formation of phosphorylase *a* and phosphorylation of MLC were transient, since both processes declined to basal levels within 30 min after the addition of NE even though tension remained elevated. NP and GTN inhibited tension, phosphorylase *a* formation, and MLC phosphorylation although inhibition of phosphorylase was greater when strips were treated with submaximal (i.e., 0.01 μM) NE concentrations. GTN was a more effective inhibitor of phosphorylase *a* formation than NP in NE-treated strips, although both agents and 8-Br-cGMP inhibited MLC phosphorylation. The guanylate cyclase inhibitor methylene blue (10 μM) effectively prevented the effects of NP and GTN. The results suggest that NP, GTN, and 8-Br-cGMP inhibit phosphorylase kinase and MLC kinase activation by lowering Ca^{2+} in the cell. This hypothesis is supported by the observations that 8-Br-cGMP inhibited the Ca^{2+}-dependent, KCl-induced phosphorylase *a* formation most markedly at reduced concentrations of extracellular Ca^{2+}. In addition, neither NP, GTN, nor 8-Br-cGMP inhibited phosphorylase *a* formation in forskolin-treated tissues, which occurred in response to cAMP-dependent phosphorylation of phosphorylase *b* kinase.

INTRODUCTION

Tissue levels of cGMP are increased in a time and concentration-dependent manner by nitro vasodilators such as NP[3] and GTN in various smooth muscle preparations (1–5). In addition, relaxation of several smooth muscle preparations by analogs of cGMP, but not 5′-GMP, occurs at concentrations in the micromolar range (6–8). Cyclic GMP-dependent protein kinase and putative substrates also have been reported to exist in smooth muscle-containing tissues (9). Although these observations suggest a role for cGMP in relaxation of vascular smooth muscle, the mechanism of action of the nucleotide is not known. We have recently investigated the mechanism of action of NP and the 8-bromo derivative of cGMP (8-Br-cGMP) in rat aorta and have suggested that 8-Br-cGMP and agents which increase cGMP levels in rat aorta inhibit contraction by reducing free Ca^{2+} concentrations in the cytoplasm (8, 10). If vascular smooth muscle relaxation mediated by cGMP is due to the lowering of free Ca^{2+} concentrations, then the activities of enzymes that are dependent on Ca^{2+} for catalytic activity should reflect the changes in Ca^{2+} levels within the cell. This approach has been used in various systems by other investigators to monitor free intracellular Ca^{2+} levels (11–14).

It is well established that the Ca^{2+}-dependent phosphorylation of the regulatory light chain of myosin is responsible for the actin-dependent activation of myosin ATPase which precedes the rapid cross-bridge cycling that leads to tension development in smooth muscle (see Ref. 15 for a review). Phosphorylation of MLC is catalyzed by a MLC kinase whose activity is regulated by calmodulin (15). In addition, it is known that an elevated

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[3] The abbreviations used are: NP, nitroprusside; GTN, glyceryl trinitrate; NE, norepinephrine; 8-Br-cGMP, 8-bromo cyclic GMP; MLC, myosin light chain; MLC-P, phosphorylated MLC; KRB, Krebs-Ringer bicarbonate buffer; FOR, forskolin; MB, methylene blue; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid.

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Ca^{2+} concentration is the primary factor that regulates the activity of nonactivated (i.e., dephosphorylated) phosphorylase *b* kinase in muscle, and that Ca^{2+} released during excitation stimulates nonactivated phosphorylase *b* kinase to catalyze the conversion of phosphorylase *b* to *a* (12, 16, 17). Here we report the effects of two nitro vasodilators, NP and GTN, and the 8-bromo analog of cGMP on the phosphorylation of MLC and the conversion of phosphorylase *b* to *a* in rat aorta. The results suggest that cGMP, presumably through cGMP-dependent protein kinase, mediates nitro vasodilator-induced relaxation, in part at least, by reducing the levels of free intracellular Ca^{2+}.

MATERIALS AND METHODS

Materials. 8-Bromoguanosine-3′,5′ cyclic monophosphate sodium salt, sodium nitroprusside, and *l*-norepinephrine bitartrate were purchased from Sigma Chemical Co. (St. Louis, MO). All norepinephrine solutions were made fresh daily with 0.9% NaCl containing 0.1% (w/v) sodium metabisulfite. Sodium nitroprusside and 8-Br-cGMP solutions were prepared daily in water and test tubes containing freshly prepared solutions of nitroprusside were wrapped with aluminum foil to prevent degradation. Forskolin was obtained from Calbiochem-Behring Corp. (La Jolla, CA), and was dissolved in 95% ethanol. Glyceryl trinitrate tablets (Nitrostat; Parke-Davis, Morris Plains, NJ) were dissolved in water. Uniformly labeled [^{14}C]glucose 1-phosphate dipotassium salt was purchased from ICN (Irvine, CA). Some ampholytes (pH 4–6) were from LKB, Inc., and others (pH 3–10) were purchased from Pharmacia. Krebs-Ringer bicarbonate buffer contained 117 mM NaCl, 4.7 mM KCl, 1.1 mM $MgSO_4$, 1.2 mM KH_2PO_4, 25 mM $NaHCO_3$, 1.5 mM $CaCl_2$ and 20 mM glucose.

Tissue preparation and incubation. Mature, male Sprague-Dawley rats (250–274 g) were sacrificed by decapitation, and the aortas were rapidly excised anteriorly below the aortic arch and posteriorly above the bifurcation into the external iliac. After carefully stripping away loose fat and connective tissue, the aortic strips were immersed in glass vials containing 5 ml of KRB buffer aerated with 95% O_2/5% CO_2 at 37°. The strips were shaken in a Dubnoff metabolic incubator (position 5) to ensure proper diffusion and were allowed to equilibrate for 90 min with three changes of KRB buffer. Following the equilibration period, strips were incubated in the absence or presence of the agents for varying lengths of time. The strips were rapidly removed from the incubation medium, touched to absorbent paper, and subsequently immersed in dichlorodifluoromethane (Freon 12) cooled in liquid N_2. The frozen aortic strips were powdered by percussion with a stainless steel mortar cooled to the temperature of liquid N_2 and stored at −70° prior to their use for biochemical assays. In other experiments, the aortic strips were prepared for tension measurements by mounting them in a muscle bath as previously described (8). Briefly, vessels were placed on glass rods, and helical strips (2–3 mm wide, 10 mm in length) were cut. The strips were attached on one end using a stainless steel hook to a micrometer while the other end was placed in a Plexiglas vise clip connected to a Statham strain gauge force transducer (20-μV sensitivity). The initial tension was set at 500–600 mg and the tissues were allowed to relax for 90 min with three changes of KRB buffer. After a final adjustment of tension to 200–300 mg, contractions were recorded using a Gould 2400 recorder at a paper speed of 0.5 mm/sec. Tissues were frozen by one of two methods: in Method A, the strips were removed from the bath as quickly as possible, blotted once, and frozen in dichlorodifluoromethane cooled in liquid N_2. This procedure required 3–4 sec. With Method B, the muscle bath was drained of KRB buffer, and acetone cooled to low temperature with crushed dry ice was added as described by Driska *et al.* (18). The strips were thawed in the acetone as it was allowed to return to room temperature and homogenized directly in a solution specifically designed for isoelectric focusing experiments. Although only Method A was used for phosphorylase determinations, both methods gave similar results with respect to MLC phosphorylation.

