Effect of NO donors on protein phosphorylation in intact vascular and nonvascular smooth muscles

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Hennan, James K., and Jack Diamond. Effect of NO donors on protein phosphorylation in intact vascular and nonvascular smooth muscles. Am J Physiol Heart Circ Physiol 280: H1565–H1580, 2001.—It is generally well accepted that nitrovasodilator-induced relaxation of vascular smooth muscle involves elevation of cGMP and activation of a specific cGMP-dependent protein kinase [protein kinase G (PKG)]. However, the protein targets of PKG and the underlying mechanisms by which this kinase leads to a relaxant response have not been elucidated. Several types of smooth muscle, including rat myometrium and vas deferens, are not relaxed by sodium nitroprusside, even at concentrations that produce marked elevation of cGMP and activation of PKG. The main objective of our studies was to compare PKG-mediated protein phosphorylation in intact rat aorta, rat myometrium, and rat vas deferens using two-dimensional gel electrophoresis. In intact rat aorta, seven PKG substrates were detected during relaxation of the tissue. None of the PKG substrates identified in the rat aorta appeared to be phosphorylated in the myometrium or vas deferens after administration of various cGMP-elevating agents. Thus the failure of the rat myometrium and rat vas deferens to relax in the face of cGMP elevation and PKG activation may be due to a lack of PKG substrate phosphorylation.

Numerous agents are capable of activating guanylyl cyclases (GC) and increasing tissue levels of cGMP in smooth muscle. These include nitrovasodilators such as sodium nitroprusside (SNP) and S-nitroso-N-acetylenicillamine (SNAP), particulate GC (pGC) activators such as atrial natriuretic peptide (ANP), and endogenous nitric oxide (NO) generated within the endothelium. Elevation of cGMP may affect cell function through a variety of mediators, including cGMP-gated ion channels, cGMP-regulated phosphodiesterases, and cGMP-dependent protein kinases [protein kinase G (PKG)]. There is strong evidence suggesting that relaxation of vascular smooth muscle by cGMP-elevating agents is mediated via activation of PKG. This protein kinase presumably regulates the activity of specific proteins involved in the relaxation process by catalyzing the transfer of the γ-phosphoryl group of ATP to the hydroxyl group of serine and threonine residues on target substrates. Several lines of evidence support this hypothesis. However, the exact identities of those PKG substrates involved in intact vascular smooth muscle relaxation and the mechanism(s) by which intracellular calcium levels are reduced remain unclear.

The early literature describing PKG-mediated phosphorylation is composed mainly of in vitro studies using broken cell preparations. Although these studies provide useful information regarding possible PKG substrates, it is not always possible to extrapolate in vitro results to an intact tissue setting. However, only a few reports have appeared in the literature investigating PKG-mediated phosphorylation in intact tissues. In 1982, using intact rabbit aorta, Rapoport et al. (37) were the first to demonstrate that SNP induced a concentration-dependent increase in incorporation of 32P into nine proteins and decrease in incorporation into two proteins. These patterns of phosphorylation were mimicked by 8-bromo-cGMP (8-BrcGMP), indicating that PKG may be involved. However, none of the proteins described in this study were identified. Since this original study, considerable research regarding the role of PKG-mediated phosphorylation in intact smooth muscle relaxation has focused on the effects of PKG on myosin light chain phosphorylation. Several pathways have been described whereby PKG can mediate a decrease in the phosphorylation of myosin light chains: inhibition of the calcium-dependent activation of myosin light chain kinase (30), stimulation of myosin phosphatase activity (16, 19, 21), and intermediate filament phosphorylation (15). Despite the evidence indicating that activation of PKG can result in dephosphorylation of myosin light chains, it is unclear whether this effect is a direct result of PKG-mediated phosphorylation or simply a result of the decrease in intracellular calcium that occurs during relaxation. Because PKG-mediated phosphorylation is well correlated with decreases in intracellular calcium levels and PKG has been shown to phosphorylate substrates that could mediate calcium uptake, such as the inositol trisphosphate (IP3)
receptor and phospholamban (7, 17), it is possible that this protein kinase mediates relaxation via activation of calcium-sequestering mechanisms rather than direct effects on myosin light chains.

More recently, in bovine carotid artery smooth muscle, PKG was shown to phosphorylate two 20-kDa proteins that share sequence homology with a heat-shock-related protein (HSP-20) (1, 3). Although the exact function of heat-shock protein (HSP) phosphorylation is unknown, it has been suggested that these proteins are important regulatory components of the actin-based cytoskeleton that can interact with intermediate filaments and, in turn, regulate vascular smooth muscle contraction and relaxation (26, 28, 38).

Despite the many studies described above investigating PKG-mediated phosphorylation, the underlying mechanisms by which PKG leads to a relaxant response remain undefined. This is due in part to a difficulty in directly correlating PKG-mediated phosphorylation with smooth muscle relaxation.

In the present study, we provide quantitative estimation of SNP-induced, PKG-mediated phosphorylation in the intact smooth muscle of the rat aorta using high-resolution two-dimensional gel electrophoresis. Studies with the soluble GC (sGC) inhibitor 1H-[1,2,4] oxadiazolo[4,3-a]quinazoline-1-one (ODQ) and with other agents such as a pGC activator (ANP) and a cGMP analog (8-BrcGMP) indicate that the substrates identified in our experiments are phosphorylated in a cGMP-dependent manner. To determine which of these substrates may be playing a physiological role in the relaxant response of the rat aorta, we also investigated PKG-mediated phosphorylation in the rat myometrium and rat vas deferens, which are not relaxed by SNP, despite significant elevations in cGMP and activations of PKG (13, 32). The results of these studies suggest that the failure of the uterus and vas deferens to relax in the face of cGMP elevation and PKG activation may be due to a lack of phosphorylation of the PKG substrates identified in the rat aorta. Relaxation of the rat myometrium by the nitrosothiol donor SNAP is accompanied by phosphorylation of several other proteins, but this phosphorylation occurs independently of cGMP elevation and is presumably mediated by a kinase other than PKG.

**EXPERIMENTAL PROCEDURES**

**Materials.** Acrylamide, urea, glycine, Tris-base, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate, and SDS were purchased from Roche Diagnostics (Laval, PQ, Canada). Ampholyte (Bio-Lyte) 3-10, N,N,N′-methylene-bis acrylamide, bromphenol blue, N,N,N′,N′-tetramethylthylenediamine, and SDS-PAGE low-molecular-weight silver stain standards were purchased from Bio-Rad Laboratories (Mississauga, ON, Canada). [γ-32P]ATP, BIOTRAK cGMP scintillation proximity assay kit, and cellulose sheets were obtained from Amershams Pharmacia Biotech (Baie d’Urfe, PQ, Canada). Bio-Max MS X-ray film, transcreen high-energy intensifying screens, and 32P-labeled o-phosphoric acid were purchased from Mandel/DuPont-NEN (Boston, MA). Kodak Photoflow was purchased from a local photography supply store. BPDEtide and ANP were purchased from Bachem (Torrance, CA). KT-5823 and ODQ were obtained from Biomol Research Laboratories (Plymouth Meeting, PA). Resolyte 4-8 was obtained from BDH (Toronto, ON, Canada). 4-(2-Aminoethyl)benzenesulfonyl fluoride was purchased from Calbiochem (San Diego, CA). Silver SNAP stain was purchased from Pierce (Rockford, IL). All remaining reagents were purchased from Sigma (Oakville, ON, Canada).

