ROLE OF CYCLIC GMP, CYCLIC GMP-DEPENDENT PROTEIN KINASE
AND PROTEIN PHOSPHORYLATION IN THE CONTROL OF
SMOOTH MUSCLE TENSION.

By

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Division of Pharmacology and Toxicology
Faculty of Pharmaceutical Sciences

We accept this thesis as conforming
to the required standard

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March, 2000
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ABSTRACT

Numerous agents are capable of activating guanylyl cyclases and increasing tissue levels of guanosine 3':5'-cyclic monophosphate (cGMP). 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 satisfied. However, the cGMP hypothesis may only apply in certain smooth muscles. One aim of the present study was to further investigate the role of cGMP in uterine contractility in an attempt to resolve some of the apparent inconsistencies regarding the importance of cGMP in this tissue.

The results of our studies in the rat myometrium confirm the previous suggestion that the rat myometrium can be classified as a "non-responsive" smooth muscle with respect to the fact that it does not relax in response to increases in the tissue levels of cGMP. Sodium nitroprusside (SNP) was shown to significantly increase cGMP levels and to activate cyclic GMP-dependent protein kinase (PKG) in our myometrial preparations but did not cause relaxation of the tissue. Therefore, a lack of PKG activation cannot be used to explain the failure of cGMP-elevating agents such as SNP to cause relaxation of the rat myometrium.

In vascular smooth muscle, it is generally well accepted that the cGMP-dependent component of relaxation involves activation of a specific PKG. The protein targets of PKG and the underlying mechanisms by which this kinase leads to a relaxant response have not been completely elucidated. The final objective of our studies was to investigate the smooth muscle substrates of PKG in intact rat aorta, rat myometrium and rat vas deferens using two-dimensional gel electrophoresis.

In intact rat aorta, seven PKG substrates and two calcium-dependent phosphorylation events were detected during relaxation of the tissue. None of the PKG substrates identified in the rat aorta could be identified in the myometrium or vas deferens following administration of numerous cGMP-elevating agents. In addition, we were unable to detect changes in phosphorylation of any other proteins. 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.
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<tr>
<td>%T</td>
<td>percent total monomer</td>
</tr>
<tr>
<td>[γ-32P]ATP</td>
<td>ATP in which the phosphorus at the γ position is radioactive</td>
</tr>
<tr>
<td>μ</td>
<td>micro (1 x 10^-6)</td>
</tr>
<tr>
<td>8-bromo-cGMP</td>
<td>8-bromoguanosine 3':5'-cyclic monophosphate</td>
</tr>
<tr>
<td>A</td>
<td>ampere</td>
</tr>
<tr>
<td>AEBSF</td>
<td>[4-(2-aminoethyl)benzenesulfonylfluoride, HCl]</td>
</tr>
<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
</tr>
<tr>
<td>ANP</td>
<td>atrial natriuretic peptide</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine 5'-triphosphate</td>
</tr>
<tr>
<td>BPDEtide</td>
<td>a peptide substrate of PKG (RKISASEFDRPLR)</td>
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<tr>
<td>cAMP</td>
<td>adenosine 3':5'-cyclic monophosphate</td>
</tr>
<tr>
<td>cGMP</td>
<td>guanosine 3':5'-cyclic monophosphate</td>
</tr>
<tr>
<td>CHAPS</td>
<td>3-[(3-cholamidopropyl)dimethyl-ammonio]-1-propanesulfonate</td>
</tr>
<tr>
<td>Ci</td>
<td>Curie</td>
</tr>
<tr>
<td>DTT</td>
<td>dithiothreitol</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylene diamine tetraacetic acid</td>
</tr>
<tr>
<td>EGTA</td>
<td>ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid</td>
</tr>
<tr>
<td>g</td>
<td>gram</td>
</tr>
<tr>
<td>g</td>
<td>relative centrifugal force</td>
</tr>
<tr>
<td>HEPES</td>
<td>N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid</td>
</tr>
<tr>
<td>hr</td>
<td>hour</td>
</tr>
<tr>
<td>IBMX</td>
<td>3-isobutyl-1-methylxanthine</td>
</tr>
<tr>
<td>IEF</td>
<td>isoelectric focusing</td>
</tr>
<tr>
<td>kDa</td>
<td>kilo Dalton</td>
</tr>
<tr>
<td>KT5823</td>
<td>(8R,9S,11S)-(−)-9-methoxy-9-methoxycarbonyl-8-methyl-2,3,9,10-tetrahydro-8,11-epoxy-1H,8H,11H-2,7b,11a-triazadibenzo(a,g)cycloocta(cde)-trinden-1-one</td>
</tr>
<tr>
<td>L</td>
<td>litre</td>
</tr>
<tr>
<td>m</td>
<td>milli (1 x 10^-3), if a prefix</td>
</tr>
<tr>
<td>M</td>
<td>molar</td>
</tr>
<tr>
<td>min</td>
<td>minute</td>
</tr>
<tr>
<td>ml</td>
<td>millilitre</td>
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</table>
mol  mole
MW  molecular weight
n  nano (1 x 10^-9)
°C  degrees Celsius
OD  optical density
ODQ  1H-[1,2,4]oxadiazolo[4,3-a]quinoxaline-1-one
p  pico (1 x 10^-12)
PAGE  polyacrylamide gel electrophoresis
PDE  phosphodiesterase
PE  phenylephrine
pI  isoelectric point
PKA  cAMP-dependent protein kinase
PKG  cGMP-dependent protein kinase
PKI  synthetic inhibitor of PKA
pGC  particulate guanylyl cyclase
SDS  sodium dodecyl sulfate
SEM  standard error of the mean
sGC  soluble guanylyl cyclase
SNAP  S-nitroso-N-acetylpenicillamine
SNP  sodium nitroprusside
TCA  trichloroacetic acid
TEMED  N,N,N',N'-tetramethylethylenediamine
V  volt
wt/vol  weight per volume
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DEDICATION

To my Mom and Dad,
and
my Sister and Brother,
for giving me the love and support to succeed

and

to my Wife and two Daughters,
I cannot imagine today or tomorrow without you.
1.0 INTRODUCTION

A review of the literature in the area of cGMP-mediated effects in smooth muscle will show that the current thinking regarding the role of this cyclic nucleotide in smooth muscle has undergone a complete reversal since the mid 1970's. Cyclic GMP was originally assumed to be a mediator of smooth muscle contraction (Goldberg et al., 1975) but is now believed to be an important mediator of smooth muscle relaxation. This cyclic nucleotide is synthesized by soluble and particulate guanylyl cyclases and is degraded by specific phosphodiesterases. Soluble guanylyl cyclase activity in smooth muscle is controlled primarily by nitric oxide (NO). This short-lived signal transduction molecule can be generated by endothelium stimulation with muscarinic agonists such as acetylcholine or by adding direct-acting nitrovasodilators such as sodium nitroprusside (SNP) and nitroglycerin. Particulate guanylyl cyclases produce intracellular increases in cGMP via extracellular receptor stimulation with natriuretic peptides such as atrial natriuretic peptide (ANP). When cGMP levels are elevated, cGMP can exert its effects through a variety of mediators including cGMP-gated ion channels, cGMP-regulated phosphodiesterases and cGMP-dependent protein kinases (PKG). During vascular smooth muscle relaxation, PKG has been described as the most important mediator of cGMP actions (Lincoln, 1989). Figure 1 illustrates the signal transduction pathways of nitrovasodilators, endogenous NO (EDRF) and ANP that lead to increases in tissue levels of cGMP through guanylyl cyclase activation. Cyclic GMP can then mediate smooth muscle relaxation via one or more of the mechanisms noted above (phosphodiesterases, PKG or ion channels).

Despite good evidence for a role for the cGMP/PKG pathway in relaxation of vascular smooth muscle, its role in regulating the relaxation of several non-vascular
Nitric oxide (NO) can be produced endogenously either by stimulation of specific receptors on the endothelium that lead to activation of constitutive nitric oxide synthase (c-NOS) and production of EDRF (NO) or by direct activation of c-NOS with its substrate L-arginine within smooth muscle cells. Endogenous NO, and NO derived from nitrovasodilators such as SNP and SNAP, selectively activate soluble guanylyl cyclase (sGC) leading to increased cGMP levels in smooth muscle. Atrial natriuretic peptide (ANP) elevates cGMP independently of sGC by activating a specific particulate guanylyl cyclase (pGC). When cGMP levels are elevated, numerous cGMP mediators including cGMP-gated ion channels, cGMP-regulated phosphodiesterases and cGMP-dependent protein kinase (PKG) can carry out cGMP actions and lead to smooth muscle relaxation. The most important of these cGMP mediators in smooth muscle is believed to be PKG. This protein kinase presumably mediates smooth muscle relaxation by phosphorylating specific target proteins that somehow regulate tension of smooth muscle.
smooth muscles remains controversial. In rat vas deferens and rat distal colon, NO donating agents such as SNP, and particulate guanylyl cyclase activators such as ANP, have been shown to markedly increase tissue levels of cGMP without relaxing the preparations (Diamond and Janis, 1978; Diamond, 1983; Suthamnatpong et al., 1993a,b; Patel et al., 1997). These results contrast with those observed in the rat aorta where small increases in cGMP produced by NO donors or natriuretic peptides are accompanied by significant relaxation. Thus, it appears that smooth muscles can be classified as either "responsive" or "non-responsive" with respect to whether or not they are relaxed by increases in tissue levels of cGMP. It has been suggested that the failure of "non-responsive" smooth muscles to relax in the face of elevated cGMP is due to an inability of the elevated cGMP to activate PKG. However, recent investigations in our laboratory have reported that in rat vas deferens and rat distal colon, PKG can be significantly activated without causing relaxation (Patel et al., 1997). If it is assumed that an elevation of cGMP and activation of PKG are responsible for vascular smooth muscle relaxation, then why do these "non-responsive" smooth muscles fail to relax?

Another more controversial "non-responsive" smooth muscle is the rat uterus. In myometrial preparations of uteri, NO donors have been shown to markedly increase tissue levels of cGMP without inhibiting spontaneous or KCl-induced contractions (Diamond, 1983). Conversely, several recent reports have described the presence of a NO/cGMP relaxation system in rat uteri and it has been linked to the maintenance of uterine quiescence during pregnancy (Yallampalli et al., 1993a,b; Izumi et al., 1993; Buhimschi et al., 1995). The results of these studies directly contrast with those observed in our earlier experiments in uterine smooth muscle. Thus, there is conflicting evidence in the literature concerning the role of cGMP in the control of uterine
relaxation. The following section will review the evidence for and against a role for cGMP in smooth muscle relaxation and provide a detailed analysis of the recent controversial data in uterine smooth muscle. A research proposal will also be presented designed to further investigate the role of cGMP in vascular smooth muscle relaxation and to attempt to resolve some of the apparent inconsistencies concerning the role of both cGMP and PKG in uterine contractility.