Phosphorylase assay. For the assay of phosphorylase *a* formation, approximately 15 mg of powdered tissue were transferred to a Duall homogenization tube (size 20, Kontes Glass Co., Vineland, NJ) and homogenized at −20° in 0.5 ml of 60% (v/v) aqueous glycerol solution containing 100 mM KF and 100 mM EDTA according to the method of Stull and Mayer (17). Then 0.3 ml of a solution containing 100 mM KF and 100 mM EDTA was added, and the sample was homogenized for an additional 3–5 sec. The pH of the EDTA solution was 7.4. The homogenate was centrifuged at 12,000 × *g* for 20 min, and the supernatant fraction was assayed for phosphorylase activity by the radiometric method of Gilboe *et al.* (19). The specific activity of [^{14}C]glucose 1-phosphate was approximately 0.25 μCi/μmol. None of the treatments used in these experiments significantly affected total enzyme activity (100 ± 9 nmol of glucose 1-phosphate/min/g wet weight; $n = 20$) and the data are expressed as the activity ratio (−5′-AMP/+5′-AMP). An increase in the activity ratio indicates an increase in the percentage of phosphorylase in the *a* form.

Myosin phosphorylation. Tissue (approximately 15 mg) was transferred to a Duall size 20 tube and homogenized at room temperature in 400 μl of homogenizing solution consisting of 8 M urea, 50 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 5 mM EDTA, 15 mM β-mercaptoethanol, 2% Nonidet P-40, 0.5% sodium dodecyl sulfate and 40 mM sodium pyrophosphate. The homogenate was centrifuged for 10 min in a Brinkmann Microfuge, and 200 μl of the supernatant fraction were transferred to a 10 × 75 mm tube to which were added ampholytes (1.6% LKB, pH 4–6, 0.4% Pharmacia, pH 3–10). After mixing, the sample was applied to a 4% acrylamide tube gel (5 mm in diameter and 11.5 cm in length) containing 8 M urea and 2% ampholytes. All samples were electrofocused for 16–18 hr at 400 V followed by 1 hr at 500 V using 10 mM ethylenediamine in the cathode chamber and 10 mM iminodiacetic acid in the anode chamber. After electrofocusing, the gels were removed and that section containing the MLC was cut out and placed on top of a 12% sodium dodecyl sulfate vertical slab gel (15 cm) containing a 3% stacking gel (1 cm). After overlaying the isoelectric focusing gel with 1.0% agarose, electrophoresis in the second dimension was performed at 40 mA/gel according to the method of O'Farrell (20). Gels were removed and fixed for 1 hr in 50% methanol and 10% acetic acid and stained with 0.25% Coomassie Blue for 0.5 hr. Gels were destained, and the proteins, identified as nonphosphorylated MLC and MLC-P by their isoelectric points and molecular weights, were then scanned using a Gelman ACD automatic computing densitometer. The amount of MLC-P was expressed as a percentage of the total phospho- and dephospho-MLC and assigned a mol P/mol MLC based on the stoichiometry of MLC phosphorylation (1 mol P/mol MLC).

Cyclic AMP-dependent protein kinase assay. The cAMP-dependent protein kinase activity ratio was determined by a modification of the method of Corbin *et al.* (21). Frozen tissue was homogenized in 0.5 ml of 10 mM potassium phosphate, pH 6.8, containing 5 mM EDTA, 0.1 mM isobutylmethylxanthine and 0.4 M NaCl. The homogenate was centrifuged for 5 min at 8000 × *g*, and 20 μl of the supernatant fraction was assayed for cAMP-dependent protein kinase activity. Results are expressed as the activity ratio (−cAMP/+cAMP).

Other methods. Cyclic GMP was determined by radioimmunoassay (22) and protein by the method of Lowry *et al.* (23) using bovine serum albumin as a standard. Statistical analyses were made using the Newman-Keuls test for multiple comparisons or the two-tailed Student's *t* test.

RESULTS

Activation of phosphorylase a formation, MLC phosphorylation, and contraction. Fig. 1 demonstrates the effect of a maximally effective concentration of 1 μM NE on the formation of phosphorylase *a*, MLC phosphorylation, and contraction in the rat aorta. Tension was maintained for up to 60 min in these experiments (panel