**Preparation and tension measurements in rat aorta.** Descending thoracic aortae were excised from rats immediately after death in a CO2 inhalation chamber. Tissues were trimmed free of loosely adhering connective tissue and fat and cut into a helical strip to expose the endothelium. The endothelium was removed by gentle rubbing with a glass rod. Prepared muscle strips of ~10 × 4 mm were suspended in isolated organ baths at 37°C with a preload of 2 g in a physiological salt solution of the following composition (mM): 4.7 KCl, 140 NaCl, 10 HEPES, 1 MgCl2, 1.5 CaCl2, 0.2 NaH2PO4, and 10 glucose. Tissue baths were aerated with 95% O2-5% CO2, which maintained a pH of ~7.4. Isometric tension was recorded with force displacement transducers (model FT03C, Grass Instruments) connected to a polygraph recorder (model 7D, Grass), as described previously (31). At predetermined times after the addition of drug, tissues were frozen with liquid nitrogen-cooled clamps and stored at ~80°C until assessment of cGMP levels and PKG activity. Percent inhibition of contraction was calculated by measuring the change in amplitude of the phenylephrine (PE)-induced contraction after addition of the drug.

**cGMP estimation.** Frozen smooth muscles from the above contractility experiments (10–20 mg) were placed in liquid nitrogen-cooled Teflon capsules (1-ml capacity; Hansen Industries; Richmond, BC, Canada) with a chilled metal pestle and pulverized in a ProMix, Dentsply dental amalgam mixer (30 s at high speed). Next, 0.75 ml of ice-cold TCA (6%, wt/vol) was added to the capsule, and the tissue was homogenized for another 30 s at high speed. The homogenate was removed and centrifuged at 2,000 g for 15 min at 4°C. TCA was extracted from the supernatant with four washes of ice-cold water-saturated ether (5 ml/wash). cGMP levels were measured using a commercially available scintillation proximity RIA kit (acetylation protocol; Amershams-Pharmacia). The TCA-insoluble pellet was stored at ~80°C for protein estimation by methods previously described (23, 25). Tissue cGMP levels were calculated as picomoles of cGMP per milligram of protein.

**cGMP-dependent protein kinase assay.** Frozen aortic strips weighing ~40 mg were pulverized in Teflon capsules by a method similar to that described for cGMP extraction, except a homogenization buffer was substituted for TCA. The buffer contained 10 mM HEPES, 1 mM EDTA, 10 mM dithiothreitol, 1 mM IBMX, 125 mM KCl, 1 mM benzamidine, 10 μg/ml leupeptin, and 10 μg/ml pepstatin. The homogenate was centrifuged at 30,000 g for 5 min, and the supernatant was assayed for soluble PKG activity, as described elsewhere (15). Briefly, PKG activity was determined by measuring the transfer of the [γ-32P]phosphoryl group of ATP to BPDEtide (RKISASEDFRPLR), which is a relatively specific substrate for PKG. The assay was carried out in a total volume of 70 μl containing 150 μM BPDEtide, 10 mM HEPES, 35 mM β-glycerophosphate, 4 mM magnesium acetate, 200 μM [γ-32P]ATP (2.5 μCi/tube), 5 μM synthetic protein kinase A inhibitor, and 0.5 mM EGTA in the absence or presence of 5 μM cGMP. The reaction was initiated by addition of 20 μl of the sample supernatant. The reaction was allowed to proceed for 10 min at 4°C and was stopped by spotting 50 μl of the reaction mixture onto 2 × 2 cm squares of phosphocellulose paper (Whatman P81). The paper was then washed four times in

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0.5% o-phosphoric acid for 10 min each. The papers were dried and then transferred to scintillation vials containing 2.5 ml of liquid scintillant. Radioactivity was counted in a Beckman LS 6000TA liquid scintillation counter. PKG activity was expressed as picomoles of phosphate incorporated into substrate per minute per milligram of protein. PKG activity was assessed by calculating the activity ratio, which is a measure of the PKG activity in the absence of exogenously added cGMP (endogenous cGMP only) divided by the PKG activity in the presence of enough exogenous cGMP (5 μM) to maximally activate the kinase. Protein levels in the supernatant were estimated using a commercially available dye-binding assay previously described (4).

Two-dimensional gel electrophoresis: tissue preparation and radioactive labeling of proteins. Smooth muscle strips from rat aorta were prepared as described above. Uteri were removed from nonovariectomized, estrogen-primed prostegereinfluenced rats, and myometrial strips were prepared by peeling the endometrium and circular muscle away from the longitudinal smooth muscle. This hormonal treatment has been shown previously to provide tissues that exhibit strong, persistent spontaneous contractions that are nonresponsive to marked increases in tissue levels of cGMP (13). Rat vas deferens were carefully removed, trimmed free of loosely adhering connective tissue, and cut open longitudinally to expose the lumen and remove all traces of sperm. All prepared muscle strips were placed in a well-oxygenated, low-phosphate physiological salt solution of the following composition (mM): 4.7 KCl, 140 NaCl, 10 HEPES, 1 MgCl₂, 1.5 CaCl₂, 0.2 NaH₂PO₄, and 10 glucose. ³²P-labeled P was then added to the buffer at a concentration of 500 μCi/ml to label cellular ATP pools. Tissues were incubated with radio-labeled phosphate for 3 h at 37°C with gentle agitation. Once cellular ATP pools were labeled, smooth muscle strips were treated as described in RESULTS. After the addition of the drug, individual smooth muscles were blotted on tissue paper and frozen with liquid nitrogen-cooled clamps and stored at –80°C for future use.

Sample preparation. Frozen smooth muscle strips labeled with ³²P were homogenized as described above with a homogenization buffer containing 10 mM EDTA, 10 mM HEPES, 1 mM benzamidamine, 1 mM 4-(2-aminoethyl)benzenesulfonyl fluoride, 0.001 mM protein kinase A inhibitor, 0.001 mM KT-5823, 100 mM sodium fluoride, 10 mM dithiothreitol, and 10 μg/ml leupeptin. The homogenate was removed and centrifuged for 1 h at 100,000 × g. The supernatant from this spin made up the soluble protein fraction for our experiments. To concentrate the protein in the collected fractions, samples were added to Amicon Microcon 10 microconcentrators. Protein levels in the final filtrates were determined using the method of Lowry et al. (23) as modified by Markwell et al. (25). Once protein levels were determined, protein samples were prepared for isoelectric focusing (IEF) by addition of urea (9 M) and Resolvy 4-8 (2%; see Materials and Methods) to provide tissues that exhibit strong, persistent spontaneous contractions that are nonresponsive to marked increases in tissue levels of cGMP (13). Rat vas deferens were carefully removed, trimmed free of loosely adhering connective tissue, and cut open longitudinally to expose the lumen and remove all traces of sperm. All prepared muscle strips were placed in a well-oxygenated, low-phosphate physiological salt solution of the following composition (mM): 4.7 KCl, 140 NaCl, 10 HEPES, 1 MgCl₂, 1.5 CaCl₂, 0.2 NaH₂PO₄, and 10 glucose. ³²P-labeled P was then added to the buffer at a concentration of 500 μCi/ml to label cellular ATP pools. Tissues were incubated with radio-labeled phosphate for 3 h at 37°C with gentle agitation. Once cellular ATP pools were labeled, smooth muscle strips were treated as described in RESULTS. After the addition of the drug, individual smooth muscles were blotted on tissue paper and frozen with liquid nitrogen-cooled clamps and stored at –80°C for future use.

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Statistical analysis. In the PKG assays of the rat aorta, PKG activity ratios in tissues treated with SNP were compared with ratios in untreated control tissues by Student’s

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t-test. To compare the data obtained from the cumulative dose-response curves of SNP and SNAP in the absence and presence of ODQ in rat aorta, repeated-measures one-way ANOVA was used. All further comparisons of contractile responses and cGMP levels were carried out using a one-way ANOVA, followed by Student-Newman-Keuls multiple comparison test. To analyze the densitometry obtained from our two-dimensional gel electrophoresis studies, values were converted to percentage of corresponding control for statistical analysis. Because each individual set of results contains its own controls, we were able to calculate this conversion and eliminate some of the inherent variation in densitometry that can occur from one set of autoradiographs to the next. After conversion, all densitometry results were compared by one-way ANOVA, followed by Student-Newman-Keuls multiple comparison test. Values are means ± SE, and results were considered significant when \( P < 0.05 \) for all comparisons.