As described above, PKG has been suggested to be the most important mediator of cGMP actions in vascular smooth muscle. This protein kinase presumably elicits an effect by phosphorylating specific target proteins that somehow regulate tension of the muscle. There are several lines of evidence in support of this hypothesis (see Lincoln, 1996; Pfeifer et al., 1999 for review). However, the exact identities of those PKG target proteins involved in vascular smooth muscle relaxation and the mechanism(s) by which calcium levels are reduced remain unclear at this time. The following discussion will provide an in-depth review of the literature surrounding the possible targets and mechanisms of action of PKG and also present a proposal for investigating the intact smooth muscle substrates of this kinase in rat aorta using two dimensional gel electrophoresis. Since the lack of relaxation of "non-responsive" smooth muscles may be due to differing abilities of PKG to phosphorylate proteins in these tissues or to the absence of crucial PKG substrates in the tissues, experiments will also be proposed that compare PKG-mediated phosphorylation in "responsive" versus "non-responsive" smooth muscles.
1.1 Nitric oxide and cGMP-elevating systems.

1.1.1 Nitric oxide and nitric oxide synthases.

The physiological significance of NO-stimulated increases in cGMP has been the subject of intense interest since the discovery that mammalian vascular endothelial cells can produce and release a signal transduction molecule capable of modulating the tone of underlying smooth muscle. This molecule, first identified by Furchgott and Zawadski in 1980 as endothelium-derived relaxing factor (EDRF), was subsequently shown to be NO (Ignarro et al., 1987a; Palmer et al., 1987). Further investigations revealed that NO is formed within the endothelium via oxidation of a terminal guanidino nitrogen on L-arginine by the enzyme NO-synthase (NOS) (see Figure 1). A co-product produced from this amino acid breakdown is L-citrulline. There are three distinct NO synthases described in mammalian systems: first, a constitutive form, expressed in neurons, that synthesizes NO in response to calcium-promoted calmodulin binding (NOS-I or n-NOS); a second constitutively expressed form found in vascular endothelial cells that also binds calmodulin in a calcium-dependent manner to synthesize NO (NOS-III or c-NOS); and a third, inducible, form found in macrophages, vascular smooth muscle cells and hepatocytes that is activated independent of calcium by cytokines (NOS-II or i-NOS).

The details of the NOS reaction mechanism are reviewed elsewhere (see Sennequier and Stuehr, 1996; Schmidt et al., 1993). In brief, four co-factors (heme, FMN, FAD and H₄biopterin) and two co-substrates (O₂ and NADPH) are required for the reaction that proceeds by the hydroxylation of L-arginine to N⁰-OH-L-arginine, followed by the oxidation of N⁰-OH-L-arginine to L-citrulline and NO. Active NO synthases occur as homodimers and calcium-activated calmodulin binding is thought to initiate the electron
transfers necessary for the phase 1 hydroxylation of L-arginine described above (Abu-Soud and Stuehr, 1993).

The field of NO research has expanded well beyond its original boundaries of smooth muscle to include almost every system in mammalian models. NO has been suggested to affect neuronal functions in non-adrenergic non-cholinergic neurotransmission, synaptogenesis, sensory input, synaptic plasticity and wakefulness. NO may play a pathophysiological role in migraine, epilepsy, stroke and neurotoxicity. NO may be intimately involved in cell-mediated immune responses as well as inflammation and autoimmunity. In addition to NO-mediated effects, impairments in NO production may have serious implications. For example, impaired NO production by c-NOS in vascular endothelial cells has been suggested to have important implications in vascular diseases such as atherosclerosis, hypertension, diabetes and reperfusion injury (see Schmidt, 1994 for a review). In the proposed experiments of this thesis, we will focus on NO-mediated relaxation of smooth muscle using nitrovasodilators. In a whole animal model or in situ model with intact endothelium, NO production in vascular endothelial cells by c-NOS acts similarly to the nitrovasodilators used in my proposed experiments.

Constitutive NO synthase in the vascular endothelium can be stimulated to produce NO and induce smooth muscle relaxation by numerous hormones and pharmacological agents including acetylcholine, histamine, noradrenaline, the calcium ionophore A23187 and ATP (Rapoport and Murad, 1983; Rapoport et al., 1983; Lincoln 1989). These agents induce NO production within the endothelium by stimulating signal transduction pathways that increase calcium levels in the cell. Active calcium-calmodulin complexes that bind to NO synthase are subsequently produced and NO
production is initiated. Following its synthesis, NO diffuses randomly away from its point of synthesis to interact with various intracellular molecules (half life - 5 seconds). When calcium concentrations within the endothelium return to resting levels, NO synthesis is turned off.

1.1.2 Nitrovasodilators.

In order to selectively study the effects of NO-mediated increases in cGMP andPKG activity in smooth muscle, the endothelial cell lining was removed as these cells can contribute small but significant changes to biochemical measures such as cGMP concentration, PKG activity and PKG-mediated phosphorylation. With the endothelium denuded studies there is an increased demand for nitrovasodilators that generate NO in a controlled manner. Difficulties associated with delivering NO gas in solution or by inhalation have also increased the demand for these agents. Nitrovasodilators, which include compounds such as sodium nitroprusside (SNP), S-nitroso-N-acetylpenicillamine (SNAP) and nitroglycerin, have been shown to act through the release of NO, which is generated by either spontaneous or enzymatic breakdown of the original agent. Nitric oxide, but not the parent compound, is believed to be responsible for the activation of soluble guanylyl cyclase, the increased production of cGMP and the vascular smooth muscle relaxation induced by these agents (see Schmidt et al., 1993 for a review). The pathways that lead to NO formation can differ significantly between individual agents and in some cases the result is different rates of NO formation and side reactions such as nitrosation and nitration. In biological systems, the exact mechanisms of NO release from SNP are not completely understood. SNP releases NO by either irradiation with light or a one-electron reduction (Feelisch, 1998). This reduction reaction may occur via interactions with thiolate anions or thiols (Rochelle et al., 1994). SNAP undergoes
spontaneous homolytic and heterolytic cleavage to give rise to NO in solution (Arnelle and Stamler, 1995). However, SNAP may also be involved in transnitrosation reactions in which NO is transferred from one thiol to another. The ability to cause transnitrosation reactions is a feature that distinguishes S-nitrosothiols such as SNAP from other classes of NO donors (Feelisch, 1998). There are several pathways capable of inducing NO release from nitroglycerin in intact tissues. These include interactions with sulfhydryl groups, reactions with NADPH-dependent cytochrome P450 pathways (McDonald and Bennet, 1993) and interactions with glutathione S-transferases (Kenkare et al., 1994).

1.1.3 Soluble guanylyl cyclase.

Most of the functions of NO as a signaling molecule are mediated by the stimulation of the heme-containing enzyme, soluble guanylyl cyclase (sGC). This heterodimeric protein is made up two subunits, one $\alpha$ and one $\beta$. Each subunit contains a common C-terminal cyclase catalytic domain that is also present in membrane-bound guanylyl cyclases and adenylyl cyclases. Four different subunits of the sGC enzyme have been identified to date, including $\alpha_1$ (73 kDa), $\alpha_2$ (82 kDa), $\beta_1$ (70 kDa) and $\beta_2$ (70 kDa) (Koesling et al., 1991). However, the only sGC heterodimer combinations known to be active are $\alpha_1\beta_1$ or $\alpha_2\beta_1$ (Koesling et al., 1998). The dimerization partner of subunit $\beta_2$ has yet to be identified. Activation of sGC by NO is initiated by NO binding to the heme iron and proceeds via breaking of the histidine-to-iron bond. The resulting conformational change in sGC structure activates the catalytic site of the enzyme leading to increased cyclization of the $\alpha$-phosphate on GTP, forming cGMP. NO-mediated activation of sGC is the predominant cGMP-elevating mechanism throughout the body. However, NO-independent activation of sGC can also occur through another
group of sGC-activating-factors which includes carbon monoxide (CO) and hydroxyl radicals (OH) (Schmidt et al., 1991). CO is generated by heme oxygenase and OH formation can occur through the peroxynitrite pathway in the presence of superoxide (Beckman et al., 1990). In certain systems these alternative sGC activators may play important roles but in smooth muscle their effects are not clearly understood.

Significant advances in the understanding of the roles that sGC and cGMP play in smooth muscle relaxation have been provided by the discovery of the selective sGC inhibitor, 1H-[1,2,4]oxadiazolo[4,3-a]-quinoxalin-1-one (ODQ). This quinoxalin derivative binds to sGC in an NO-competitive manner and leads to an irreversible inhibition of stimulated sGC activity possibly by oxidation of the heme iron (Schrammel et al., 1996). Other less selective inhibitors of sGC such as methylene blue have been used in the past to inhibit increases in cGMP; however, this compound has also been shown to inhibit nitric oxide synthase activity in the endothelium (Mayer et al., 1993) and prostacyclin synthesis in endothelial cells (Martin et al., 1989).

1.1.4 Cyclic GMP-independent, NO-induced smooth muscle relaxation.

Nitric oxide is thought to relax smooth muscle by stimulating sGC, elevating cGMP and activating PKG which leads to phosphorylation of several intracellular substrates that somehow regulate smooth muscle tension. More recent studies have shown that NO, itself, may directly relax smooth muscle by activating membrane channels such as calcium-dependent potassium channels and causing hyperpolarization (Bolotina et al., 1994). Since this original discovery, several reports of direct calcium-activated potassium channel activation with NO have been described in human myometrium (Bradley et al., 1998), rat pulmonary artery (Zhao et al., 1997), rabbit carotid artery (Plane et al., 1998) and in vascular and tracheal smooth muscle of the
guinea pig (Bialecki and Stinson-Fisher, 1995). It has been suggested that a thiol-containing domain in calcium-activated potassium channels may interact with NO to accelerate disulfide bond formation and modulate channel opening (Stamler, 1994). It is important to note that calcium-activated potassium channel activity may also be effected by PKG-mediated phosphorylation (Alioua et al., 1998; Fukao et al., 1999). Despite the evidence for a cGMP-independent component in NO-induced relaxation of smooth muscle, a large portion of the relaxant response remains cGMP-dependent.