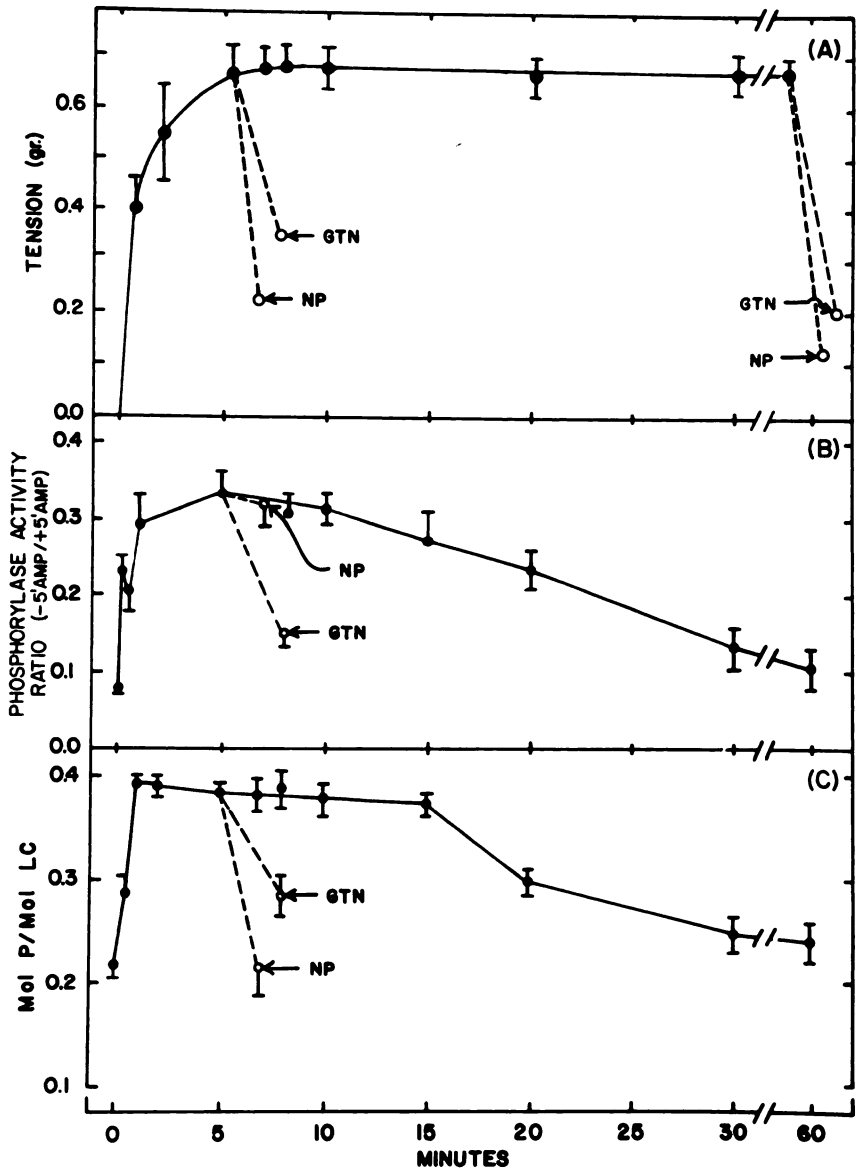

FIG. 1. *Time course of activation of contraction (panel A), phosphorylase a formation (panel B), and MLC phosphorylation (panel C) after the addition of 1 μM NE*

Rat aortic strips were mounted in muscle baths and equilibrated for 90 min in KRB at 37° as described in Materials and Methods. Strips were frozen in dichlorodifluoromethane cooled to the temperature of liquid N_2 as described in method A in the text. Phosphorylase *a* formation and MLC phosphorylation were determined as described. The open circles and dashed lines indicate the effects of NP (1 μM) and GTN (1 μM) on tension, phosphorylase *a* formation, and MLC phosphorylation.

A) while phosphorylase *a* (panel B) and phosphorylated MLC (panel C) declined to basal levels within 30 min. Tension, phosphorylase *a* formation, and MLC phosphorylation in untreated tissues did not vary during the 60-min incubation (data not shown). MLC phosphorylation reached a maximum of 0.4 mol P/mol LC after the addition of NE. Experiments were performed to test whether dephosphorylation of MLC occurred during homogenization. First, ^{32}P-labeled MLC was added to the homogenate, and the degree of dephosphorylation was examined with time. Second, rat aortas were incubated with ^{32}P for 120 min to label ATP pools. NE was added for 5 min, and homogenates were prepared and allowed to stand for various periods of time before electrophoresis. No significant degree of dephosphorylation of MLC was observed with either procedure (data not shown). Thus, the stoichiometry of phosphorylation observed in this tissue appeared to represent true levels of MLC phosphorylation during contraction. The open circles and dashed lines demonstrate the effects of 1 μM NP and 1 μM GTN on tension, phosphorylase *a* formation, and MLC phosphorylation. Both NP and GTN produced a rapid decline in MLC phosphorylation and tension when added 5 min after contraction compared with nontreated contracted strips. In addition, GTN produced a rapid fall in phosphorylase *a* formation while NP had little or no effect on phosphorylase *a* formation when added at 5 min. When GTN and NP were added to contracted strips at 60 min, a rapid fall in tension was observed within 2 min after the addition of either drug, although no effect

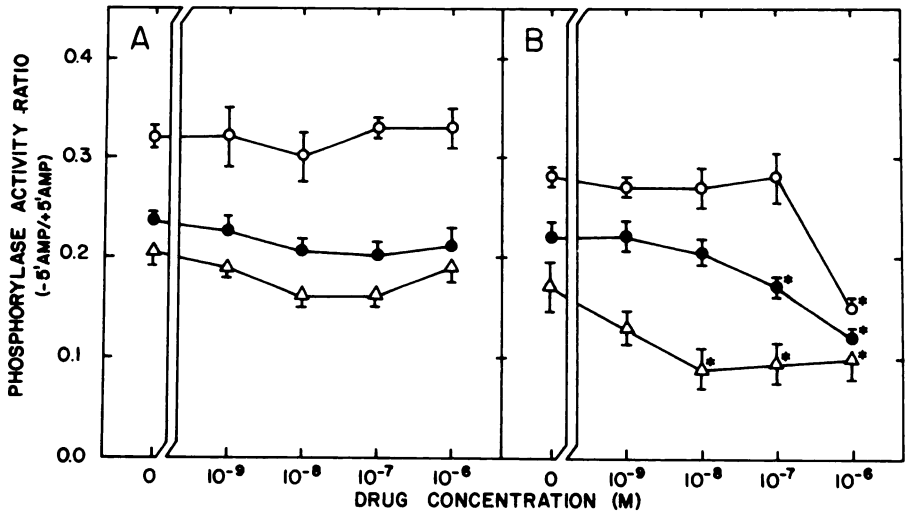

FIG. 2. *Concentration-dependent effects of NP (panel A) and GTN (panel B) on NE-stimulated phosphorylase a formation*

Rat aortic strips were equilibrated in glass vials for 90 min in KRB at 37° and then stimulated with 1.0 (○), 0.1 (●), or 0.01 (△) μM NE. After 5 min, strips were treated with various concentrations of NP or GTN for 2 or 3 min, respectively. At this point, the tissues were frozen and phosphorylase *a* formation was determined. Each point represents the mean ± standard error for 4–8 samples. The data were analyzed using the Newman-Keuls test for multiple comparisons. Asterisks denote statistical significance from appropriate NE-stimulated control at $p < 0.05$.

on MLC phosphorylation was observed, since the levels of MLC-P had returned to basal values. Neither GTN nor NP was able to lower MLC phosphorylation below that which was seen at 60 min (data not shown), confirming the findings of transient MLC phosphorylation seen in other tissues (14, 24). The increase in MLC phosphorylation and phosphorylase *a* by NE in rat aorta was blocked by phentolamine, but not propranolol, consistent with the premise (25) that α-adrenergic receptor activation mediated these responses (data not shown).