RESULTS

Contractility and cGMP levels in rat aorta preparations. The effects of SNP (1 and 30 \( \mu \)M), ANP (100 nM), 8-Br-cGMP (100 nM), and SNAP (1 \( \mu \)M and 1 mM) on PE-induced contractions (1 \( \mu \)M) and cGMP levels in rat aorta are shown in Fig. 1. Representative traces from these experiments are also illustrated. SNP, ANP, 8-Br-cGMP, and SNAP produced a complete relaxation of PE-induced contractions at the doses listed above. SNP (1 and 30 \( \mu \)M), ANP (100 nM), and SNAP (1 \( \mu \)M and 1 mM) generated significant increases in cGMP levels in association with their relaxant response. An additional experiment with 1 \( \mu \)M ANP produced complete relaxation of PE-induced contractions (105.7 ± 3.1\%) and a further significant increase in the level of cGMP (to 4.3 ± 0.2 pmol/mg protein, \( n = 3 \)). To determine the importance of cGMP in the relaxations mediated by SNP and SNAP, the specific inhibitor of sGC, ODQ (25 \( \mu \)M), was added to the bath 20 min before the addition of PE. ODQ completely reversed the relaxant response induced by 1 \( \mu \)M SNP and partially blocked the relaxation induced by 30 \( \mu \)M SNP and 1 \( \mu \)M SNAP (Fig. 1). ODQ had no effect on the relaxant response produced by 1 mM SNAP. Figure 1 shows that ODQ completely blocked the elevation of cGMP produced by all doses of SNP and SNAP, despite the fact that 30 \( \mu \)M SNP, 1 \( \mu \)M SNAP, and 1 mM SNAP produced significant relaxations. These results indicate that cGMP is an important mediator of the relaxant responses of SNP and SNAP in the rat aorta. However, they also suggest that at least a portion of SNP- and SNAP-induced relaxation in the rat aorta may be mediated via mechanisms independent of cGMP. cGMP-indepen-
ANOVA. ODQ (P SE). *Significant difference between the absence and presence of ODQ. Values are means of 4 aortic strips from 4 different rats; error bars, 6 length of the dose-response period. In the inability of an ODQ-mediated blockade of cGMP to further investigate absence and presence of ODQ.

To further investigate laxant response to SNAP than to SNP. dent mechanisms mediate a larger portion of the relaxant response to SNAP than to SNP.

Cumulative dose responses to SNP and SNAP in the absence and presence of ODQ. To further investigate the inability of an ODQ-mediated blockade of cGMP elevation to completely block SNP- and SNAP-induced relaxations, cumulative dose-response curves to SNP and SNAP were determined in the absence and presence of ODQ (25 μM). As shown in Fig. 2, ODQ produced a significant shift in the relaxant response induced by these cGMP-elevating agents. In Fig. 2A, ODQ markedly reduced the relaxation caused by 10 nM–1 μM SNP, although as the concentration of SNP increased above 1 μM, a large portion of the relaxant response continued to occur, despite sGC inhibition by ODQ. In Fig. 2B, ODQ only reduced SNAP-induced relaxation significantly at 100 nM SNAP. These results confirm the above conclusions that SNP- and SNAP-induced relaxations may be partially mediated by mechanisms independent of cGMP. They also indicate that SNAP-induced relaxation has a greater cGMP-independent component, making it less sensitive to sGC inhibition by ODQ.

Effect of SNP on PKG activity ratios in rat aorta. Total tissue levels of PKG and the effect of 30 μM SNP on PKG activity ratios were measured in rat aorta using the updated PKG assay described previously (13). Strips of rat aorta were suspended in tissue baths as described in EXPERIMENTAL PROCEDURES, allowed to equilibrate, and then contracted with 1 μM PE for a total of 7 min or contracted with PE for a total of 7 min with 30 μM SNP added for the last 3 min. As shown in Table 1, SNP produced a significant increase in the PKG activity ratio. No significant change in total PKG activity (i.e., in the presence of added cGMP) was observed between control and SNP-treated muscles. Thus the increase in the SNP-induced relaxation was due to activation of specific PKG by increases in endogenous cGMP and not to an increase in nonspecific (cyclic nucleotide-independent) protein kinase activity.

Separation of proteins from intact smooth muscle using high-resolution two-dimensional gel electrophoresis. Figure 3 shows a typical silver stain of a soluble smooth muscle protein extract from an untreated, intact rat myometrial preparation. This extract was separated on an 11% total monomer second-dimension SDS-polyacrylamide gel. The sensitivity of detection for this silver stain is rated at ~0.25 ng. The molecular mass markers indicate that the proteins in this gel range from ~10 to 140 kDa. The isoelectric point (pI) ranges from ~4 to 7.8.

Table 1. Effect of SNP on cGMP-dependent protein kinase activity in PE-contracted rat aorta

<table>
<thead>
<tr>
<th>PKG Activity, pmol PO₄₃⁻·min⁻¹·mg⁻¹</th>
<th>−cGMP</th>
<th>+cGMP</th>
<th>Activity Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>4.4 ± 0.5</td>
<td>44.2 ± 8.0</td>
<td>0.10 ± 0.01</td>
</tr>
<tr>
<td>SNP (30 μM)</td>
<td>12.1 ± 1.4*</td>
<td>38.2 ± 2.7</td>
<td>0.32 ± 0.02*</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 4. SNP, sodium nitroprusside; PE, phenylephrine; PKG, protein kinase G. Control and SNP-treated tissues were compared using Student's t-test. *Significant difference from control (P < 0.05).
Similar separations were also obtained in the rat aorta and rat vas deferens.

Effect of SNP on protein phosphorylation in intact rat aorta smooth muscle. Representative autoradiographs showing soluble smooth muscle protein phosphorylation from untreated control tissues and from tissues treated with 1 μM PE for 7 min, 1 μM PE for 7 min plus 30 μM SNP for the last 3 min, and 25 μM ODQ for 20 min plus 1 μM PE for 7 min plus 30 μM SNP for the last 3 min are shown in Fig. 4. These gels represent the patterns of PKG-mediated phosphorylation separated on 11% second-dimension SDS-polyacrylamide gels. Additional gels of 7.5 and 15% total monomer were also used to better resolve some of the proteins of interest identified in Fig. 4 (autoradiographs not shown). Ten proteins, the phosphorylation levels of which are altered in the presence of SNP, have been identified on the autoradiographs (A–J). Corresponding molecular mass markers and pI markers have been added to indicate some of the characteristics of these proteins.

Quantitative assessments of the degree of phosphorylation in the autoradiographs described above are shown in Table 2. SNP resulted in a significant increase in 32P incorporation into proteins A–G, whereas it produced a significant decrease in 32P incorporation into proteins H–J. By comparing the pattern of phosphorylation in an untreated aorta with that of a PE control, it was found that the level of phosphorylation of proteins I and J increased significantly in the presence of the contractile agent, PE, presumably in a calcium-dependent manner (Table 2). Furthermore, when muscles were relaxed with SNP, presumably accompanied by a decrease in cytoplasmic calcium, proteins I and J were dephosphorylated (Table 2), despite the fact that SNP increases cGMP levels and activates PKG. In contrast, protein H did not increase in phosphorylation in the presence of PE, suggesting that the SNP-induced decrease in phosphorylation of this protein is not a calcium-dependent process but may be somehow directly mediated by PKG (see below).