1.1.5 Particulate guanylyl cyclases.

A second form of guanylyl cyclase capable of elevating cGMP and relaxing smooth muscle are the particulate guanylyl cyclases (pGC). These membrane bound enzymes consist of two to four monomers with each monomer containing an extracellular natriuretic peptide receptor site with a transmembrane segment and an intracellular C-terminal catalytic cyclase domain. To date, six isoforms of pGC have been cloned (see Foster et al., 1999 for review). Two of these isoforms (pGC-A and pGC-B) are distributed throughout the body whereas the remaining four are either localized or still under investigation (Foster et al., 1999). Four different pGC activators have also been described, including ANP, BNP, CNP and guanylin. For my studies, I will focus on the atrial natriuretic peptide, ANP. This low molecular weight protein is synthesized by atrial cells and has a blood pressure lowering effect due to its ability to relax vascular smooth muscle. Upon binding of ANP to the extracellular receptor of pGC, a conformational shift is induced, resulting in an interaction between two catalytic domains (internal dimerization) of the enzyme (Foster et al., 1999). The intracellular cyclase domain is consequently activated and increased cyclization of the α-phosphate of GTP produces cGMP.
1.2 Evidence for and against a role for cGMP in smooth muscle relaxation.

The Yin Yang hypothesis of Goldberg et al. (1975) proposed that adenosine 3',5'-cyclic monophosphate (cAMP) was a mediator of smooth muscle relaxation and cGMP was a mediator of smooth muscle contraction. However, shortly after it was proposed, researchers began to produce results that did not match with the concept that cGMP was involved in smooth muscle contraction. Some of the first evidence against this hypothesis was provided by Diamond and co-workers, who demonstrated that cGMP levels were unchanged or decreased in spontaneous and KCl-induced contractions of rat uteri (Diamond and Hartle, 1974, 1976; Diamond and Holmes, 1975). Further investigations revealed that cGMP elevations during smooth muscle contraction occurred as much as 15 seconds after the initiation of contraction, suggesting that cGMP accumulation was a result of, rather than the cause of, contraction (Diamond and Hartle, 1976). As other researchers began to discover that cGMP might not be a mediator of smooth muscle contraction, Shultz et al. (1977) and Katsuki et al. (1977) proposed that cGMP accumulation was involved in smooth muscle relaxation, and thus the Yin Yang hypothesis was incorrect.

In order to confirm that cGMP is, in fact, the mediator of smooth muscle relaxation several criteria must be satisfied. These criteria were described for another cyclic nucleotide, cAMP, by Sutherland and co-workers in 1968 and summarized by Nakatsu and Diamond in 1989 with respect to cGMP. They include the following:

1.) The vasorelaxant drug must be capable of stimulating the activity of guanylyl cyclase in broken cell preparations of the target smooth muscle.
2.) Drug-induced relaxations of the smooth muscle must be well correlated with increases in the tissue levels of cGMP in both a time-dependent and dose-dependent manner.

3.) Agents that prevent the breakdown of cGMP, such as the phosphodiesterase inhibitor M&B22948 (zaprinast), should potentiate smooth muscle relaxation by cGMP-elevating agents.

4.) Agents that interfere with the synthesis of cGMP, such as ODQ, should inhibit the relaxing effects of the drug.

5.) Analogs of cGMP, such as 8-bromo-cGMP, should be able to mimic smooth muscle relaxations induced by cGMP-elevating agents.

1.2.1 Criterion one.

Ever since cGMP was proposed as a mediator of smooth muscle relaxation, volumes of literature began to appear in support of this new hypothesis. In order to present the results that satisfy the criteria above, only key representative studies will be described. Numerous smooth muscle relaxing agents are now available that activate sGC and satisfy criterion one. They include S-nitrosothiols such as SNAP and S-nitrosoglutathione, organic nitrates such as nitroglycerin, sydnonimines such as 3-morpholino-sydnonimine (SIN-1), NONOates such as diethylamine NO (DEA/NO) and SNP. All of these agents activate sGC by releasing NO. The literature confirming the ability of NO donors to activate sGC has been extensively reviewed by Waldman and Murad in 1987 and by Koesling and Friebe in 1999. A relatively new compound capable of activating sGC, independent of NO, is 3-(5′-hydroxymethyl-2′-furyl)-1-benzyl-indazol (YC-1). YC-1 increases cGMP levels and mimics many of the known effects of NO and NO donors, including inhibition of vascular smooth muscle contraction (Mulsch et al.,
1997). Further support for criterion one is gained from the ability of the smooth muscle relaxant, ANP, to activate pGC (Winquist et al., 1984). The evidence in support of criterion one clearly demonstrates that agents capable of increasing cGMP do so by activating guanylyl cyclase.

1.2.2 Criterion two.

Nitrovasodilator-induced smooth muscle relaxation was originally shown to correlate with increases in cGMP by Diamond and co-workers using rat uteri and canine femoral artery (Diamond and Holmes 1975; Diamond and Hartle, 1976) with additional evidence provided by Katsuki et al. (1977) in bovine trachea, guinea pig trachea and guinea pig taenia cecum. Cyclic GMP elevations in bovine coronary arteries induced by nitroglycerin, SNP and sodium nitrite were subsequently shown to be well correlated with relaxation in dose- and time-dependent manners (Kukovetz et al., 1979). Additional experiments in bovine coronary arteries (Gruetter et al., 1981), bovine mesenteric artery (Axelsson et al., 1979), rat thoracic aorta (Keith et al., 1982) and in guinea pig ileum (Keith et al., 1983) demonstrated that cGMP elevations precede relaxation and are well correlated with smooth muscle relaxation. However, a few of these early studies also described data not supporting a correlation between cGMP level and percent relaxation. Axelsson et al. (1979) and Keith et al. (1983,1983) reported that nitroglycerin induced rapid, significant elevations of cGMP within 15 seconds that returned to control levels by 2 minutes. However, relaxation was well maintained after the decrease in cGMP concentration. Similar evidence not supporting criterion two was demonstrated in bovine coronary arteries, where vessels without endothelium did not relax in response to acetylcholine-induced increases in cGMP (Holzmann, 1982). More recent studies utilizing the selective guanylyl cyclase inhibitor ODQ have also
demonstrated poor correlations between cGMP concentration and percent relaxation in rat mesenteric and rabbit carotid artery (Plane et al., 1996; Plane et al., 1998). A detailed analysis of the earlier non-supporting results by Nakatsu and Diamond (1989) concludes that the cGMP hypothesis is not necessarily disproved by these results and suggests several possible reasons for the lack of linearity between cGMP levels and percent relaxation including insufficient cGMP detection sensitivity, localized high concentration cGMP pools, cGMP-independent mechanisms of relaxation and the extent of pre-contraction with stimulant drugs. The discovery of EDRF and its identification as NO and the many studies correlating this smooth muscle relaxation pathway with cGMP elevation provide good support for criterion two. Several investigators made significant contributions to elucidating this mechanism including Holzmann (1982), Diamond and Chu (1983), Rapoport and Murad (1983) and Ignarro et al. (1984). Although there is strong evidence in support of a correlation between cGMP elevation and smooth muscle relaxation (criterion two), caution must be noted that this is not enough to prove a causal relationship. Certainly, the non-supporting data described above and the evidence in non-vascular smooth muscles that follows attest to this fact.

1.2.2.1 Non-supporting evidence in non-vascular smooth muscle.

In the smooth muscles of the rat vas deferens, distal colon and uterus, very large increases in cGMP content, produced by drugs such as SNP, nitroglycerin, ANP and NO, do not correlate well with the mediation of relaxation (Diamond and Janis, 1978; Diamond, 1983; Suthamnatpong et al., 1993a,b; Patel et al., 1997). Elevations of cGMP by SNP were as high as 16-fold in rat vas deferens, but this did not relax or prevent phenylephrine-induced contractions (Diamond and Janis, 1978). In the rat colon, NO and ANP produce variable effects depending on whether the proximal or distal segment
of the organ is studied. Distal portions treated with NO or ANP fail to relax in the presence of elevated cGMP, whereas proximal portions do undergo NO-induced and ANP-induced relaxation (Suthamnatpong et al., 1993a,b; Patel et al., 1997). The results of these studies in rat vas deferens and distal colon suggest that certain smooth muscles can be designated as "non-responsive" in comparison to other smooth muscles in which the majority of evidence supports a role for cGMP as a mediator of relaxation.

In rat uterus, the role of cGMP in regulating smooth muscle tension is somewhat controversial. Myometrial preparations have been shown to develop 2-fold and 6-fold increases in cGMP induced by 0.5 and 5 mM SNP, respectively, without any inhibition of spontaneous contractions (Diamond, 1983). This early study lead to the classification of the uterus as another "non-responsive" smooth muscle. More recently, additional evidence was reported to support the classification of the uterus as "non-responsive". Kuenzli et al. (1996) demonstrated that elevations in cGMP induced by another NO donor, S-nitroso-L-cysteine, were not accompanied by inhibition of spontaneous contractions in pregnant or non-pregnant guinea pig myometrium, although drug-induced contractions were inhibited by the compound. The blockade of the cGMP elevation by methylene blue did not block this relaxant effect of S-nitroso-L-cysteine, indicating that cGMP elevation was not required for relaxation of drug-induced contractions.

In contrast to these results, a number of recent reports have suggested that cGMP may play an important role in the control of uterine motility. For example, ANP has been shown to completely inhibit spontaneous contractions of myometrial strips from estrogen-treated virgin rats and from sterile horns of 10-14 day pregnant rats (Potvin and Varma, 1990). This tocolytic effect of ANP was abolished in myometria
exposed to exogenous or placentally-produced progesterone and this loss of effect was concluded to be the result of decreases in particulate guanylyl cyclase activity and cGMP elevation (Potvin and Varma, 1990; Potvin et al., 1991).

In another series of studies, Yallampalli et al. (1993a & b), Izumi et al. (1993) and Buhimschi et al. (1995) have reported that a NO/cGMP relaxation system is present in human and rat pregnant uteri, and that it plays an important role in inhibiting spontaneous contractions. These studies also concluded that the NO/cGMP pathway may be responsible for maintaining a quiescent uterus during pregnancy and that a decrease in uterine responsiveness to the relaxant effects of NO at term may lead to the initiation of labor. The latter conclusion is in direct contrast to the results of Potvin and Varma (1990) and Potvin et al. (1991) who reported that progesterone administration and/or late stage pregnancy were accompanied by decreases in particulate guanylyl cyclase activity and cGMP elevation.

Thus, there is conflicting evidence in the literature concerning the role of cGMP in the control of uterine motility and the effects of changes in hormonal status on this process. One objective of the experiments proposed in this thesis will be to further investigate the importance of cGMP in relaxation of uterine smooth muscle.