Effects of NP and GTN on phosphorylase a formation. Fig. 2 shows the effects of NP (panel A) and GTN (panel B) on phosphorylase *a* formation stimulated by NE. In most experiments, a small but statistically insignificant inhibition of phosphorylase *a* formation produced by 0.01 μM NE was observed with NP (Fig. 2A). In contrast, GTN caused significant inhibition of phosphorylase *a* formation at all three concentrations of NE (Fig. 2B). However, higher concentrations of GTN were required to inhibit phosphorylase *a* formation when higher concentrations of NE were used to stimulate phosphorylase *a* formation. Similar results were observed (data not shown) when the same experiments were performed on aortic strips maintained under tension in a muscle bath. We have performed a number of experiments comparing the effects of agents (e.g., NE, NP, GTN, and 8-Br-cGMP) on both phosphorylase *a* formation and MLC phosphorylation in free-floating tissues and tissues under tension. Differences in the responses of the tissues in either situation have not been observed. Likewise, Aksoy *et al.* (24) found no differences in MLC phosphorylation in free-floating versus suspended carotid artery pieces. Because of the simplicity and the greater number of experiments that could be performed with tissues in glass vials as opposed to those in muscle baths, we chose to incubate tissues in glass vials for the remaining studies. Only when contractile data were desired did we determine phosphorylase *a* formation and MLC phosphorylation in tissues under tension.

Since both NP and GTN are potent smooth muscle relaxants, particularly when rat aorta is stimulated to contract with submaximal concentrations of NE (8, 26), the lack of an inhibitory effect of NP on phosphorylase *a* formation stimulated by NE was investigated further. As shown in Table 1, both NP and GTN increased cGMP levels in a concentration-dependent fashion. NP, however, was the more effective of the two drugs in raising cGMP levels in rat aorta. Cyclic AMP levels were unaffected by either agent (data not shown). Nevertheless, at 1 μM NP, a significant activation of the cAMP-dependent protein kinase was observed, suggesting that the large increase in cGMP (approximately 1 μM) produced by

TABLE 1

Effects of NP and GTN on cGMP levels and cAMP-dependent protein kinase activity ratios in rat aorta

Aortas were incubated as described in Materials and Methods. All strips were stimulated with 0.01 μM NE for 5 min and then treated with various concentrations of NP for 2 min or GTN for 3 min. Control strips were stimulated with NE alone and frozen after 7 min. No significant differences in cGMP levels or cAMP-dependent protein kinase activity ratios were observed between strips stimulated with NE for 7 or 8 min. The data are presented as the mean ± standard error. The data were analyzed using Student's *t* test. * Denotes statistical significance from untreated muscle at $p < 0.01$; ** $p < 0.005$.

Drug	Concentration	cGMP levels	cAMP-dependent protein kinase activity ratio (−cAMP/+cAMP)
	μM	*pmol/mg protein*	
None		0.75 ± 0.08	0.386 ± 0.024
NP	0.01	0.91 ± 0.11	0.413 ± 0.023
	0.10	5.44 ± 1.52*	0.421 ± 0.035
	1.00	47.55 ± 15.90*	0.513 ± 0.033**
GTN	0.01	0.71 ± 0.07	0.380 ± 0.018
	0.10	2.10 ± 0.34*	0.415 ± 0.039
	1.00	5.82 ± 0.76*	0.451 ± 0.038

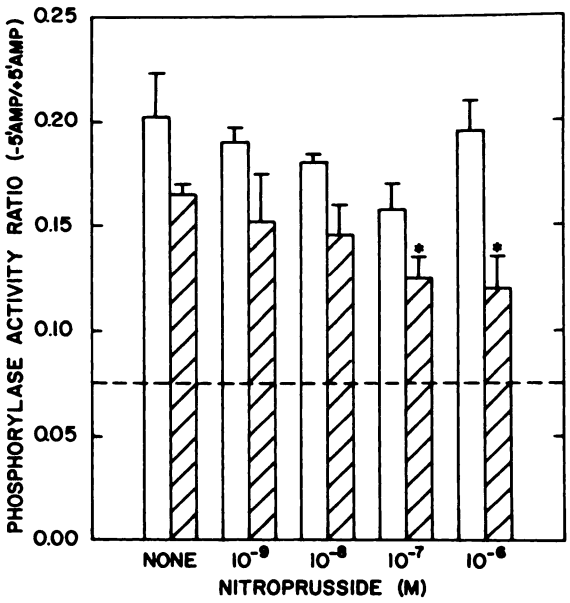

FIG. 3. *Effects of NP pretreatment on NE-stimulated phosphorylase a formation*

Aortic strips were treated with NP for 3 min and then stimulated with 0.1 μM NE (□) or 0.01 μM NE (▨) for 5 min and frozen. Phosphorylase *a* formation was then determined. The dashed line demonstrates the phosphorylase activity ratio with no treatment. Each point represents the mean ± standard error for 4–8 samples. The data were analyzed using the Newman-Keuls test for multiple comparisons. Asterisks denote statistical significance from 0.01 μM NE-treated control at $p < 0.05$.

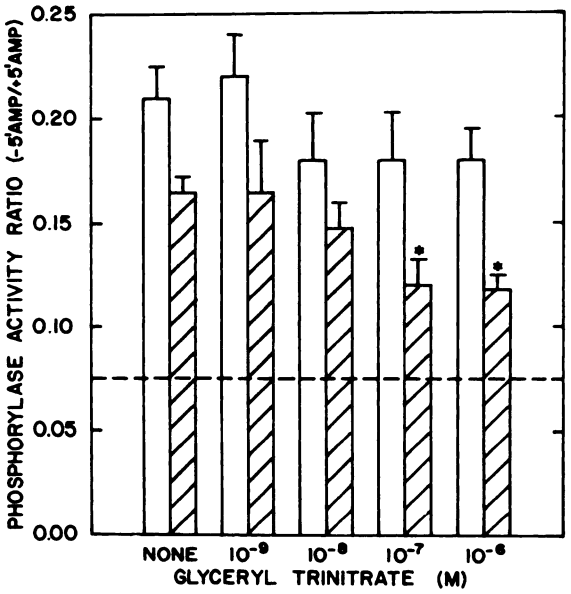

FIG. 4. *Effects of GTN pretreatment on NE-stimulated phosphorylase a formation*

Aortic strips were treated with GTN for 3 min and then were stimulated with 0.1 (□) or 0.01 μM NE (▨) for 5 min and frozen. Phosphorylase *a* formation was then determined. The dashed line demonstrates the phosphorylase activity ratio with no treatment. Each point represents the mean ± standard error for 4–8 samples. The data were analyzed using the Newman-Keuls test for multiple comparisons. Asterisks denote statistical significance from 0.01 μM NE-treated control at $p < 0.05$.