To determine whether the changes in phosphorylation induced by SNP in proteins A–H were, in fact, PKG mediated, we compared phosphorylation patterns in control untreated aortae, SNP-treated aortae, and aortae pretreated with the sGC inhibitor ODQ. ODQ effectively inhibited the increase in phosphorylation of proteins A–G and the decrease in phosphorylation of proteins H–J (Table 2). This is consistent with the conclusion that proteins A–G are substrates of PKG in intact smooth muscle. It also supports the conclusion that proteins I and J are phosphorylated in a calcium-dependent manner, because SNP-induced dephosphorylation of proteins I and J is also blocked by ODQ, which prevents the relaxation and, presumably, the fall in cytoplasmic calcium caused by SNP. Further analysis of the decrease in phosphorylation of protein H revealed that its neighboring protein, which is identified above as protein B (and which exhibited increased phosphorylation), may, in fact, be the same as protein H. PKG-mediated phosphorylation of protein H appears to induce a shift in pI, giving rise to protein B. In preliminary studies using several doses of SNP, it was found that the decrease in phosphorylation of protein H is directly proportional to the increase in protein B (data not shown). The fact that protein H is already phosphorylated in our control gels suggests that this protein may undergo additional phosphorylation by PKG at a separate site, leading to the pI shift observed in our gels.

Fig. 3. Representative 11% SDS-polyacrylamide gel of a soluble smooth muscle protein sample extracted from an intact rat myometrial preparation and separated using 2-dimensional gel electrophoresis. To determine the molecular masses of the proteins resolved, the molecular mass standards must be shifted upward by the depth of the well seen at top left. pI, isoelectric point.

Fig. 4. Representative autoradiographs of soluble protein phosphorylation in rat aortic smooth muscle separated on 11% 2nd-dimension SDS-polyacrylamide gels. Intact tissue strips were incubated with 32P for 3 h at 37°C and then left untreated to act as control or treated with 1 μM PE for 7 min, 1 μM PE for 7 min + 30 μM SNP for the last 3 min, and 25 μM ODQ for 20 min + 1 μM PE for 7 min + 30 μM SNP for the last 3 min. At the end of the treatment protocol, muscle strips were blotted on tissue paper and frozen between liquid nitrogen-cooled clamps. The soluble protein fraction from each tissue was separated using 2-dimensional gel electrophoresis. Gels were dried and exposed to X-ray film for 24 h. Ten proteins of interest to our study have been identified on the autoradiograph as A–J.
Effect of ANP, 8-BrcGMP, and SNAP on protein phosphorylation in intact rat aorta smooth muscle. To provide further evidence that the substrates identified in rat aorta during SNP-induced relaxation were, in fact, mediated by increases in cGMP and PKG activity, we next investigated the phosphorylation patterns induced by two cGMP-elevating agents (ANP and SNAP) and a PKG activator (8-BrcGMP). Because ANP and 8-BrcGMP do not rely on sGC to increase cGMP and activate PKG, ODQ was not used in this group of experiments. Individual tissues were treated with 1 μM PE for 9 min plus ANP (10 μM) for the last 5 min and 1 mM 8-BrcGMP for 15 min, followed by 1 μM PE for 5 min. Autoradiographs were generated from 7.5, 11, and 15% total monomer second-dimension SDS-polyacrylamide gels. The same 10 proteins that were identified during SNP-induced relaxation showed altered phosphorylation in the presence of ANP and 8-BrcGMP (autoradiographs not shown).

As shown in Table 3, ANP resulted in a significant increase in phosphorylation of proteins A–F and produced a significant decrease in phosphorylation of proteins H–J. Only protein G failed to show a significant change, although the level of phosphorylation does appear to have increased. 8-BrcGMP induced a significant increase in 32P incorporation into proteins B, D, and F and significantly decreased 32P incorporation into proteins I and J (Table 3). All the remaining proteins identified in Table 3 show a change in the level of phosphorylation during 8-BrcGMP-induced relaxation, but because of large variations in densitometry between experiments, the changes are not statistically significant. In general, the levels of phosphorylation induced by ANP and 8-BrcGMP were noticeably lower than those observed with SNP. Because ANP is not as good an elevator of cGMP (Fig. 1), this may explain the decreased magnitude of PKG-mediated phosphorylation seen with this agent. Problems associated with the ability of 8-BrcGMP to cross the smooth muscle cell membrane and contact PKG could explain the lower levels of phosphorylation induced by this cGMP analog. Despite the lower levels of phosphoryla-

### Table 2. Effect of SNP, in the absence and presence of ODQ, on 32P incorporation into intact rat aortic smooth muscle proteins

<table>
<thead>
<tr>
<th>Protein</th>
<th>Mol Mass, kDa</th>
<th>pI</th>
<th>Control (1 μM for 7 min)</th>
<th>PE (1 μM for 4 min) + SNP (30 μM for 3 min)</th>
<th>ODQ (25 μM for 20 min) + PE + SNP</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>22</td>
<td>5.8</td>
<td>11.1 ± 0.5</td>
<td>13.0 ± 0.3</td>
<td>4.61 ± 1.9†</td>
</tr>
<tr>
<td>B</td>
<td>21</td>
<td>6.3</td>
<td>0.6 ± 0.2</td>
<td>1.0 ± 0.4</td>
<td>2.7 ± 0.7†</td>
</tr>
<tr>
<td>C</td>
<td>26</td>
<td>6.6</td>
<td>0.5 ± 0.1</td>
<td>0.7 ± 0.3</td>
<td>2.2 ± 0.7†</td>
</tr>
<tr>
<td>D</td>
<td>56</td>
<td>7.5</td>
<td>2.2 ± 0.1</td>
<td>3.0 ± 1.0</td>
<td>13.1 ± 4.5†</td>
</tr>
<tr>
<td>E</td>
<td>56</td>
<td>7.4</td>
<td>1.3 ± 0.6</td>
<td>1.3 ± 0.4</td>
<td>7.4 ± 1.1†</td>
</tr>
<tr>
<td>F</td>
<td>56</td>
<td>7.3</td>
<td>1.2 ± 0.5</td>
<td>1.2 ± 0.2</td>
<td>6.2 ± 2.1†</td>
</tr>
<tr>
<td>G</td>
<td>16</td>
<td>6.9</td>
<td>0.5 ± 0.1</td>
<td>0.7 ± 0.3</td>
<td>2.0 ± 0.9</td>
</tr>
<tr>
<td>H</td>
<td>22</td>
<td>6.4</td>
<td>9.6 ± 2.1</td>
<td>7.1 ± 1.7</td>
<td>3.2 ± 0.8†</td>
</tr>
<tr>
<td>I</td>
<td>21</td>
<td>5.2</td>
<td>6.8 ± 1.3</td>
<td>11.3 ± 3.2</td>
<td>4.1 ± 1.6†</td>
</tr>
<tr>
<td>J</td>
<td>21</td>
<td>5.3</td>
<td>11.2 ± 1.3</td>
<td>14.9 ± 3.3</td>
<td>6.8 ± 3.1†</td>
</tr>
</tbody>
</table>

Values (means ± SE) are presented as background-corrected optical density, integrated over all pixels in the spot; n = 5 or 6 experiments, with each experiment including a control, PE, PE + SNP, and 1H-[1,2,4]oxadiazolo [4,3-a] quinoxaline-1-one (ODQ) + PE + SNP. Densitometry values are converted to percentage of corresponding control for statistical analysis. This conversion eliminates some of the variations in densitometry that occur from one experiment to the next (see EXPERIMENTAL PROCEDURES). Groups were compared by one-way ANOVA, followed by Student-Newman-Keuls multiple comparison test. Molecular mass (mol mass) and isoelectric point (pI) were calculated from standards run concurrently. *Significant difference from control (P < 0.05); †significant difference from PE treated (P < 0.05); ‡significant difference from the absence and presence of ODQ in SNP-treated muscles.