1.2.3 Criterion three.

Several phosphodiesterase (PDE) enzymes are involved in cGMP signaling. These enzymes hydrolyze cGMP and cAMP to 5'-nucleotides or are activated by cGMP to hydrolyze cAMP. In mammals, 19 different genes have been described that encode for ten distinct PDE gene families (types): PDE1 to PDE10 (Dousa, 1999; Conti and Jin, 1999; Soderling et al., 1999). Types 5, 6 and 9 selectively breakdown cGMP with type 5 being abundant in smooth muscle. Types 3, 4, 7, and 8 are selective for cAMP and the
remaining enzymes accept both cyclic nucleotides as substrates (Dousa, 1999). The
type 5 PDE inhibitor, M&B22948 (zaprinast), has been an effective tool for satisfying
criterion three. In 1982, this compound was shown to reduce the contractile responses
of bovine coronary artery and increase cGMP levels (Holzmann, 1982). Similar
relaxations and increases in cGMP were produced in bovine intrapulmonary arteries and
veins with zaprinast (Ignarro et al., 1987b) and in pig coronary arteries with the PDE
inhibitor, methyl-isobutylxanthine (Lorenz and Wells, 1983). From these reports it would
appear that criterion three is satisfied with respect to PDE-mediated increases in cGMP
being capable of mediating smooth muscle relaxation. However, in one particular study
less conclusive evidence was found in support of a role for cGMP using zaprinast. In
this study, Schoeffter et al. (1987) demonstrated that in rat aorta zaprinast significantly
increases cGMP levels while producing a small (10%) relaxation (Schoeffter et al.,
1987). Similar levels of cGMP produced by SNP resulted in approximately 50%
relaxations. In addition, the presence of zaprinast during SNP-induced relaxations had
no effect on the relaxant response (Schoeffter et al., 1987). It is important to note that
subsequent investigations in our lab revealed that zaprinast may have non-specific
effects on other enzymes including phospholipase C (Diamond, unpublished data).

1.2.4 Criterion four.

Several drugs have been used in the past to inhibit cGMP synthesis including
methylene blue, hemoglobin, cyanide and 6-anilino-5,8-quinolinedione (LY83583). Methylene blue blocks cGMP synthesis by inhibiting sGC and is the most commonly
used drug from this group (Gruetter et al., 1979). The first studies utilizing methylene
blue were carried out in bovine coronary artery (Gruetter et al., 1981) and guinea pig
ileum (Keith et al., 1983) with similar results. Methylene blue significantly decreased
both the elevation in cGMP and percent relaxation induced by nitrovasodilators. As mentioned earlier, methylene blue has recently been demonstrated to have several non-specific effects on other enzymes in addition to its ability to inhibit sGC. As a result, more recent experiments evaluating the effects of blocking cGMP synthesis through the EDRF/NO relaxation pathway and with the novel inhibitor, ODQ, provide the best evidence in support of criterion four and the cGMP hypothesis. In acetylcholine-, ATP- and calcium ionophore A23187-mediated vascular smooth muscle relaxation, EDRF/NO is generated within vascular endothelial cells by c-NOS and diffuses into underlying smooth muscle to activate sGC and elevate cGMP. Several studies have demonstrated that inhibition of acetylcholine, calcium ionophore A23187 and ATP signaling with agents such as LY83583, quinacrine, bromphenacyl bromide and 5,8,11,14-eicosatetraynoic acid (ETYA) inhibits both the relaxation and the increase in cGMP levels induced by these agents which subsequently blocks the relaxation (Furchgott, 1983; Rapoport and Murad, 1983; Ignarro et al., 1984; Diamond, 1987). It is important to note that although the inhibitors used above did block the signal transduction events leading to c-NOS activation in the endothelium, the non-specific, multiple effects of these drugs make it difficult to determine the exact pathway of inhibition.

Experiments utilizing the selective sGC inhibitor, ODQ, provide strong support for the role of cGMP in smooth muscle relaxation. In precontracted rat aorta, ODQ attenuates relaxations induced by DEA/NO, nitroglycerin and by NO generated after the induction of c-NOS (Moro et al., 1996; Olson et al., 1997). In bovine pulmonary artery, both cGMP elevation and relaxation were antagonized by ODQ in a concentration-dependent manner (Brunner et al., 1996). Criterion four is clearly satisfied by these results.
1.2.5 Criterion five.

In the final criterion, it is suggested that analogs of cGMP, such as 8-bromo-cGMP, should be able to mimic smooth muscle relaxation induced by cGMP-elevating agents. The first demonstration of 8-bromo-cGMP-induced smooth muscle relaxation reported that 1 to 100 μM concentrations induced concentration-dependent relaxations of rat and rabbit aorta, as well as rat uterus and rat vas deferens (Shultz et al. 1979). Several investigations that followed showed 8-bromo-cGMP-induced relaxation of numerous blood vessels (Rapoport et al., 1982; Lincoln, 1983). The results of these studies satisfy the final criterion described by Sutherland et al. (1968) and complete a large amount of evidence that supports a role for cGMP as a mediator of smooth muscle relaxation. Throughout the above review results not supporting the cGMP hypothesis have also been described. In many cases, these opposing results occur in non-vascular smooth muscles. In the original cGMP hypothesis proposed by Schultz et al. (1977) and Katsuki et al. (1977) it was assumed that cGMP mediated the relaxation of all smooth muscles. However, the results described in the non-vascular smooth muscles above indicate that the cGMP hypothesis may only apply to certain types of smooth muscle such as vascular tissues. A further analysis of the downstream effects mediated by an increase in cGMP should confirm whether or not the cGMP hypothesis does not apply to the "non-responsive" non-vascular smooth muscles mentioned above.

1.3 Cyclic GMP-dependent protein kinase (PKG).

There is good evidence in the literature favoring a role for PKG in cGMP-mediated relaxation. PKG is believed to regulate 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, threonine and tyrosine residues on target substrates.
PKG exists as a dimer and is activated in a two step process. First, cGMP binds at site 1 eliciting a charge shift within PKG that initiates displacement of a pseudosubstrate inhibitory domain (I) from the catalytic domain (C) giving rise to a partially active form of the kinase that has approximately 50% of the activity found in a fully activated, cGMP bound PKG. The displacement of the pseudosubstrate inhibitory domain primes site 2 for cGMP binding and when site 2 is saturated PKG becomes fully active (Scott, 1991). The reaction can be depicted as follows:

\[
(\text{IC})_2 + 4\text{cGMP} \leftrightarrow (\text{IC})_2 \cdot \text{cGMP}_2 + 2\text{cGMP} \leftrightarrow (\text{IC})_2 \cdot \text{cGMP}_4
\]

inactive \hspace{1cm} partially active \hspace{1cm} fully active

Two separate isoenzymes of PKG have been cloned and expressed in mammalian cells, type I and type II (Wernet et al., 1989; Sandberg et al., 1989). Type I is a homodimer that displays alternate mRNA splicing to produce two isoforms Iα and Iβ with molecular weights of 76 and 78 kDa, respectively (Wernet et al., 1989; Sandberg et al., 1989). These two isoforms of PKG are cytosolic and found in abundance in smooth muscle cells, platelets and Purkinje cells (Lincoln et al., 1988). It is important to note that type Iβ requires ten-fold higher levels of cGMP for activation than type Iα (Schmidt et al., 1993). Type II PKG is a membrane-bound, 86 kDa enzyme, found mainly in intestinal epithelial cells (deJonge, 1981).

1.4 Evidence that increased PKG activity is well correlated with an increase in cGMP and smooth muscle relaxation.
The activity of PKG is measured in the absence and presence of added cGMP and data are expressed as activity ratios. Until recently, it has been extremely difficult to accurately measure the activation state of PKG in intact smooth muscles, and very few studies have been published to date in which PKG activity has been determined following induction of smooth muscle relaxation. Early studies in rat aorta by Fiscus et al. (1983, 1985) did report SNP-, acetylcholine- and ANP-induced increases in PKG activity that correlated well with elevated cGMP and smooth muscle relaxation. However, the substrate utilized in these kinase assays (histone H2B) was not very specific for PKG, increasing the chance of cGMP-independent phosphorylation of the substrate. An updated PKG assay, described by Jiang et al. (1992), utilizes a newly developed PKG substrate named BPDEtide (RKISASEFDRPLR) which is 16-fold more specific for PKG than histone H2B. The specificity of this substrate for PKG was revealed when the addition of the cAMP-dependent protein kinase (PKA) inhibitor, PKI, did not affect the basal phosphorylation of BPDEtide (Colbran et al., 1992). In the original assay, basal histone H2B phosphorylation decreased 40% with the addition of PKI (Colbran et al., 1992). Using this updated PKG assay, Patel and Diamond (1997) have demonstrated that PKG activity is increased during nitrovasodilator-induced rabbit aorta smooth muscle relaxation. PKG activity was measured with various concentrations of SNP and nitroglycerin for varying times and PKG activity was found to increase in a concentration- and time-dependent manner. Although this does not prove a causal relationship between cGMP elevation, PKG activation and smooth muscle relaxation, it is consistent with the proposed role of PKG as a mediator of cGMP actions.

Further evidence in support of the involvement of PKG in cGMP-mediated vasodilation is provided by Cornwell and Lincoln (1989). In this study, ANP and 8-
bromo-cGMP were shown to lower intracellular calcium levels in primary rat aortic cells using a calcium-sensitive dye, fura-2. Since the removal of intracellular calcium induces smooth muscle relaxation, this was concluded to be an indicator of the ability of these agents to relax aortic cells. In the same study, following several cell passages, the levels of PKG were found to be diminished, and these passaged cells were unresponsive to stimulation with ANP and 8-bromo-cGMP. Addition of PKG to the cytosol through osmotic lysis restored the calcium lowering actions of these drugs, confirming that PKG is required for the calcium sequestering and assumed relaxant responses of these agents. In an additional study, using still another approach, Francis et al. (1988) demonstrated that the relative potencies of a series of cyclic nucleotide analogs as smooth muscle relaxants correlated well with their relative potencies as activators of partially purified PKG. These data support a role for PKG as well as cGMP in smooth muscle relaxation.

1.5 PKG activity in "non-responsive" non-vascular smooth muscles.

In the rat vas deferens and distal colon, SNP and ANP increased cGMP levels without relaxing the preparations (Patel et al., 1997). The possibility was considered that the lack of relaxation observed in these tissues, in spite of a marked elevation of cGMP, may be due to a failure of cGMP to activate PKG. However, Patel et al. (1997) demonstrated significant activations of PKG in both smooth muscles. Therefore, a failure to activate PKG cannot be the cause of the inability of cGMP-elevating agents to mediate smooth muscle relaxation. Although these experiments do not answer the question as to why these smooth muscles remain "non-responsive" with respect to increases in cGMP, they do indicate that PKG is activated during an elevation in cGMP in these tissues.
The literature above provides good evidence that PKG is, in fact, a mediator of cGMP actions and possibly smooth muscle relaxation. PKG presumably acts by phosphorylating protein targets within the cell that somehow regulate the tension of the muscle (for example by decreasing cytoplasmic calcium). The next logical step is to determine what these PKG substrates are and their role in smooth muscle relaxation. As vascular smooth muscles appear to show the best correlation between cGMP elevation, PKG activation and relaxation, they are the prime tissue to begin investigating the phosphorylation targets of PKG. In the non-responsive smooth muscles of the rat vas deferens and distal colon, where cGMP elevation and PKG activation are well correlated but fail to produce relaxation, it would also be of interest to determine which PKG targets are phosphorylated, because the absence of crucial kinase substrates could explain the non-responsive nature of these tissues.