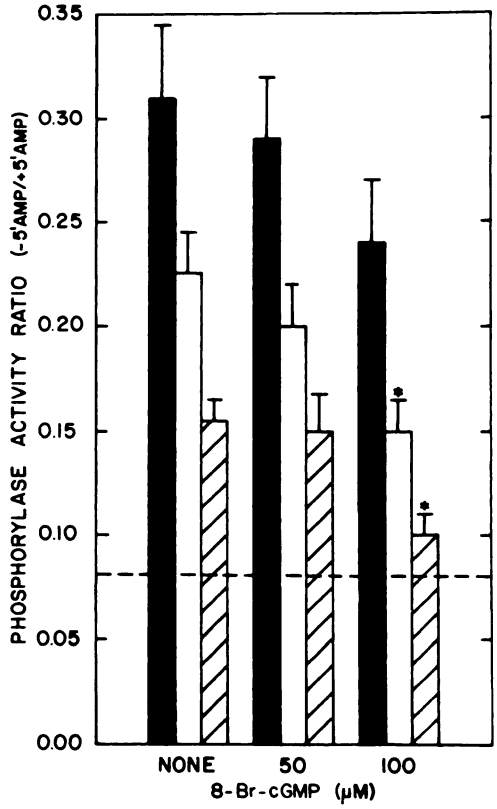

FIG. 5. *Effects of 8-Br-cGMP pretreatment on NE-stimulated phosphorylase a formation*

Aortic strips were treated with 8-Br-cGMP for 15 min and then were stimulated with 1.0 (■), 0.1 (□) or 0.01 μM NE (▨) for 5 min and frozen. Phosphorylase *a* formation was then determined. The dashed line demonstrates the phosphorylase activity ratio with no treatment. Each point represents the mean ± standard error for 4–8 samples. The data were analyzed using the Newman-Keuls test for multiple comparisons. Asterisks denote statistical significance from appropriate NE-treated control group at $p < 0.05$.

this agent could be sufficient to activate cAMP-dependent protein kinase in intact cells. It is known that this concentration of cGMP activates cAMP-dependent protein kinase *in vitro* (27). Thus, one possible explanation for the lack of inhibitory effects of NP at 1 μM (Fig. 2A) could be the activation of aortic phosphorylase *b* kinase catalyzed by the cAMP-dependent protein kinase. On the other hand, the lack of inhibition of phosphorylase *a* formation at 0.1 μM NP (Fig. 2A) was apparently due to another action of this drug, since 1.0 μM GTN, which produced a similar increase in cGMP as 0.1 μM NP, prevented phosphorylase *a* formation by NE (Table 1).

Effects of NP, GTN, and 8-Br-cGMP pretreatment on phosphorylase a formation. Because phosphorylase *a* formation began to decline after 10 min with NE (Fig. 1), it was difficult to interpret experiments in which agents such as 8-Br-cGMP must be incubated with strips for several minutes before effects can be observed. Thus, strips were incubated with NP or GTN for 3 min and 8-Br-cGMP for 15 min before treatment with NE. These results are shown in Figs. 3, 4, and 5. NP inhibited phosphorylase *a* formation produced by 0.01 μM but not by 0.1 μM NE (Fig. 3). The inhibition produced by 0.1 μM NP after stimulation with 0.01 μM NE approached

50% of the NE-stimulated activity. This was in contrast to the results shown in Fig. 2 where NP was added to tissues for 2 min after the stimulation of phosphorylase *a* formation by NE. This suggests that a short-lived metabolite of NP may counteract the effects of NP within 2 min, but not after 8 min of NP treatment. Likewise, GTN was effective in reducing phosphorylase *a* formation caused by NE (Fig. 4). Unlike the experiments described for Fig. 2, NP and GTN were present for 8 min before the tissues were frozen. It was possible that cGMP levels in strips pretreated with GTN for 3 min followed by NE for 5 min were different from the levels in strips treated for 3 min with GTN after 5 min with NE, thus accounting for the greater inhibition of phosphorylase *a* formation by GTN in Fig. 2 compared to Fig. 4. Thus, we measured cGMP content under the conditions described for both experiments and determined that cGMP levels in the preincubation experiment (Fig. 4) were only 64% of those in the experiments described in Fig. 2. This is consistent with the greater efficacy of GTN in Fig. 2 compared to Fig. 4. The 8-bromo analog of cGMP also inhibited phosphorylase *a* formation, especially at 0.01 μM NE (Fig. 5). 5′-GMP and 8-Br-5′-GMP had no effect on NE-stimulated phosphorylase *a* formation, indicating specificity for the 3′,5′-cyclic form of GMP (data not shown). These results suggest that agents which raise cGMP as well as the analog of cGMP itself were capable of inhibiting NE-induced phosphorylase *a* formation at submaximal concentrations of NE.

Effects of NP, GTN, and 8-Br-cGMP treatment on MLC phosphorylation. As was the case for phosphorylase *a* formation, NE-stimulated phosphorylation of MLC was transient (Fig. 1). Thus, strips were incubated with NP or GTN for 3 min and 8-Br-cGMP for 15 min before treatment with 1 μM NE. As shown in Fig. 6, all three relaxing agents inhibited MLC phosphorylation in a concentration-dependent fashion. Unlike the case with phosphorylase, NP was more potent than GTN in reducing MLC phosphorylation.

Effects of Ca^{2+} on 8-Br-cGMP inhibition of phosphorylase a formation. The activation of aortic phosphorylase is a balance between the Ca^{2+}-stimulated activation of phosphorylase *b* kinase and the activity of phosphorylase phosphatase. The data presented thus far suggest an effect of NP, GTN, and 8-Br-cGMP on either the inhibition of the Ca^{2+}-activated protein kinases (i.e., MLC kinase and phosphorylase kinase) or enhanced dephosphorylation of the substrates (e.g., MLC and phosphorylase *a*). In order to investigate the effects of cGMP on the inhibition of Ca^{2+}-activated phosphorylase *a* formation, aortic strips were depleted of Ca^{2+} by incubation in Ca^{2+}-free solution containing 1 mM EGTA with or without a submaximal concentration of 8-Br-cGMP (50 μM). The Ca^{2+}-free KRB containing EGTA was then replaced with a KRB buffer containing 117 mM KCl plus various concentrations of Ca^{2+} for 5 min. As shown in Fig. 7, a greater inhibition of KCl-induced phosphorylase *a* formation by 8-Br-cGMP was observed at the lower concentrations of extracellular Ca^{2+} than at the higher concentrations (77% inhibition at 0.375 mM, 52% at 0.75 mM, 45% at 1.5 mM, and 26% at 3.0 mM).