### Table 3. Effect of ANP and 8-BrcGMP on 32P incorporation into intact rat aortic smooth muscle proteins

<table>
<thead>
<tr>
<th>Protein</th>
<th>Mol Mass, kDa</th>
<th>pI</th>
<th>Control (1 μM for 9 min)</th>
<th>PE (1 μM for 5 min) + ANP (10 μM for 4 min)</th>
<th>8-BrcGMP (1 mM for 15 min) + PE (1 μM for 5 min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>22</td>
<td>5.8</td>
<td>11.1 ± 0.5</td>
<td>13.0 ± 0.3</td>
<td>4.61 ± 1.9†</td>
</tr>
<tr>
<td>B</td>
<td>21</td>
<td>6.3</td>
<td>0.6 ± 0.2</td>
<td>1.0 ± 0.4</td>
<td>2.7 ± 0.7†</td>
</tr>
<tr>
<td>C</td>
<td>26</td>
<td>6.6</td>
<td>0.5 ± 0.1</td>
<td>0.7 ± 0.3</td>
<td>2.2 ± 0.7†</td>
</tr>
<tr>
<td>D</td>
<td>56</td>
<td>7.5</td>
<td>2.2 ± 0.1</td>
<td>3.0 ± 1.0</td>
<td>13.1 ± 4.5†</td>
</tr>
<tr>
<td>E</td>
<td>56</td>
<td>7.4</td>
<td>1.3 ± 0.6</td>
<td>1.3 ± 0.4</td>
<td>7.4 ± 1.1†</td>
</tr>
<tr>
<td>F</td>
<td>56</td>
<td>7.3</td>
<td>1.2 ± 0.5</td>
<td>1.2 ± 0.2</td>
<td>6.2 ± 2.1†</td>
</tr>
<tr>
<td>G</td>
<td>16</td>
<td>6.9</td>
<td>0.5 ± 0.1</td>
<td>0.7 ± 0.3</td>
<td>2.0 ± 0.9</td>
</tr>
<tr>
<td>H</td>
<td>22</td>
<td>6.4</td>
<td>9.6 ± 2.1</td>
<td>7.1 ± 1.7</td>
<td>3.2 ± 0.8†</td>
</tr>
<tr>
<td>I</td>
<td>21</td>
<td>5.2</td>
<td>6.8 ± 1.3</td>
<td>11.3 ± 3.2</td>
<td>4.1 ± 1.6†</td>
</tr>
<tr>
<td>J</td>
<td>21</td>
<td>5.3</td>
<td>11.2 ± 1.3</td>
<td>14.9 ± 3.3</td>
<td>6.8 ± 3.1†</td>
</tr>
</tbody>
</table>

Values (means ± SE) are presented as background-corrected optical density, integrated over all pixels in the spot; n = 5 or 6 experiments, with each experiment including a control, PE, PE + atrial natriuretic peptide (ANP), and 8-bromo-cGMP (8-BrcGMP) + PE. Densitometry values are converted to percentage of corresponding control for statistical analysis. This conversion eliminates some of the variations in densitometry that occur from one experiment to the next (see EXPERIMENTAL PROCEDURES). Groups were compared by 1-way ANOVA, followed by Student-Newman-Keuls multiple comparison test. Molecular mass (mol mass) and isoelectric point (pI) were calculated from standards run concurrently. *Significant difference from control (P < 0.05); †significant difference from PE treated (P < 0.05); ‡significant difference from the absence and presence of ODQ in SNP-treated muscles.
PKG-mediated phosphorylation in the rat aorta was also assessed in the presence of the nitrosothiol donor SNAP. Aortic segments were treated with 1 mM PE for 7 min plus 1 mM SNAP for the last 3 min and 25 μM ODQ for 20 min plus 1 mM PE for 7 min plus 1 mM SNAP for the last 3 min. SNAP altered the phosphorylation of the same 10 proteins (A–J) identified during SNAP-induced relaxation in the aorta (autoradiographs not shown). Pretreatment with ODQ resulted in a blockade of SNAP-induced changes in protein phosphorylation. Table 4 shows the quantitative assessments of the degree of PKG-mediated phosphorylation of some of these proteins during SNAP-induced relaxation. Because 7.5 and 15% gels were not analyzed in this study, quantitative densitometry analyses were limited to proteins A–C and H–J. SNAP induced a significant increase in 32P incorporation into proteins A–C and produced a significant decrease in 32P incorporation into proteins H and I. The level of phosphorylation of protein J was decreased with SNAP, but because of large variations in the densitometry between experiments, this decrease was not statistically significant. Pretreatment with ODQ significantly reversed the SNAP-induced changes in phosphorylation of proteins A–C, H, and J. It also appeared to reverse the effect of SNAP on protein I, although, again because of the variability, ODQ + PE + SNAP is not significantly different from PE + SNAP. ODQ + PE + SNAP is not significantly different from PE alone, which indicates that ODQ has reversed the phosphorylation to levels similar to those found in the absence of SNAP. The results in Table 4 demonstrate that SNAP mimics SNP-induced PKG-mediated phosphorylation during rat aorta smooth muscle relaxation. Because no new PKG substrates were identified in the presence of SNAP, it appears that the cGMP-mediated component of SNAP-induced relaxation occurs via pathways similar to SNP.

Protein phosphorylation in the particulate fraction of rat aorta. In all the two-dimensional gel electrophoresis experiments in the rat aorta, we also investigated PKG-mediated phosphorylation in the particulate fraction. Despite achieving good solubilization of particulate fraction proteins, we were unable to identify any significant changes in protein phosphorylation from the autoradiographs (data not shown). Several in vitro investigations have demonstrated PKG-mediated phosphorylation of numerous membrane-bound proteins in smooth muscle (6, 39). However, we were unable to detect these phosphorylation events in the intact tissue setting.

Effects of SNAP and SNP on protein phosphorylation in intact myometrial smooth muscle. The rat myometrium can be classified as a “nonresponsive” smooth muscle, in that it does not relax in response to an elevation of cGMP and activation of PKG by agents such as SNP (8, 13). However, it does relax in the presence of SNAP through an unknown mechanism that may involve a cGMP-independent component (13). Although cGMP elevation does not appear to be responsible for SNAP-induced relaxation of rat myometrium, SNAP is an excellent elevator of cGMP in this tissue. Therefore, we utilized SNAP and SNP to investigate the patterns of PKG-mediated phosphorylation in the myometrium. In the introduction, it was proposed that if one or more of the PKG substrates identified in the aorta was absent or not phosphorylated during an elevation of cGMP and activation of PKG in the rat myometrium, that protein may be of particular importance in cGMP-mediated smooth muscle relaxation. Autoradiographs showing intact myometrial phosphorylation in a control, untreated tissue, tissues treated with 5 mM SNP for 5 min, tissues treated with 1 mM SNAP for 5 min, and tissues treated with 25 μM ODQ for 20 min, followed by 1 mM SNAP for 5 min are shown in Fig. 5. From these autoradiographs, we were unable to detect any SNAP- or SNP-induced changes in phosphorylation of the seven proteins identified as

Table 4. Effect of SNAP, in the absence and presence of ODQ, on 32P incorporation into intact rat aortic smooth muscle proteins