1.6 In vitro PKG-mediated phosphorylation.

PKG-mediated phosphorylation can be monitored by incorporating radiolabeled inorganic phosphate $^{32}$P into the ATP pool of smooth muscle cells. When the cells are homogenized and PKG is activated the patterns of in vitro phosphorylation induced by PKG can be assessed by separating proteins with one- or two-dimensional polyacrylamide gel electrophoresis and exposing gels to x-ray film for autoradiography. Although in vitro preparations are less technically demanding to use compared to intact tissue preparations, caution must be used in interpreting these results as they may not reflect the normal situation in intact tissues. Several proteins have been identified using these in vitro techniques. Casnelli et al. (1980) demonstrated increased phosphorylation of four particulate fraction proteins in rat and rabbit aorta with molecular weights (MW) of 250 kDa, 130 kDa, 85 kDa and 75 kDa. Additional analysis in the
rabbit aorta revealed that the proteins described above are also phosphorylated by PKA (Parks et al., 1987). The 130 kDa protein was later suggested to be the plasmalemmal calcium pump itself (Furukawa and Nakamura, 1987) or an associated enzyme such as phosphatidylinositol kinase that indirectly stimulates calcium-ATPase activation (Vrolix et al., 1988). Subsequent studies showed that ANP stimulated the phosphorylation of three proteins with MW's of 225 kDa, 132 kDa and 11 kDa, with the latter not having been described previously (Sarcevic et al., 1989). The 11 kDa protein was suggested to be phospholamban, a relatively well known PKA substrate capable of modulating the activity of sarcolemmal calcium-ATPase and stimulating calcium uptake into the sarcoplasmic reticulum (Sarcevic et al., 1989; Huggins et al., 1989). The 250 kDa protein was later shown to be a splice variant of the type 1 inositol 1,4,5-trisphosphate (IP₃) receptor which when phosphorylated by PKG stimulates sarcoplasmic reticulum calcium-ATPase activity (Yoshida et al., 1991; Yoshida et al., 1999; Fujita et al., 1999).

A more complete analysis of the in vitro substrates of PKG was provided by Li et al. (1996). In this study, heat and acid treatments were used to remove other endogenous protein kinase activity and to partially purify the proteins. The result was detection of 34 PKG substrates, 3 of which were previously identified. These 34 PKG substrates were separated in several different fractions, and it was reported that 17 of the substrates were heat stable, 9 were heat stable and also acid soluble, and the remaining were detected in a final chromatographic separation (Li et al., 1996). Further investigation into the PKG-specificity of these substrates revealed that 7 proteins (40, 33, 28, 25, 24, 23, 22 kDa) from the heat stable group were poorly phosphorylated by PKA and not at all by PKC (Li et al., 1996). In addition, these seven proteins were the most abundant of all the substrates identified (Li et al., 1996). Most of the remaining
substrates showed little specificity for PKG over PKA or PKC. In this study, no attempt was made to identify any of the proteins. Some of the proteins identified above as PKG-substrates have also been verified as substrates in intact tissue experiments. A review of this work appears below.

1.7 PKG-mediated phosphorylation in intact tissues: proposed mechanisms of relaxation.

Over the last 15 years a growing body of literature has emerged investigating PKG-mediated phosphorylation in intact tissues. In 1982, using intact tissue, Rapoport et al. demonstrated that SNP induced a concentration-dependent increase in incorporation of $[^{32}\text{P}]$ phosphate into nine proteins and a decrease in incorporation into two proteins. It is unclear if the phosphorylation of these substrates occurs through a cGMP-dependent or independent mechanism, as no inhibitors of sGC were used prior to the addition of SNP. However, the patterns of phosphorylation were mimicked by exposure to 8-Bromo-cGMP. The specificity of these substrates for PKG is questionable as PKA was also shown to partially mimic the patterns of phosphorylation. No attempt was made to quantitate the increases in phosphorylation observed in this study and none of the proteins of interest were identified.

Considerable research regarding the role of PKG-mediated phosphorylation in intact smooth muscle has focused on the effects of PKG on myosin light chain phosphorylation. Using one dimensional gel electrophoresis and non-labeled tissue samples, Johnson and Lincoln (1985) demonstrated that SNP, GTN and 8-Br-cGMP inhibit phosphorylase a formation and myosin light chain phosphorylation in vivo. Myosin light chains have an approximate molecular weight of 20 kDa and isoelectric points (PI) of between 5.2 and 5.3. The presence of phosphate was determined by
molecular weight shifts on a low percentage acrylamide gel. However, it is unclear if the effects of these drugs are direct actions of PKG or simply a result of the decrease in intracellular calcium that occurs during smooth muscle relaxation. In similar studies using intact rabbit aorta and intact rabbit femoral arteries, ANP and 8-bromo-cGMP, respectively, were demonstrated to inhibit the extent of myosin light chain phosphorylation during transient calcium release by either inhibiting the calcium-dependent activation of myosin light chain kinase or by stimulating phosphoprotein phosphatase activity (Paglin et al., 1988; McDaniel et al., 1992; Lincoln and Cornwell, 1993; Lee et al., 1997). In yet another analysis of PKG-mediated inhibition of myosin light chain phosphorylation in porcine coronary artery, Ishibashi et al. (1995) identified six PKG substrates phosphorylated in response to nitroglycerin-induced relaxation including myosin light chain (20 kDa - PI 5.2), desmin (57 kDa - PI 5.4), synemin (230 kDa - PI 5.3) and three unidentified proteins all at a molecular weight of 27 kDa with PI's of 5.7, 5.9 and 6.1. The fact that PKG had phosphorylated two intermediate filament proteins, desmin and synemin, lead this group to conclude that myosin light chain phosphorylation may not be directly inhibited or decreased by activation of myosin light chain phosphatase, but instead cross-bridge attachments may be blocked by intermediate filament phosphorylation leading to a decrease in tone.

In a very recent report, it has been shown that PKG is targeted to the smooth muscle cell contractile apparatus by a leucine zipper interaction with the myosin binding subunit of myosin phosphatase (Surks et al., 1999). A disruption of this interaction prevents the dephosphorylation of myosin light chain, suggesting that this interaction plays an important role in the relaxation process (Surks et al., 1999). Since PKG has been demonstrated to increase myosin phosphatase activity in the experiments
described above, it is possible that this novel interaction co-localizes PKG with its substrate. In contrast, Nakamura et al. (1999) has shown that myosin light chain phosphatase phosphorylation by PKG does not lead to an active form of the enzyme. PKG-mediated phosphorylation of a small acidic protein (17-18 kDa), telokin, has been demonstrated to enhance myosin phosphatase acivity, which supports the above studies suggesting a role of phosphatase activation in PKG-mediated relaxation (Wu et al., 1998). Taking into account all the results described above concerning changes in myosin light chain phosphorylation during relaxation, it is more than likely that this dephosphorylation event plays an important role in the control of smooth muscle tone. Whether the dephosphorylation of myosin light chain is simply a result of the decrease in intracellular calcium during relaxation or a result of PKG-mediated phosphorylation remains unknown. The fact that PKG has been shown to phosphorylate myosin light chain phosphatase, myosin light chain kinase and intermediate filaments that interfere with cross-bridge formation indicates that a combination of calcium-mediated decreases and PKG-mediated decreases in myosin light chain phosphorylation could contribute to the final relaxation response.

Numerous studies with NO, cGMP-elevating agents, cGMP analogs and PKG have identified calcium-activated potassium channels as principal contributors to the smooth muscle relaxant response induced by these agents (Tare et al., 1990; Hamaguchi et al., 1991; Robertson et al., 1993; Bolotina et al., 1994; Archer et al., 1994). Although NO has been shown to activate calcium-activated potassium channels without the presence of cGMP, several reports have indicated that a portion of channel activation requires cGMP elevation and PKG activation. Alioua et al. (1995) and Fukao et al. (1999) have shown direct PKG phosphorylation of the α-subunit of the channel in
broken cell preparations from tracheal smooth muscle and HEK293 cells, respectively. The approximate molecular weight of the α-subunit is 125 kDa. To further support this relaxation pathway, PKG-mediated phosphorylation of calcium-activated potassium channels has been described in whole *Xenopus* oocytes genetically altered to express the channel (Alioua et al., 1998). However, it remains to be determined if these channels can be activated in intact smooth muscles during cGMP-mediated relaxation.

In 1991, Cornwell et al. used one dimensional gel electrophoresis of proteins from vascular smooth muscle cells to show that phospholamban phosphorylation is increased following the activation of PKG, and that this increase in phosphorylation is well correlated with an increase in sarcoplasmic reticulum calcium-ATPase activity. In this paper, confocal laser scanning microscopy was also used to show co-localization of PKG with calcium-ATPase pumps on the sarcoplasmic reticulum. In 1994, Komalavilas and Lincoln reported that PKG phosphorylates the IP$_3$ receptor *in vivo* with high affinity. The IP$_3$ receptor subunit has an approximate molecular weight of 260 kDa on SDS-PAGE. This intact tissue phosphorylation is supported by several *in vitro* studies described above in which the IP$_3$ receptor was also described as a PKG substrate. Since PKG may be co-localized to the sarcoplasmic reticulum, it is possible that it may be targeting the IP$_3$ receptor as well as phospholamban. The IP$_3$ receptor is also a substrate for PKA, PKC and calcium-calmodulin-dependent protein kinase, thus raising the question as to the functional importance of a PKG-mediated phosphorylation of this substrate. Lincoln and colleagues also reported in 1995 that PKG phosphorylates, and is anchored to, vimentin in vascular smooth muscle cells (Pryzwansky et al., 1995). This 57 kDa protein is located in the perinuclear regions of the cell, which may explain how PKG is co-localized to the sarcoplasmic reticulum. The catalytic fragment of PKG does
not bind to vimentin; therefore, PKG can remain active in phosphorylating its substrates while anchored (Pryzwansky et al., 1995).