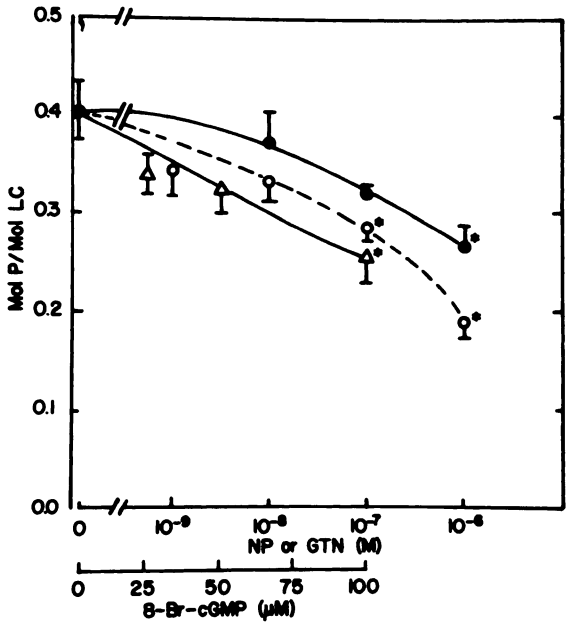

FIG. 6. *Effects of NP, GTN, and 8-Br-cGMP on MLC phosphorylation*

Aortic strips were incubated at 37° for 90 min in KRB and treated with NP (○) for 3 min, GTN (●) for 3 min, or 8-Br-cGMP (△) for 15 min before the addition of 1 μM NE. After 5 min, the tissues were frozen and MLC phosphorylation was determined. Each point represents the mean ± standard error for 4–8 samples. The data were analyzed using the Newman-Keuls test for multiple comparisons. Asterisks denote statistical significance from NE-treated control at $p < 0.05$.

Effects of NP, GTN, and 8-Br-cGMP on forskolin-stimulated phosphorylase a formation. One possible mechanism of action of NP, GTN, and 8-Br-cGMP to decrease phosphorylase *a* formation is to stimulate phosphorylase *a* to *b* conversion, presumably by the stimulation of a phosphorylase phosphatase. To investigate this possibility, strips were treated with FOR to stimulate phosphorylase *a* formation. We have previously shown that FOR increases cAMP levels and cAMP-dependent protein kinase activity ratios and relaxes the contracted rat aortic strip (26). In this study, we observed that FOR produced a rapid increase in phosphorylase *a* levels which remained elevated for 15 min (data not shown). Because FOR is a potent relaxing agent in rat aorta, the cAMP-dependent activation of phosphorylase kinase in smooth muscle presumably occurred without the elevation of intracellular Ca^{2+} levels (26). As shown in Fig. 8, FOR at 1.0 and 10 μM increased phosphorylase *a* formation by approximately 2- and 3-fold, respectively. Neither NP (0.1 μM for 2 min), GTN (1 μM for 3 min), nor 8-Br-cGMP (100 μM for 10 min) inhibited the FOR-stimulated formation of phosphorylase *a*. One μM FOR was chosen because this concentration produced an increase in phosphorylase *a* levels approximately equal to that of 0.01 μM NE. These results suggest that the decrease in NE-induced phosphorylase *a* levels caused by the nitro compounds and 8-Br-cGMP was not a result of enhanced dephosphorylation of phosphorylase *a*, and occurred pri-

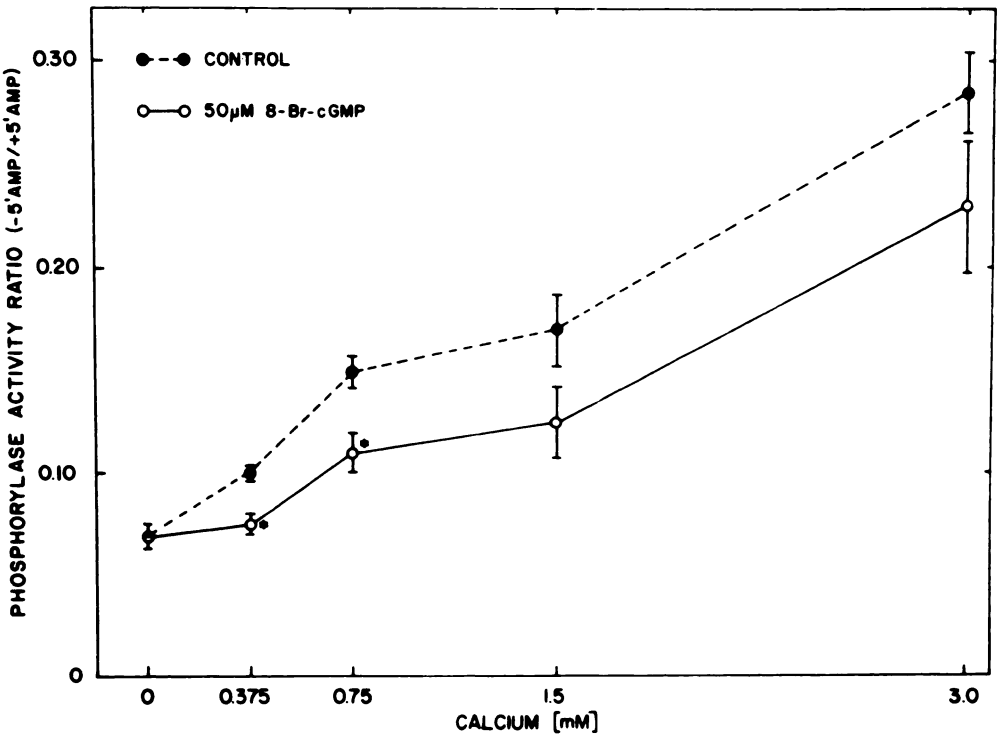

FIG. 7. *Effects of 8-Br-cGMP on KCl-stimulated phosphorylase a formation*

Rat aortic strips were incubated for 90 min in KRB at 37° and then for 20 min in Ca^{2+}-free KRB. During the last 5 min of the final incubation, 1 mM EGTA was added to chelate remaining extracellular Ca^{2+}. The Ca^{2+}-free KRB was then removed and phosphorylase *a* formation was stimulated with 117 mM K^+-KRB plus various concentration of extracellular Ca^{2+}. Tissues treated with 50 μM 8-Br-cGMP were incubated with the nucleotide 15 min prior to stimulation with high K^+-KRB. After treatment for 5 min with K^+-KRB, the tissues were frozen and phosphorylase *a* formation was determined. Each point represents the mean ± standard error for 4–8 samples. The data were analyzed using Student's *t* test. Asterisks denote statistical significance from appropriate paired control-treated tissue at $p < 0.05$.

marily because of decreased phosphorylase kinase activity due to diminished concentrations of intracellular Ca^{2+}.