<table>
<thead>
<tr>
<th>Protein</th>
<th>Mol Mass, kDa</th>
<th>pI</th>
<th>Control (1 μM for 7 min)</th>
<th>PE (1 μM for 4 min) + SNAP (1 mM for 3 min)</th>
<th>ODQ (25 μM for 20 min) + PE + SNAP</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>22</td>
<td>5.8</td>
<td>2.6 ± 0.2</td>
<td>6.1 ± 1.1</td>
<td>17.1 ± 1.3†</td>
</tr>
<tr>
<td>B</td>
<td>21</td>
<td>6.3</td>
<td>0.6 ± 0.2</td>
<td>1.8 ± 0.3</td>
<td>8.6 ± 1.5†</td>
</tr>
<tr>
<td>C</td>
<td>26</td>
<td>6.6</td>
<td>1.0 ± 0.4</td>
<td>4.9 ± 1.1</td>
<td>13.5 ± 3.4†</td>
</tr>
<tr>
<td>H</td>
<td>22</td>
<td>6.4</td>
<td>3.2 ± 1.4</td>
<td>4.4 ± 1.2</td>
<td>0.9 ± 0.3†</td>
</tr>
<tr>
<td>I</td>
<td>21</td>
<td>5.2</td>
<td>6.3 ± 1.3</td>
<td>13.2 ± 1.1</td>
<td>9.0 ± 2.2†</td>
</tr>
<tr>
<td>J</td>
<td>21</td>
<td>5.3</td>
<td>7.6 ± 1.8</td>
<td>14.3 ± 2.1</td>
<td>10.1 ± 2.3</td>
</tr>
</tbody>
</table>

Values (means ± SE) are presented as background-corrected optical density, integrated over all pixels in the spot; n = 5 or 6 experiments, with each experiment including a control, PE, PE + 8-nitroso-N-acetylpenicillamine (SNAP), and ODQ + PE + SNAP. Densitometry values are converted to percentage of corresponding control for statistical analysis. This conversion eliminates some of the variations in densitometry that occur from one experiment to the next (see EXPERIMENTAL PROCEDURES). Molecular mass and pI were calculated from standards run concurrently. Groups were compared by 1-way ANOVA, followed by Student-Newman-Keuls multiple comparison test. †Significant difference from control (P < 0.05); ‡significant difference from PE treated (P < 0.05); ††significant difference between the absence and presence of ODQ in SNAP-treated muscles.
PKG-MEDIATED PHOSPHORYLATION IN INTACT SMOOTH MUSCLE
PKG substrates in the aorta. In fact, it appears as though the PKG substrates in the aorta are absent in the rat myometrium. In a comparison between control and SNAP-treated autoradiographs, four new proteins with phosphorylation levels that were altered in the presence of SNAP are identified and labeled K–N (Fig. 5). Interestingly, these increases in phosphorylation induced by SNAP (1 mM) were unaffected by an inhibition of sGC with ODQ (Fig. 5). The degree of phosphorylation of SNAP-induced phosphorylation of four new proteins identified in the rat myometrium, we also investigated whether these proteins were phosphorylated in the presence of SNP. As shown in Fig. 5, SNP did not produce any change in phosphorylation of proteins K–N. The quantitative assessments of phosphorylation confirm that no measurable change in phosphorylation occurred in the presence of SNP (Table 5). The patterns of phosphorylation induced by 8-BrcGMP were also investigated in the rat myometrium, but again we were unable to detect the PKG substrates identified in the aorta or changes in 32P incorporation compared with controls (autoradiographs not shown).

8-BrcGMP did not increase the phosphorylation of proteins K–N (data not shown).

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Table 6. Effect of SNP and 8-BrcGMP on $^{32}$P incorporation into intact rat vas deferens smooth muscle proteins

<table>
<thead>
<tr>
<th>Protein</th>
<th>Mol Mass, kDa</th>
<th>$pI$</th>
<th>Control (3 μM for 30 s)</th>
<th>SNP (1 mM for 2 min) + PE (3 μM for 30 s)</th>
<th>8-BrcGMP (1 mM for 15 min) + PE (3 μM for 30 s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>22</td>
<td>5.8</td>
<td>0.5 ± 0.2</td>
<td>0.8 ± 0.04</td>
<td>0.4 ± 0.05</td>
</tr>
<tr>
<td>B</td>
<td>21</td>
<td>6.3</td>
<td>0.4 ± 0.1</td>
<td>0.3 ± 0.04</td>
<td>0.3 ± 0.05</td>
</tr>
<tr>
<td>C</td>
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<td>6.6</td>
<td>0.3 ± 0.1</td>
<td>0.3 ± 0.04</td>
<td>0.3 ± 0.05</td>
</tr>
<tr>
<td>H</td>
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<td>5.1 ± 1.8</td>
<td>7.3 ± 2.6</td>
<td>5.8 ± 2.0</td>
</tr>
<tr>
<td>I</td>
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<td>3.6 ± 2.2</td>
<td>4.6 ± 2.1</td>
<td>3.6 ± 1.9</td>
</tr>
<tr>
<td>J</td>
<td>21</td>
<td>5.3</td>
<td>3.8 ± 2.1</td>
<td>6.4 ± 2.2</td>
<td>5.2 ± 1.6</td>
</tr>
<tr>
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</tr>
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<td>0.3 ± 0.03</td>
<td>0.2 ± 0.05</td>
</tr>
<tr>
<td>M</td>
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<td>7.5</td>
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<td>0.3 ± 0.05</td>
</tr>
<tr>
<td>N</td>
<td>28</td>
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<td>4.1 ± 0.6</td>
<td>5.2 ± 1.7</td>
<td>3.7 ± 1.2</td>
</tr>
</tbody>
</table>

Values (means ± SE) are presented as background-corrected optical density, integrated over all pixels in the spot; $n = 4$ or 5 experiments, with each experiment including a control, PE, SNP + PE, and 8-BrcGMP + PE. Densitometry values are converted to percentage of corresponding control for statistical analysis. This conversion eliminates some of the variations in densitometry that occur from one experiment to the next (see EXPERIMENTAL PROCEDURES). Molecular mass and $pI$ were calculated from standards run concurrently. Groups were compared by 1-way ANOVA, followed by Student-Newman-Keuls multiple comparison test. *Significant difference from PE treated ($P < 0.05$).

Relaxation and the concentration of SNP required to show PKG-mediated phosphorylation of the proteins identified in Table 2. At 100 nM SNP, we were unable to detect significant changes in protein phosphorylation, although the smooth muscle was ~100% relaxed (Fig. 2A). In preliminary experiments at 1 μM SNP, PKG-mediated phosphorylation was detected in the same seven proteins described in Table 4 (data not shown). However, with the variability inherent in these experiments, we were unable to show statistical significance. This apparent dissociation also occurred with ANP, 8-BrcGMP, and SNAP. Despite having to employ higher doses in our two-dimensional gel electrophoresis experiments, the apparent increase in protein phosphorylation detected with 1 μM SNP indicates that the patterns of phosphorylation are not changed by increasing the dose. In addition, the use of ODQ to confirm that the identified proteins are cGMP dependent ensures that the phosphorylation we are observing is PKG mediated and not due to nonspecific kinase activation.

**DISCUSSION**

A detailed analysis of the literature concerning the role of cGMP in mediating vascular smooth muscle relaxation confirms that the criteria necessary to determine that cGMP is involved in this effect have been reasonably well satisfied (27). In vascular smooth muscle, it is generally accepted that the cGMP-mediated component of relaxation involves activation of a specific PKG. Activation of PKG by relaxant drugs has been reported to occur in several vascular preparations (33), and recent experiments in our own laboratory have demonstrated that relaxation of rabbit aorta by nitrovasodilators is well correlated with elevation of cGMP and activation of PKG (31). In the present study, relaxation of rat aortic strips by 30 μM SNP was accompanied by a threefold activation of PKG. In contrast to these results in vascular preparations, elevation of cGMP and activation of PKG by SNP is not accompanied by relaxation in some types of smooth muscle, including rat vas deferens (32) and rat myometrium (8, 13), leading to the classification of the latter types of muscles as nonresponsive to cGMP elevation. PKG presumably elicits an effect in vascular smooth muscle by phosphorylating specific target proteins that somehow regulate tension in the smooth muscle. As noted in the introduction, the protein targets of PKG and the underlying mechanisms by which this kinase leads to a relaxant response have not been completely elucidated. The main objective of the present study was to investigate the substrates of PKG in intact smooth muscles using two-dimensional gel electrophoresis. To assess the physiological significance of any PKG substrates identified, experiments were performed to compare PKG-mediated phosphorylation in responsive vs. nonresponsive smooth muscles to determine whether the absence of crucial PKG substrates or different abilities of PKG to phosphorylate these substrates might explain the lack of relaxation in nonresponsive smooth muscles.