In another study using two dimensional gel electrophoresis and labeling of intact tissues, Bergh et al. (1995) compared the cyclic nucleotide-dependent vasorelaxation of human umbilical artery smooth muscle (HUASM) with that of bovine carotid artery smooth muscle (BCASM). HUASM fails to relax in response to a SNP-mediated increase in cGMP and can be classified as "non-responsive", whereas BCASM relaxes well. By comparing the phosphorylation patterns from the two different tissues they found that two 20 kDa proteins were phosphorylated in BCASM and not in HUASM. In a subsequent publication, these proteins were sequenced and found to share sequence homology with heat shock protein 20 (HSP20), a recently identified protein (Beall et al., 1997). These heat shock proteins have also been shown to be substrates of PKA in intact BCASM (Beall et al., 1999). The exact function of the HSP family of proteins is unknown, but it has been suggested that they assist in the assembly, disassembly, stabilization and transport of intracellular proteins (Beall et al., 1997). Recent evidence suggests that HSP's 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 (Beall et al., 1997). Additional studies have shown that

Despite good evidence in support of a role for PKG in mediating the effects of cGMP that lead to smooth muscle relaxation, it is still unclear which mechanisms of relaxation activated by PKG-mediated phosphorylation result in relaxation. This is due in part to a difficulty in directly correlating PKG-mediated phosphorylation with smooth muscle relaxation. In the research proposed in this thesis, phosphorylation patterns in
cGMP-responsive and non-responsive tissues will be compared extensively following treatment with cGMP-elevating agents. The absence or relatively low levels of phosphorylation in non-responsive smooth muscles may give us an indication of the requirement for such phosphorylated proteins in cGMP-induced relaxation.

1.8 Summary and rationale for proposed experiments.

The criteria have been satisfied to support a role for cGMP in vascular smooth muscle relaxation. The discovery of the intracellular messenger NO and the enzymes that lead to its endogenous production have led to the recognition of the importance of cGMP signal transduction. Despite evidence for a role for cGMP in vascular smooth muscle, numerous studies in the non-vascular smooth muscles of rat vas deferens, distal colon and uterus indicate that smooth muscles may be classified as either "responsive" or "non-responsive" with respect to whether or not they are relaxed by increases in cGMP. Recent controversy concerning the role of cGMP in rat uterus indicates that more studies are required to resolve some of the apparent inconsistencies in this smooth muscle.

PKG activity is well correlated with increases in cGMP in both responsive, vascular smooth muscles and non-responsive, rat vas deferens and distal colon smooth muscles. Thus, a lack of PKG activation cannot be used to explain the failure of rat vas deferens and distal colon to relax in response to an elevation of cGMP. It remains to be determined if a lack of PKG activation in the presence of cGMP in rat uterus can be used to explain the failure of this smooth muscle to relax. PKG is believed to mediate its effects by phosphorylating specific proteins that somehow regulate smooth muscle tension. In the literature review above, numerous possible mechanisms and substrates were described that may or may not contribute to the relaxant response induced by PKG.

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in smooth muscle. Only a few of the studies were able to demonstrate phosphorylation of PKG substrates in intact tissue. Clearly, more research is needed in the area of PKG-mediated phosphorylation, especially with respect to identifying which events are important to the control of smooth muscle tension.

1.8.1 Specific objectives of proposed research.

Most of the overall objectives have been alluded to in preceding sections. The specific objectives are as follows:

1.) Measure contractile responses and cGMP levels in rat myometrial preparations following treatment with SNP, ANP, L-arginine and SNAP. Also measure contractile responses to the cGMP analog, 8-bromo-cGMP. Determine whether or not changes in hormonal status can alter the ability of these agents to induce inhibition of spontaneous contractions in the myometrium. Investigate the importance of cGMP in the actions of those agents capable of relaxing myometrium using the sGC inhibitor, ODQ.

2.) Measure myometrial PKG activity ratios in the presence of SNP-induced elevations of cGMP to determine if the non-responsive nature of the uterus occurs due to a lack of PKG activation.

3.) Measure contractile responses and cGMP levels in rat thoracic aortae exposed to SNP, ANP and SNAP. Also measure contractile responses to 8-bromo-cGMP. Investigate the importance of cGMP in relaxant responses using the sGC inhibitor, ODQ.

4.) Measure PKG activity ratios in rat aortae in response to SNP to determine total levels for comparison with other smooth muscles.
5.) Use high resolution two-dimensional gel electrophoresis to characterize the patterns of PKG-mediated phosphorylation in intact rat aorta following relaxation with SNP, ANP, 8-bromo-cGMP and SNAP. Confirm PKG-mediated phosphorylation with the sGC inhibitor, ODQ, where appropriate. Quantitate the degree of phosphorylation using densitometry.

6.) Use high resolution two-dimensional gel electrophoresis to characterize the patterns of PKG-mediated phosphorylation in intact rat vas deferens, distal colon, proximal colon and uterus following treatment with SNP, 8-bromo-cGMP and SNAP. Confirm PKG-mediated phosphorylation using ODQ where appropriate. Compare patterns of phosphorylation in non-vascular smooth muscles with those obtained in vascular smooth muscles to determine which protein(s), if any, show phosphorylation in responsive versus non-responsive muscles.
2.0 MATERIALS AND METHODS

2.1 Chemicals and materials.

Chemicals and materials were purchased from the following sources:

Amersham Pharmacia Biotech, Inc. (Baie d'Urfe, Quebec, Canada)

$[^{32}P]$ Adenosine 5'-triphosphate, BIOTRAK® cGMP scintillation proximity assay kit, cellulose sheets, isoelectric focusing carbamoylation standards, $[^{32}P]$ orthophosphate.

Bachem California (Torrence, CA, U.S.A.)

Cyclic GMP-dependent protein kinase substrate (BPDEtide), atrial natriuretic peptide (rat ANP, 1-28 amino acids).

BDH Inc. (Vancouver, BC, Canada)

Sodium hydrogen carbonate, d-glucose, sodium dihydrogen orthophosphate dihydrate, magnesium chloride, sodium hydroxide, sodium fluoride, ethylene glycol-bis($\beta$-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA), resolyte 4-8.

Biomol Research Laboratories, Inc. (Plymouth Meeting, PA, U.S.A.)

1H-[1,2,4]oxadiazolo[4,3-a]quinoxaline-1-one (ODQ), KT5823.

Bio-Rad Laboratories Ltd. (Mississauga, Ontario, Canada)

Urea, silver stain, SDS-PAGE low range molecular weight standards, Biolyte® 3/10 ampholyte, N,N'-methylene-bis acrylamide, bromophenol blue, N,N,N',N'-tetramethyl-ethylenediamine (TEMED), Coomassie® brilliant blue R-250.

Calbiochem-Novobiochem Corporation (San Diego, CA, U.S.A.)

[4-(2-aminoethyl)benzenesulfonylfluoride, HCl] (AEBSF).

Dow Corning Corporation (Midland, MI, U.S.A.)

High vacuum grease.
Eastman Kodak Company (Rochester, NY, U.S.A.)
Kodak Photo-Flo® 200 solution.

Agarose, calcium chloride, copper sulfate, o-phosphoric acid, potassium chloride, potassium phosphate monobasic, sodium carbonate, sodium chloride, magnesium sulfate, hydrochloric acid, potassium hydroxide, glacial acetic acid, ethanol, methanol, isopropanol, ethyl ether, Scintiverse® scintillation fluid.

Halocarbon Laboratories (River Edge, NJ, U.S.A.)
Halothane.

Hip Hing Oil Factory Ltd. (Kowloon, Hong Kong)
Lion and Globe peanut oil.

Medigas (Vancouver, BC, Canada)
5% carbon dioxide/95% oxygen.

NEN® Life Science Products (Boston, MA, U.S.A.)

Pierce Chemical Company (Rockford, IL, U.S.A.)
Gelcode® SilverSNAP stain kit.

Roche Diagnostics (Laval, Quebec, Canada)
Acrylamide, glycine, tris(hydroxymethyl)aminomethane (Tris-base), sodium dodecyl sulfate (SDS), 3-[(3-cholamidopropyl)dimethyl-ammonio]-1-propanesulfonate (CHAPS), bovine serum albumin.
Sigma-Aldrich Canada (Oakville, Ontario, Canada)

Adenosine 5’-triphosphate, benzamidine, L-arginine, dithiothreitol, ethylene diamine tetraacetic acid (EDTA), Folin and Ciocalteu’s phenol reagent, guanosine 3’:5’-cyclic monophosphate (cGMP), 3-isobutyl-1-methylxanthine, leupeptin, pepstatin, β-glycerophosphate, protein kinase inhibitor (PKI), sodium nitroprusside (SNP), Triton® X-100, Nonidet P-40, progesterone, β-estradiol, magnesium acetate, crocin scarlet 7B, S-nitroso-N-acetylpenicillamine (SNAP), N-acetyl-D-penicillamine, trichloroacetic acid (TCA), pepstatin A, inorganic phosphate assay kit, mixed bed resin, β-mercaptoethanol, dimethyl sulfoxide, HEPES, 8-bromoguanosine 3’:5’-cyclic monophosphate, charybdotoxin, sodium pyrophosphate, phenylephrine, glycerol.

Whatman Ltd. (Maidstone, Kent, England)

Phosphocellulose paper (P81).

2.2 Animals.

Male and female Sprague Dawley rats weighing 250-300 g were obtained from the Animal Care Facility, University of British Columbia and housed 2 to 3 animals per cage. All rats had free access to food and water.

2.3 Source, preparation and tension measurements in rat myometrium.

Prior to removal of uterine segments, rats were subjected to various hormonal treatments. Groups A-D were ovariectomized under halothane anaesthesia, accompanied by 80% N₂O and 20% O₂. A small incision was made on the hind quarter directly above the ovary, the ovary and ovarian artery were tied off and the ovary was removed. Wound clips were used to seal the openings and rats were given 7 days to recover before subcutaneous hormone injections were initiated. Group A received one injection of 200 µl of vehicle (peanut oil) 48 hours prior to sacrifice. Group B received
one injection of 100 μg β-estradiol 48 hours prior to sacrifice. Group C received one injection of progesterone (3 mg/kg) a day for three days. Group C animals were sacrificed 24 hours after the third injection. Group D received the same initial treatment as group B; however, following the first 48 hours each rat received one injection of progesterone (3 mg/kg) a day for three days. Timed pregnant rats were received on the 17th day of gestation and sacrificed on the 18th day. One additional treatment employed in later studies included non-ovariectomized rats injected with group D hormones. All animals were sacrificed in a CO₂ inhalation chamber. Uteri were removed, trimmed free of loosely adhering connective tissue and fat, and cut longitudinally along the vascularized side to expose the endometrium. Next, the endometrium and circular smooth muscle were peeled away from the longitudinal smooth muscle of the myometrium. Myometrial strips of approximately 6 mm by 3 mm were suspended in isolated organ baths at 37°C with a preload of 0.5 g in a physiological salt solution with the following composition (mM): KCl, 4.7; NaCl, 118; MgSO₄, 1.19; KH₂PO₄, 1.19; CaCl₂, 2.5, glucose, 11; and NaHCO₃, 25. Tissue baths were aerated with 95% O₂, 5% CO₂ which maintained a pH of approximately 7.4. Isometric tension was recorded with force displacement transducers (Grass Model FT03C) which were connected to a Grass Model 7D polygraph recorder. Tissues were given 20 minutes to equilibrate and achieve steady state contractions prior to the addition of agents. Contractile responses were measured in each hormonal group following the addition of SNP (5 mM), ANP (100 nM), L-arginine (1 mM) or 8-bromo-cGMP (100 μM). Additional studies using non-ovariectomized, group D hormonally influenced rats investigated the contractile responses to SNAP in the absence and presence of ODQ. Control untreated tissues received vehicle where appropriate. Five
minutes after the addition of drug, tissues were frozen with liquid nitrogen-cooled clamps and stored at -80°C prior to assessment of cGMP levels and PKG activity. In one additional study, myometrial strips were pretreated with 50 nM charybdotoxin, a specific inhibitor of calcium-activated potassium channels, to assess whether SNAP-induced relaxation was mediated by cGMP-independent membrane channel activation. For cumulative dose-response curves, SNAP was added to the bath in increasing concentrations in the absence or presence of ODQ. Percent inhibition of spontaneous contractions was calculated by measuring the change in the amplitudes of the contractions following addition of the drug.