Effects of methylene blue on phosphorylase a formation and MLC phosphorylation. It has been shown that MB inhibits nitro vasodilator-induced increases in cGMP (4). Table 2 shows the effects of MB on phosphorylase *a* formation and MLC phosphorylation in strips treated with NP or GTN. GTN inhibited phosphorylase *a* formation and MLC phosphorylation in NE-treated strips. This effect of GTN was largely reversed by 10 μM MB. Furthermore, the NP-induced inhibition of MLC phosphorylation was prevented by MB. The reversal by MB of the effects of GTN and NP on phosphorylase *a* formation and MLC phosphorylation was accompanied by a decrease in cGMP levels. MB had no effect on the capacity of 8-Br-cGMP to inhibit phosphorylase formation and MLC phosphorylation (data not shown). Although it was possible that MB produced nonspecific effects which were unrelated to the prevention of cGMP elevations, the results are consistent with the premise that cGMP is involved in the inhibition of MLC phosphorylation by NP and GTN and the inhibition of phosphorylase *a* formation by GTN.

DISCUSSION

Several investigators have used phosphorylase *a* formation as an indicator of intracellular Ca^{2+} levels in various tissues after hormone and drug treatments (11–14, 25). Rat aortic phosphorylase *a* formation also appears sensitive to the intracellular levels of Ca^{2+} since (a) the α-adrenergic and Ca^{2+}-linked agent NE stimulates phosphorylase *a* formation coincident with contraction and myosin phosphorylation, and (b) the omission of Ca^{2+} from the physiological saline solution prevents the stimulation of phosphorylase *a* formation by KCl. Similar results were observed by Namm (25) in the rabbit aorta. Whether vascular smooth muscle phosphorylase *b* kinase is identical or even similar to that of skeletal, cardiac, and hepatic tissue has not been established. Nevertheless, the factors regulating phosphorylase *a* formation, namely Ca^{2+} and cAMP (as demonstrated by the experiments with FOR), suggest that vascular smooth muscle phosphorylase *b* kinase is regulated in a similar fashion as that in other tissues.

The results presented in this study suggest that inorganic and organic nitrates and the 8-bromo analog of cGMP can inhibit the activation of Ca^{2+}-sensitive enzymes in vascular smooth muscle. Several experimental findings suggest that this is due to the reduction of cytoplasmic Ca^{2+} levels. First, NP and GTN inhibit tension after 5 and 60 min of treatment with NE even though MLC phosphorylation at 60 min is not significantly different from that in tissues not treated with NE (Fig. 1). If these compounds decrease the activity of MLC kinase through phosphorylation as has been proposed

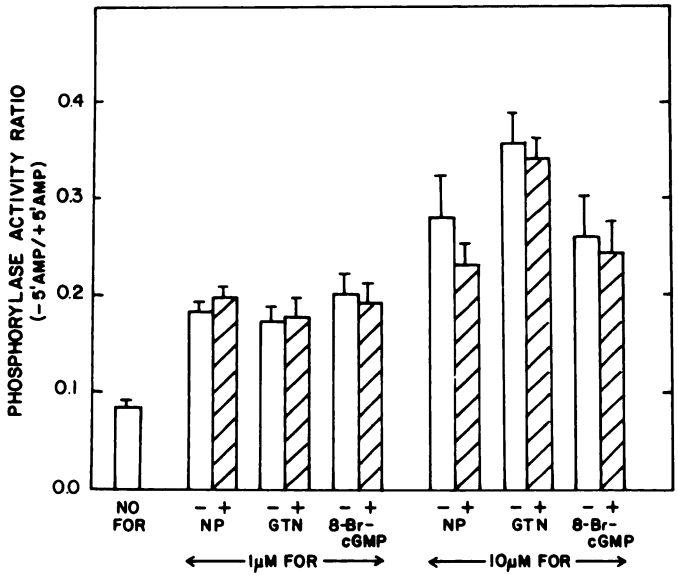

FIG. 8. *Effects of NP, GTN, and 8-Br-cGMP on phosphorylase a formation in forskolin-treated strips*

Rat aortic strips were incubated in KRB at 37° for 90 min and then stimulated with either 1.0 or 10 μM forskolin. After 5 min, 0.1 μM NP, 1 μM GTN, and 100 μM 8-Br-cGMP were added for 2, 3, or 10 min, respectively. The muscles were then frozen, and phosphorylase *a* formation was determined. Muscles not receiving NP, GTN, or 8-Br-cGMP were forskolin-stimulated for 7, 8, and 15 min, respectively. The data represent the mean ± standard error for 4–7 separate experiments.

for the cAMP-dependent protein kinase by Adelstein *et al.* (28), then one would expect relaxation to occur only when MLC is phosphorylated (i.e., 5 min). Because tension at 60 min is still dependent on the presence of Ca^{2+} (8), the common mechanism in the reduction of tension at 5 and 60 min would seem to be the reduction in the levels of cytoplasmic Ca^{2+} and not dephosphorylation of MLC. Second, the inhibition of phosphorylase *a* formation is greater at the lower concentrations of NE than at higher concentrations. This is especially apparent with GTN (Fig. 2). Studies in other laboratories (29) have shown that greater amounts of Ca^{2+} accumulate in the cell with higher concentrations of NE (i.e., 1 to 10 μM) than with lower concentrations (i.e., 0.01 μM). Therefore, the results described in Fig. 2 for GTN inhibition of phosphorylase *a* formation could be due to differences in levels of intracellular Ca^{2+}. The involvement of cGMP in the action of GTN is suggested by the findings that the inhibition of phosphorylase *a* formation by GTN is prevented by MB, a compound known to inhibit cGMP formation (Ref. #4 and Table 2). Third, 8-Br-cGMP prevents the KCl-stimulated formation of phosphorylase *a* more effectively at reduced concentrations of extracellular Ca^{2+} than at higher ones (Fig. 7). We found a similar situation with contraction, where it was observed that 8-Br-cGMP prevented KCl-induced tension development more effectively at lower concentrations of extracellular Ca^{2+} than at higher ones (8).

An alternate explanation of the data is that NP, GTN, and 8-Br-cGMP stimulate dephosphorylation of substrates such as phosphorylase *a*. Previously, we had shown that FOR stimulates cAMP production, activates cAMP-dependent protein kinase, and relaxes aorta without affecting cGMP levels (26). In addition, our data suggested that FOR reduces, rather than elevates, intracellular Ca^{2+} levels in rat aorta (26). In various muscle preparations, other cAMP-elevating agents have been shown to stimulate phosphorylase *b* kinase through phosphorylation (14–17), and it seems probable, therefore, that FOR is acting in an analogous manner in vascular smooth muscle. Thus, if NP, GTN, or 8-Br-cGMP were stimulating dephosphorylation of phosphorylase *a*, it would be expected that FOR-stimulated phosphorylase *a* formation would be inhibited by these compounds. Since phosphorylase *a* formation stimulated by FOR was not inhibited by these agents (Fig. 8), it seems unlikely that NP, GTN, and 8-Br-cGMP activate dephosphorylation of phosphorylase *a* by an effect on a phosphatase.