The results of our two-dimensional gel electrophoresis studies provide statistical verification of PKG-mediated phosphorylation in intact smooth muscles. Previous studies have shown changes in protein phosphorylation during an elevation of cGMP, but we have taken the additional steps to quantitatively assess the degree of phosphorylation using densitometry and to use statistical analyses to compare the levels between different treatment groups. In rat aorta, we identified 10 proteins with phosphorylation levels that are significantly altered in the presence of SNP (A–J, Table 2). By comparison of muscles undergoing different treatments, two of these phosphorylation events appeared to be calcium dependent (I and J) and seven others were identified as cGMP dependent, catalyzed by PKG (A–G). The PKG substrates were confirmed as cGMP dependent on the basis of studies with the sGC inhibitor ODQ. To our knowledge, this is the first demonstration of the ability of an sGC inhibitor to...
prevent the activation of PKG by cGMP-elevating agents such as SNP. As described in results, the apparent decrease in phosphorylation of protein H after SNP treatment probably represents a shift in the pI of this protein, which is then detected as protein B. Protein H is endogenously phosphorylated in our control gels and appears to be phosphorylated by PKG at a separate site, which leads to the pI shift (from 6.4 to 6.3) observed in our gels. Similar shifts in pI have been described during smooth muscle myosin light chain phosphorylation (42). In that study, the pI of myosin light chain changed from 5.1 to 5.0 during a myosin light chain kinase-mediated addition of phosphate (42). To further confirm the cGMP-dependent nature of our seven PKG substrates, we also measured PKG-mediated phosphorylation with a pGc activator (ANP), a nitrosothiol donor (SNAP), and a cGMP analog (8-BrcGMP). Each of these PKG-activating agents resulted in patterns of PKG-mediated phosphorylation similar to those found with SNP. Thus the substrates identified above appear to be phosphorylated in a cGMP-dependent manner, presumably via activation of PKG. However, it is also possible that protein kinase A may be cross-activated by the high concentrations of 8-BrcGMP used in our tissues. Further studies are required to rule out this possibility. One piece of evidence that argues against this possibility is our earlier observation that high concentrations of 8-BrcGMP failed to inhibit spontaneous contractions in rat myometrium (13). If 8-BrcGMP were activating protein kinase A, we would have expected the myometrium to relax, since it is very sensitive to the relaxant effects of cAMP-elevating agents.

As described above, the nonvascular smooth muscles of the rat myometrium and rat vas deferens do not relax in response to SNP-induced increases in cGMP and PKG activity. Our two-dimensional gel electrophoresis results provide the first evidence as to whether downstream PKG-mediated phosphorylation (or lack of phosphorylation) can be used to explain the lack of relaxation in these types of smooth muscle. In the rat myometrium, we were unable to identify any of the seven PKG substrates identified in the rat aorta. In fact, it is possible that these PKG substrates are not present in the myometrium, inasmuch as no traces of them could be detected from our gels. Endogenous protein phosphorylation was slightly lower in certain areas of the autoradiographs from myometrial preparations. Therefore, we performed preliminary experiments where 150 µg of protein from myometrial extracts were separated using two-dimensional gel electrophoresis. Normally, only 50 µg of protein were used in our experiments, but despite having triple the amount of protein in these gels, no traces of the PKG substrates were found. Endogenous phosphorylation levels in these high-protein experiments were well above those observed in the rat aorta, indicating that our lack of protein detection was not simply due to low levels of phosphorylation. Because our myometrial tissues were not precontracted before the addition of the cGMP-elevating agents above, we were also unable to show changes in the phosphorylation of the calcium-dependent proteins, I and J. However, low levels of phosphorylation of these proteins indicate that they are present in this smooth muscle. The possibility was considered that our lack of PKG substrate detection could be a result of lower total levels of PKG. However, a comparison of total PKG activities in the rat aorta and rat myometrium indicates that the kinase levels are similar in these two smooth muscles (Table 2) (13). Another possible explanation for the absence of PKG-mediated phosphorylation may reside in variations in the levels of phosphatases. If phosphatases are more concentrated in the myometrium than in the aorta, this could explain the absence of PKG-mediated phosphorylation in this nonresponsive tissue. However, we have no information on the relative amounts of phosphatases present in these tissues. Our preliminary experiments with increased protein loads tend to eliminate this possibility, since phosphorylation levels in these autoradiographs were well above those observed in the aorta. However, no detectable increases in PKG-mediated phosphorylation were observed. With the assumption that phosphorylation of one or more of the proteins we identified in the rat aorta is responsible for relaxation, the lack of relaxation in the myometrium could be attributed to the absence of such protein(s).

In our analysis of the changes in phosphorylation induced by SNAP in the myometrium, we identified four new phosphorylated proteins that were not found in the rat aorta. These phosphorylation events were not altered in the presence of ODQ, suggesting that they may occur in a cGMP-independent manner. Although the elevation of cGMP by SNAP in the myometrium is not completely reversed by ODQ, it is markedly reduced, which should result in some level of reduction in PKG-mediated phosphorylation. Because the levels of phosphorylation of these four proteins are similar to, or even further elevated, in the presence of ODQ, it seems likely that these phosphorylation events occur independently of cGMP and PKG. It has already been shown that SNAP-induced relaxation in the myometrium is unaffected by a significant blockade of cGMP elevation by ODQ (13). Thus our finding of cGMP-independent, SNAP-induced phosphorylation correlates well with these earlier results. The fact that we have detected cGMP-independent protein phosphorylation in the presence of SNAP suggests that the mechanisms of relaxation caused by this NO donor may involve activation of another kinase. However, the identity of this kinase is unknown. NO has been shown to increase the activity of p38 mitogen-activated protein kinase (5). However, cGMP and PKG were shown to be required for this effect (5). It is possible that the low level of cGMP still present in the myometrium during sGC inhibition with ODQ could allow sufficient PKG activation to permit activation of the p38 kinase. However, further investigation is required to implicate such a pathway in smooth muscle.

Rat vas deferens has also been reported to be a nonresponsive smooth muscle, in that it does not relax in response to increases in the tissue levels of cGMP.
and PKG activity induced by SNP (32). Interestingly, the vas deferens does relax in the presence of 8-BrcGMP (41), and one of the objectives of the present study was to determine whether this relaxant response involves activation of PKG. None of the seven proteins previously identified in the rat aorta as PKG substrates were phosphorylated during 8-BrcGMP-induced relaxation or during cGMP elevations induced by SNP. One calcium-dependent protein, possibly a myosin light chain, did show a significant decrease in phosphorylation in the presence of 8-BrcGMP, which is consistent with the fact that this agent can cause relaxation of PE-contracted rat vas deferens. Unlike the myometrium, some of the rat aorta PKG substrates appear to be present in our rat vas deferens autoradiographs. In particular, protein H is easily identified from these autoradiographs, although no change in the densitometry of this spot was observed between control and relaxed smooth muscles. Similar to our results in the rat myometrium, the overall levels of phosphorylation seem to be lower in the rat vas deferens than in the rat aorta. As a result, preliminary experiments with higher protein loads of rat vas deferens extracts were also analyzed using our two-dimensional gel electrophoresis method. Despite the higher levels of protein used, no drug-induced changes in phosphorylation were observed in these autoradiographs. However, the presence of some of the PKG substrates identified in the aorta was more clearly resolved. If the proteins identified in the rat aorta are, in fact, present in the rat vas deferens, the inability of PKG to phosphorylate these substrates in an intact tissue may be due to the fact that it cannot gain access to the compartments in which these substrates are located. However, it is also possible that one or more of the substrates are not present in the vas deferens. Further investigations would be required to differentiate between these possibilities.