2.4 Preparation and tension measurements in rat aorta.

Descending thoracic aortae were excised from rats immediately following sacrifice 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 approximately 10 mm by 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): KCl 4.7, NaCl 140, Hepes 10, MgCl₂ 1, CaCl₂ 1.5, NaH₂PO₄ 0.2, glucose 10. Tissue baths were aerated with 95% O₂, 5% CO₂ which maintained a pH of approximately 7.4. Isometric tension was recorded with force displacement transducers (Grass Model FT03C) which were connected to a Grass Model 7D polygraph recorder. Tissues were given 40 minutes to equilibrate prior to the addition of agents. After equilibration, strips were contracted with 1.0 μM PE for five min and then washed four times for a further 40 min. Tension was continually adjusted to maintain a 2 g preload. Next, muscles were again contracted with 1.0 μM PE for 4 min and then relaxed with various cGMP-elevating
agents including SNP (1 and 30 μM), ANP (100 nM, 1 μM and 10 μM), 8-bromo-cGMP (100 μM), and SNAP (1 μM and 1 mM). In several experiments, the soluble guanylyl cyclase inhibitor, ODQ, was added 20 minutes prior to the addition of relaxing agents. At pre-determined times following the addition of drug, tissues were frozen with liquid nitrogen-cooled clamps and stored at −80°C until assessment of enzyme activity (cGMP level and PKG activity). For cumulative dose-response curves, SNP and SNAP were added to the bath in increasing concentrations in the absence or presence of ODQ. Percent inhibition of contraction was calculated by measuring the change in amplitude of the PE-induced contraction following addition of the drug.

2.5 Cyclic GMP estimation.

Frozen smooth muscles from the above tissue bath experiments (10 – 20 mg) were placed in liquid nitrogen-cooled teflon capsules (1 ml capacity) (Hansen Industries Ltd., Richmond, B.C., Canada) with a chilled metal pestle and pulverized in a ProMix™, Dentsply dental amalgam mixer (20 seconds at high speed). Next, 0.75 ml of ice-cold trichloroacetic acid (TCA) (6% w/v) was added to the capsule and the tissue was homogenized for another 20 seconds at high speed. The homogenate was removed and the capsule was washed with an additional 0.25 ml of TCA. The total homogenate (1 ml) was then centrifuged at 2000 g for 15 minutes at 4°C. The supernatant was removed and the TCA was extracted with 4 washes of ice-cold water-saturated ether (5 ml per wash). Residual ether remaining after the final wash was evaporated by placing samples in a hot, 70°C water bath. Cyclic GMP levels were measured using a commercially available scintillation proximity radioimmunoassay kit (acetylation protocol). The TCA-insoluble pellet was stored at −80°C for protein estimation by the
method of Lowry et al. (1951) as modified by Markwell et al. (1981). Tissue cGMP levels were calculated as pmol cGMP per mg protein.

2.6 Cyclic GMP-dependent protein kinase assay.

Frozen myometrial and aortic strips weighing approximately 40 mg were pulverized in teflon capsules similar to the method used in the cGMP extraction above, except a homogenization buffer was substituted for TCA. The buffer contained the following (mM): HEPES, 10; EDTA, 1; DTT, 10; IBMX, 1; KCl, 125; benzamidine, 1; plus leupeptin, 10 µg/ml; and pepstatin, 10 µg/ml. The homogenate was centrifuged at 30,000 g for 5 minutes and the supernatant was assayed for soluble PKG activity. A phosphocellulose paper assay was used to measure the phosphotransferase activity of PKG utilizing a method modified from that described by Jiang et al. (1992). PKG activity was determined by measuring the transfer of the [γ-³²P] phosphoryl group of ATP to BPDEtide (RKISASETDRPLR), which is a relatively specific substrate for PKG (Colbran et al., 1992). 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 per tube), 5 µM synthetic PKA inhibitor (PKI), 0.5 mM EGTA, and the absence or presence of 5 µM cGMP. The reaction was initiated by adding 20 µl of the sample supernatant. The reaction was allowed to proceed for 10 minutes at 4°C and was stopped by spotting 50 µl of the reaction mixture onto 2 cm x 2 cm squares of phosphocellulose paper (Whatman P81). The paper was then washed 4 times in 0.5% o-phosphoric acid for 10 minutes each. The papers were dried and then transferred to scintillation vials containing 2.5 ml liquid scintillant. Radioactivity was counted in a Beckman LS 6000TA liquid scintillation counter. PKG activity was expressed as pmol phosphate incorporated into substrate per min per mg protein. PKG activation 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 based on the method of Bradford (1976).

2.7 Two-dimensional gel electrophoresis.

2.7.1 Tissue preparation and radioactive labeling of proteins.

Smooth muscle strips from rat aorta, myometria and vas deferens were prepared as follows. Descending thoracic aortae were excised, trimmed free of loosely adhering connective tissue and fat, and cut longitudinally to expose the endothelium. The endothelium was removed with gentle rubbing with a glass rod. Uteri were removed from non-ovariectomized, group D hormonally-influenced rats and myometrial strips were prepared similar to the method described above for measurement of tension. 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): KCl 4.7, NaCl 140, Hepes 10, MgCl$_2$ 1, CaCl$_2$ 1.5, NaH$_2$PO$_4$ 0.2, glucose 10. Next, [$^{32}$P]-labeled inorganic phosphate was added to the buffer at a concentration of 500 μCi/ml to label cellular ATP pools. Tissues were incubated with radiolabeled phosphate for 3 hours at 37°C with gentle agitation. When cellular ATP pools were labeled, smooth muscle strips were treated as follows. Aortic strips were contracted with 1 μM phenylephrine (PE) for 4 min and then either relaxed with the addition of 30 μM SNP (3 min), 1 mM SNAP (3 min), 10 μM ANP (5 min) or left in the presence of PE alone to act as a control. In muscles treated with 8-
bromo-cGMP, the analog was added to the buffer 15 min prior to contraction with PE. Controls not contracted with PE were also prepared. Additional experiments with SNP and SNAP had the soluble guanylyl cyclase inhibitor, ODQ, added to the buffer 20 minutes prior to the addition of PE. Myometrial strips were relaxed with the addition of 5 mM SNP (5 min), 1 mM SNAP (5 min) and 1 mM 8-bromo-cGMP (10 min) or left untreated to act as control. No stimulants were used to precontract the tissues. For the experiments with SNP and SNAP, additional studies were done to investigate the effect of ODQ. Vas deferens strips were pretreated with 1 mM SNP and 1 mM 8-bromo-cGMP for 2 min and 15 min, respectively, and then contracted with 3 μM PE for 30 seconds. Following the treatment protocols above, individual smooth muscles were blotted on tissue paper and frozen with liquid nitrogen-cooled clamps and stored at −80°C for future use.

2.7.2 Sample preparation.

Frozen smooth muscle strips labeled with [³²P] were placed in liquid nitrogen-cooled teflon capsules (1 ml capacity) (Hansen Industries Ltd., Richmond, B.C. Canada) with a chilled metal pestle, and pulverized in a ProMix, Dentsply dental amalgam mixer (30 seconds at high speed). Next, 700 μl of ice-cold homogenization buffer was added to the capsule and the tissue was homogenized for another 30 seconds at high speed. The homogenization buffer (pH 7.4) contained the following (mM): EDTA 10, Hepes 10, benzamidine 1, AEBSF 1, protein kinase A inhibitor (PKI) 0.001, KT5823 0.001, sodium fluoride 100, dithiothreitol (DTT) 10 and leupeptin 10 μg/ml. The contents of this homogenization buffer have previously been shown to reduce the levels of phosphorylation and dephosphorylation that could take place during homogenization and sample preparation (Garrison, 1978). As an extra precaution, we added the PKG-
inhibitor KT5823 to minimize any in vitro PKG-mediated phosphorylation that may occur following homogenization. The homogenate was removed and centrifuged for 1 hour at 100,000 g. The supernatant from this spin made up the soluble protein fraction for our experiments. To prepare the particulate protein fraction the remaining pellet was re-homogenized in an ice-cold teflon capsule for 30 seconds with 500 µl of a homogenization buffer similar to the one used above except for the following additions: 1% Triton X-100 and 50 mM sodium pyrophosphate. Triton X-100 and sodium pyrophosphate were shown by Baltensberger et al. (1990) to be good conditions for the extraction of membrane bound PKG-phosphorylated substrates. This homogenate was vortexed repeatedly for 1 hour and then centrifuged at 100,000 g for 1 hour. The supernatant was collected as the particulate fraction. In order to concentrate the protein in both of 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. (1951) as modified by Markwell et al. (1981). After protein levels were determined, both protein fractions were prepared for isoelectric focusing by adding urea (9 M) and Resolyte 4-8 (2%).