Although the data presented in this paper provide substantial evidence for a cGMP-mediated inhibition of MLC phosphorylation and phosphorylase *a* formation due to an effect on Ca^{2+} levels in aortic cells, there is some discrepancy between these data and those published earlier on the effects on NP and cGMP on contraction. In particular, NP was found to be a potent relaxing agent at all concentrations of NE used to contract rat aortic strips, even though those strips contracted with submaximal concentrations of NE were

TABLE 2

Effects of methylene blue on phosphorylase a formation and MLC phosphorylation

Aortic strips were equilibrated for 90 min in KRB at 37°. Before the addition of NE, strips were incubated in the absence or presence of MB (10 μM) for 10 min. Phosphorylase *a* formation was determined after the addition of 0.1 μM NE while in separate experiments MLC phosphorylation was determined after the addition of 1.0 μM NE. The effects of NP (0.1 μM) or GTN (1.0 μM) on phosphorylase *a* formation and MLC phosphorylation were determined after a 3-min incubation with the agents. The data are presented as the mean ± standard error for 3–9 experiments. ND, not determined. * Denotes statistical significance between strips not treated with MB and MB-treated strips using Student's *t* test at $p < 0.05$.

Treatment	Phosphorylase (−5′AMP/+5′ AMP) methylene blue		MLC phosphorylation methylene blue		cGMP methylene blue	
	−	+	−	+	−	+
			mol P/mol LC		*pmol/mg protein*	
None	0.11 ± 0.01	ND	0.23 ± 0.02	0.25 ± 0.02	0.84 ± 0.11	1.13 ± 0.02
NE	0.23 ± 0.02	0.25 ± 0.02	0.39 ± 0.01	0.41 ± 0.02	0.66 ± 0.09	ND
NE + GTN	0.11 ± 0.01	0.21 ± 0.02*	0.28 ± 0.02	0.39 ± 0.02*	8.29 ± 1.14	1.68 ± 0.35*
NE + NP	0.19 ± 0.01	0.18 ± 0.01	0.23 ± 0.02	0.42 ± 0.02*	7.74 ± 1.46	2.81 ± 0.46*

more sensitive to NP (8). Although MLC phosphorylation induced by maximal NE concentrations was inhibited by NP (Fig. 7), phosphorylase *a* formation was not significantly affected in all cases. One explanation for these findings is that, at the highest concentration of NP tested, enough cAMP-dependent protein kinase is activated by cGMP (Table 1) to catalyze the conversion of phosphorylase *b* kinase to the activated form. This would make phosphorylase *b* kinase more sensitive to Ca^{2+} and thus offset the Ca^{2+}-lowering action of cGMP. This idea is supported by our observations that 1 μM NP alone stimulates phosphorylase *a* formation (−5′-AMP/+5′-AMP = 0.145 ± 0.01). Nevertheless, this does not explain why NP and GTN, which produce similar increases in cGMP levels at 0.1 and 1.0 μM, respectively (see Table 1), have differing actions on phosphorylase *a* formation (Fig. 2). It is conceivable that NP or some short-lived intracellular metabolite of NP counteracts the action of cGMP on phosphorylase *a* formation. The presence of a short-lived metabolite which counteracts the effect of cGMP on phosphorylase could explain the data shown in Figs. 2 and 3 where NP, after a 2-min exposure to the aorta, had little effect on phosphorylase *a* formation, while after 8 min NP was more effective in preventing phosphorylase *a* formation. In addition to this, the activation of phosphorylase kinase may occur at lower concentrations of intracellular Ca^{2+} than the activation of contraction (via myosin light chain kinase activation). Thus, in order to inhibit phosphorylase *b* kinase, Ca^{2+} levels would need to be lowered below those necessary to inhibit MLC phosphorylation. This is supported in part by the studies of Silver and Stull (30), which suggest that inhibition of contraction is more sensitive to changes in Ca^{2+} levels than inhibition of phosphorylase activation. And finally, it has been suggested that not all the effects of NP are mediated by cGMP. For example, NP stimulates cGMP production but does not markedly relax guinea pig ileum and is only slightly cross-tolerant with GTN in this tissue (31). The differences in the effects of NP on MLC phosphorylation and phosphorylase *a* formation tend to support the suggestion that NP produces a variety of actions not all of which are mediated by cGMP.

Recently aequorin has been used to measure Ca^{2+} levels (32), and it was shown that the intracellular Ca^{2+} concentration in aortic smooth muscle cells was much greater immediately following the stimulation of contraction by agonists than during the ensuing tonic tension phase. Ca^{2+} transients in carotid arteries are also proposed to exist based on the physiological studies of Aksoy *et al.* (24). The transient increases in myosin phosphorylation and phosphorylase *a* formation observed in Fig. 1 appear to support the existence of Ca^{2+} transients in contracted smooth muscle and indicate that the maintenance of tension is probably sensitive to relatively low concentrations of intracellular Ca^{2+}. The fact that GTN appears to be more effective in inhibiting phosphorylase *a* formation in a strip preincubated 5 min with NE (Fig. 2), then preventing activation of phosphorylase *a* formation by NE (Fig. 4), also indicates that the level of Ca^{2+} within the cell affects the capacity of these agents to inhibit phosphorylase *a* formation. Thus, more free Ca^{2+} must be removed from the cytoplasm to inhibit phosphorylase kinase in the experiments where GTN is incubated with tissues before stimulation than in tissues which are stimulated first with NE and then treated with GTN. Furthermore, because cGMP levels appear to decline shortly after stimulation with GTN (Refs. 5 and 31 and our data), the combination of higher Ca^{2+} levels and declining cGMP levels within the first 5 min with NE probably results in less Ca^{2+} being removed from the cytoplasm.

The mechanism by which elevation of cGMP leads to a reduction in the free intracellular levels of Ca^{2+} is not known. It appears to be more complex than the simple inhibition of Ca^{2+} influx (8). In addition, it is not clear whether there are different pools of Ca^{2+} which can regulate different cellular processes such as phosphorylase *a* formation and myosin light chain phosphorylation. A differential effect of cGMP on one or more pools of Ca^{2+} might also explain the effects of cGMP on the various Ca^{2+}-regulated proceses. Further investigations are needed to determine the effects of 8-Br-cGMP, NP, and GTN on other Ca^{2+}-sensitive processes, and to measure directly the effects of these agents on Ca^{2+} levels in the intact cells.

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