As mentioned in RESULTS, a dissociation between the concentration of SNP required to cause relaxation and the concentration of SNP required to show PKG-mediated phosphorylation of proteins A–J was discovered early in our investigations of PKG-mediated phosphorylation in the rat aorta. Attempts to increase the sensitivity of detection of our method were unsuccessful, and, as a result, the majority of our two-dimensional gel electrophoresis studies were performed with doses of SNP above those required to cause relaxation. Despite having to use higher doses, we believe that our detailed analysis of protein phosphorylation with several different activators of PKG in the absence and presence of the sGC inhibitor ODQ provides a reasonable assessment of the cGMP-dependent, PKG-mediated phosphorylation in intact smooth muscle. Although it is possible that high doses of NO donors can be toxic to cells and initiate pathways independent of cGMP elevation, the short duration of incubation with these agents used in our studies and the confirmation of the PKG substrates as cGMP-dependent using ODQ eliminate the likelihood that such events contribute to the patterns of phosphorylation observed in our studies.

Although no attempts were made to clearly identify the proteins of interest found in our rat aorta experiments, the literature does provide some possible identities. The two proteins exhibiting calcium-dependent phosphorylation, J and K, have molecular mass of ~21 kDa and pI of ~5.2 and 5.3, respectively. Numerous reports investigating in vivo smooth muscle phosphorylation have identified proteins with similar molecular mass and pI that exhibit a decrease in phosphorylation during relaxation (15, 16, 19, 21, 30, 43). In the experiments of Paglin et al. (30) and Ishibashi et al. (15), these proteins were identified as myosin light chains by their comigration with appropriate standard proteins extracted from rabbit aorta and bovine trachea, respectively. In other experiments, these two proteins were identified as myosin light chains on the basis of their molecular mass and pI, as described by Silver and Stull (42). Given the similarities between our results and those reported in the studies described above, we have concluded that proteins H and I in our studies are probably myosin light chains.

The proteins identified as A and B in our experiments with molecular mass of 22,000 and 21,000 and pI of 5.8 and 6.3, respectively, may have been identified as PKG substrates previously (3, 37). Bergh et al. (3) identified two proteins with molecular mass of 20 kDa that showed mobilities in two dimensions similar to those of proteins A and B. In subsequent experiments, these proteins were identified as HSP-20. Rapoport et al. (37) also identified two proteins with molecular mass of 24,000 and pI of 6.5 and 6.2 that showed increased phosphorylation after SNP-induced relaxation. Although the molecular mass and pI are slightly different in this study, an analysis of the autoradiographs of Rapoport et al. (37) indicates that these proteins may be the same as those described above in our experiments and by Bergh et al. (3). Considering these results, it is possible that proteins A and B in our studies are HSP. If PKG does mediate the phosphorylation of HSP during intact smooth muscle relaxation, it is unknown what function these activated proteins may have. Interestingly, in the study of Bergh et al. (3), the HSP were reported to be phosphorylated in a vascular smooth muscle relaxed by SNP but not in a vascular smooth muscle that did not relax in response to SNP. It is important to note that Bergh et al. (3) were unable to demonstrate activation of PKG in either of these tissues. It has been suggested that HSP are important regulatory components of the actin-based cytoskeleton that can interact with intermediate filaments and, in turn, regulate vascular smooth muscle contraction and relaxation (1). Other HSP, such as α-crystallin, are colocalized in the Z-band of cardiac muscle, the counterpart of which in smooth muscle is dense bodies (2). Phosphorylation of HSP in dense bodies of smooth muscle may lead to a blockade of cross-bridge attachments and, as a consequence, relaxation (11). In cell culture, Pryzwansky and colleagues (36) reported that PKG phosphorylates, and is an-
chored to, the soluble protein vimentin in vascular smooth muscle cells. However, we were unable to show PKG-mediated vimentin phosphorylation in the soluble protein fraction of our intact smooth muscle experiments. It is possible that PKG-mediated phosphorylation in cell culture differs significantly from that in the intact tissue setting.

Numerous studies have identified possible PKG substrates in the particulate fraction of smooth muscle. Casnellie et al. (6) demonstrated particulate fraction PKG-mediated phosphorylation of four proteins in vascular smooth muscle cell fractions treated with cGMP (250, 130, 85, and 75 kDa). In rat aorta smooth muscle cells, Sarcevic et al. (39) showed similar in vitro particulate fraction PKG-mediated phosphorylation of three proteins (225, 132, and 11 kDa) in the presence of ANP. Despite achieving good solubilization of our particulate fractions, we were unable to detect any significant changes in phosphorylation of membrane-bound proteins. The high-molecular-mass proteins described above (250 and 225 kDa) were subsequently identified as splice variants of the IP$_3$ receptor. More recent studies have demonstrated that the IP$_3$ receptor is, in fact, a substrate of PKG in intact vascular smooth muscle cells (17). Because of the high molecular mass of this protein (~260 kDa), we were unable to resolve it on our gels. As a result, we cannot eliminate the PKG-mediated phosphorylation of this protein as a possible mediator of intact smooth muscle relaxation. From these comparisons, it is clear that until substrates of PKG have been demonstrated to be phosphorylated in the intact tissue setting, their role in smooth muscle relaxation is subject to controversy.

To accompany our analysis of PKG-mediated phosphorylation in the rat aorta, contractile responses were assessed in response to treatment protocols similar to those described in our two-dimensional gel electrophoresis experiments. Because numerous recent reports have provided evidence for the presence of cGMP-independent components in NO-mediated smooth muscle relaxation (34, 35, 45), we performed a number of experiments utilizing ODQ to investigate the role of cGMP in the NO-mediated relaxation of the rat aorta. SNP, SNAP, ANP, and 8-BrcGMP produced complete relaxations of PE-induced contractions in our rat aorta experiments. Coincident with their relaxant responses, SNP, SNAP, and ANP generated significant increases in cGMP levels. ODQ completely blocked the elevation of cGMP induced by SNP and SNAP and concomitantly reversed a large portion of the relaxant responses to these agents. These data suggest that cGMP is an important mediator of the relaxant responses of SNP and SNAP in the rat aorta. However, a partial relaxation still occurred at the higher concentrations of SNP and SNAP, despite a complete blockade of cGMP elevation, indicating that at least a portion of the relaxant responses to these agents may be mediated via mechanisms independent of cGMP. Cumulative dose-response curves to SNP and SNAP (in the absence and presence of ODQ) further demonstrated the dissociation between cGMP elevation and relaxation in the rat aorta. For both agents, the dissociation becomes more apparent as the concentration of drug is increased. A comparison between the dissociations that occur with SNP and SNAP reveals that SNAP seems to have a larger cGMP-independent component to its relaxant response. Because SNAP has been reported to be a better NO donor than SNP (24), this may be the reason for its larger NO-mediated, cGMP-independent component. Nevertheless, despite the presence of an apparent cGMP-independent component in SNP- and SNAP-induced relaxation of rat aorta, cGMP is still responsible for a predominant part of the relaxant responses in this tissue. The complete relaxations induced by ANP and 8-BrcGMP in Fig. 1, which occur without the generation of NO, also support this conclusion.

In summary, PKG-mediated phosphorylation of seven proteins was found to accompany relaxation of intact rat aorta strips by cGMP-elevating agents and 8-BrcGMP. We were unable to detect phosphorylation of any of these proteins in rat myometrium and vas deferens after treatment with concentrations of SNP that markedly elevated cGMP levels and activated PKG in these tissues. The lack of PKG-mediated phosphorylation may explain the absence of relaxation seen in the latter tissues. Further experiments are required to identify which protein(s) is involved in the relaxation of vascular smooth muscle.

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