2.7.3 First dimension - isoelectric focusing (IEF).

First dimension IEF tube gels were prepared based on a combination of methods taken from three main publications (O'Farrell, 1975; Hochstrasser et al., 1988; and Dunn and Corbett, 1996). In addition, several new modifications were developed in our laboratory to complete the procedure. To prepare the IEF gel mixture, 10 g of urea was dissolved in 6.5 ml deionized water and 3 ml of stock acrylamide (30% total monomer, 2.6% crosslinking monomer). When dissolved, urea was deionized by gentle mixing with 200 mg of mixed bed resin for 1 hour. This solution was filtered and degassed
before the addition of 1 ml of a detergent solution (0.3 g CHAPS, 900 μl deionized water and 100 μl Nonidet P-40), 700 μl of Resolyte 4-8 and 300 μl BioRad Ampholyte 3-10. To start polymerization, 40 μl of 10% wt/vol ammonium persulfate (freshly prepared) and 20 μl of TEMED were added to the mixture. Isoelectric focusing gels were cast in 18 cm long glass capillary tubes (1.5 mm i.d.) prewashed with a 1/200 dilution of Kodak Photoflow 200. Photoflow 200 coats the surface of the capillary tube and facilitates easy removal of tube gels. Tuberculin syringes (1 ml) were connected to the top of each capillary tube with rubber tubing and placed in 12x75 mm disposable culture tubes. The gel solution was slowly pipetted into the culture tubes and the capillary tubes were filled by suction to a height of 14 cm. Gels were allowed to polymerize for 2 hours. Prior to attaching the tube gel adapter to the cooling core, the red tube gel adaptor gasket was lubricated with high vacuum gasket grease (Dow Corning®). Inconsistent sealing between the tube gel adaptor and the cooling core with dry gaskets led to the addition of this lubricating step. Now the tube adaptors were attached to the cooling core and the apparatus was placed in the electrophoresis tank filled with 4 L of the lower anode buffer, 10 mM degassed phosphoric acid. Protein samples were now added to the top of the capillary tubes (50 μg in 20 μl) using BioRad protein electrophoresis pipette tips. Samples were added slowly to avoid disturbing the unpolymerized region at the top of the tube gel. Samples were subsequently overlaid with 10 μl of overlay solution (8 M urea, 1% Resolyte 4-8) and then filled to the top with upper cathode buffer, 20 mM degassed NaOH. When all samples had been added, the upper buffer chamber was filled with the cathode buffer. Isoelectric focusing was carried out at 10°C with a constant voltage of 100 V for 30 mins, 500 V for 1 hour and 1000 V for 17 hours. By maintaining a temperature of 10°C we found less smearing with higher molecular weight
proteins in the first dimension. Pharmacia carbamylyte calibration IEF standards were monitored to identify the optimum separating conditions. Tube gels were extruded with a tube gel extrusion needle attached to a 10 ml syringe filled with double distilled water. The upper and lower regions of the tube gel were rimmed by inserting the needle between the tube gel and the glass capillary wall while forcing water through the needle. Extruded tube gels were lightly coated with transfer buffer containing 5% glycerol, 0.07 M Tris-HCl (pH 6.8), 2.5% SDS and immediately frozen on dry ice. Tube gel freezing was found to be an effective method of precipitating urea in the gel (Dunn and Burghes, 1983). Elimination of this step results in significant protein smearing in the second dimension. Carbamylyte calibration standard tube gels were stained for 1 hour with gentle agitation in the following solution: 27% isopropanol, 10% acetic acid, 30 mM copper sulfate, 0.85 mM crocein scarlet 7B and 0.5 mM Coomassie Brilliant Blue R-250. Gels were subsequently destained in 40% methanol, 10% acetic acid until the background cleared. Based on the stained bands of the calibration tube gels, we were able to calculate pi values for our samples when the second dimension was complete.

2.7.4 Second dimension - sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

Frozen tube gels were thawed and equilibrated for 10 mins in transfer buffer containing 1% β-mercaptoethanol. The addition of a tube gel freezing step and an increase in the concentration of SDS for the second dimension running buffer allow for a shorter equilibration time, while maintaining high protein transfer rates into the second dimension. Eight full size 20 cm SDS-PAGE gels of 1.5 mm width were prepared based on the methods of Laemmli (1970) by adding 165 ml double distilled water to 112.5 ml 1.5 M Tris-HCl (pH 8.8), 4.5 ml 10% wt/vol SDS and 165 ml stock acrylamide (30% total...
This solution was degassed and polymerization was initiated by the addition of 2.25 ml 10% wt/vol ammonium persulfate and 0.225 ml TEMED. No stacking gels were used for any of our experiments. A small volume of water saturated sec-butanol was used to overlay the gels during 1.5 hours of polymerization. Prior to the addition of tube gels, the upper surfaces of the SDS-PAGE gels were washed several times with double distilled water to remove all traces of sec-butanol. A single well comb was added to one edge of each gel to allow for the addition of a molecular weight standard. Next, tube gels were carefully applied to the surface of the second dimension gels while making sure no air bubbles appeared between the two gel surfaces. A small volume of 1% agarose, 0.1% bromophenol blue in transfer buffer was used to cement the tube gels in place and to provide a dye line during electrophoresis. Second dimension running buffer of the following composition (2.5 L), 49.5 mM Tris-base, 383.6 mM glycine and 6.9 mM SDS (0.2%), was carefully added to the upper buffer chamber to avoid dislodging the tube gels from the surface of the second dimension gels. All remaining running buffer was placed in the lower electrophoresis tank. The majority of published SDS-PAGE electrophoresis methods utilize 0.1% SDS in the running buffer. We found that increasing this concentration to 0.2% greatly facilitated protein transfer into the second dimension slab gel from the tube gel, allowed for decreased tube gel equilibration times and resulted in significant increases in resolution in the second dimension. Electrophoresis was carried out at 4 mA/gel for 12 hours followed by 20 mA/gel for approximately 6 hours or until the dye line reached the bottom of the gel. Temperature during the run was maintained at approximately 10°C.
2.7.5 Detection methods and quantification.

Following electrophoresis, gels were placed into fixative (30% ethanol, 10% acetic acid) for 3 hours. At the end of this period, all gels were silver-stained according to the protocol supplied by Pierce for the Gelcode SilverSnap Stain Kit. At the end of the stain protocol, gels were soaked in a 4% glycerol solution for 1 hour and then dried overnight in the fume hood clamped between two sheets of cellophane. A base board heater was placed at the entrance to the fume hood to create a temperature of approximately 30°C during drying. Dried gels were trimmed free of excess cellophane and placed in x-ray cassettes. Autoradiographs were taken at -70°C over a 24 hour period for aorta samples and over a 48 hour period for myometria and vas deferens samples. BioMax MS x-ray film and BioMax Transcreen HE high energy intensifying screens were used in this process. Autoradiographs were developed using an automated Kodak M35A X-OMAT processor. Quantitative assessments of the degree of phosphorylation based on densitometry of the autoradiographs were carried out using the Biolmage Visage Computer System and a Videk digital camera. Integrated intensity is the background corrected optical density (OD) integrated over all pixels in the spot. In order to account for experimental variations among autoradiographs within a single set of data, a quantitative ratio of the integrated intensities between the spots of two images was calculated. This quantitative ratio was then applied as a multiplicative factor to normalize any systematic differences in general darkness that existed between the phosphorylation patterns (Nugues, 1993). This normalization step was carried out by the Biolmage Software system used for our densitometry analyses.
2.8 Protein estimation.

Frozen pellets from the cGMP extraction were assayed for protein content using the method of Lowry et al. (1951) as modified by Markwell et al. (1981). In order to dissolve the pellets, they were resuspended in 1 ml of a modified reagent A containing (%): Na₂CO₃, 2.0; NaOH, 2.4; sodium tartrate, 0.16; sodium lauryl sulfate, 1.0 and vortexed to evenly distribute the protein contents. The concentration of NaOH in the above reagent A has been increased from 0.4% to 2.4% to aid in the disruption of the pellet. Between 10 and 50 μl of the pellet suspension was diluted to 1 ml with distilled water in the first step of the assay. Next, 3 ml of reagent C (100:1 ratio of reagent A:4% CuSO₄) was added. After 10 minutes, 0.3 ml of the final reagent D (Folin and Ciocalteau phenol reagent 1N) was added and the solution was immediately vortexed and allowed to stand at room temperature for 45 min. The absorbance of light at 690 nm was determined and protein concentration was estimated by creating a bovine serum albumin standard curve. Protein estimations of samples used in two-dimensional gel electrophoresis experiments were also measured using the above method, but no pellet disruption step was necessary. 5 to 10 μl of concentrated homogenate was diluted to 1 ml with distilled water to start the assay. Protein in the PKG soluble fraction was estimated using a commercially available assay based on the method of Bradford (1976).

2.9 Preparation of drug solutions.

Solutions of PE, SNP and 8-bromo-cGMP were prepared immediately prior to use in distilled water and protected from light. KT5823, ODQ and SNAP were dissolved in dimethyl sulfoxide, with the latter being protected from light. ANP was prepared in 0.05 M acetic acid and frozen at -70°C in aliquots until required. Charybdotoxin was
dissolved in a 150 mM NaCl solution. All remaining drug solutions were prepared in distilled water.

2.10 Statistical analysis.

In the myometrial experiments investigating the effects of SNP, ANP and L-arginine on contractile responses and cGMP levels, each drug-treated myometrial strip had its own respective control which allowed treatments to be compared to controls by a paired t-test. In the PKG assays of the myometrium and aorta, PKG activity ratios in tissues treated with SNP were compared to untreated control ratios by Student's t-test. The effect of charybdotoxin on SNAP-induced relaxation of the myometrium and cGMP levels in myometrial strips from pregnant rats were also compared using Student's t-test. 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. In Table 1 a Dunn's test was used following an ANOVA. To analyze the densitometry profiles obtained from our two-dimensional gel electrophoresis studies, values were converted to percent 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. Throughout the thesis, data are presented as mean ± standard error of the mean (SEM) and results were considered significant when P<0.05, for all comparisons. SigmaStat for Windows Version 1.0 (Jandel Scientific, San Mateo, CA, U.S.A.) software was used for statistical analyses.
3.1 Contractile responses, cGMP levels and PKG activity in rat myometrial preparations.

3.1.1 Effects of hormonal status on spontaneous myometrial contractions.

Spontaneous contractile activity varied slightly among the five groups of animals tested. Figure 2 illustrates representative tracings from each group. In the non-treated ovariectomized rats (group A), spontaneous contractile activity lasted approximately 60 minutes and was characterized by rapid, small amplitude contractions. In the ovariectomized estrogen-treated animals (group B), the spontaneous activity was inconsistent and often tapered off within the first 20 minutes of the experiment. When contractions did occur they were of greater amplitude than those observed in group A. Spontaneous contractile activity was similar in the ovariectomized, progesterone-treated (group C) and ovariectomized estrogen-primed/progesterone-treated (group D) animals. In both groups, there was a high degree of spontaneous activity with strong and regular contractions lasting up to 60 minutes. Treatments with progesterone alone and with progesterone following estrogen-priming were performed because it has previously been shown that pre-treatment with estrogen increases the number of available receptors for progesterone (Toft and O'Malley, 1972). Thus, group D may be under a stronger influence of progesterone than group C. In the 18-day pregnant rats (group E), contractions were markedly reduced in amplitude compared to groups B-D and spontaneous activity was often irregular. Despite these differences in the spontaneous contractile activity of the myometrial tissues from the groups above, they were consistent in their lack of response to the cGMP-elevating agents and cGMP analog used (Figure 2).
Figure 2. Effects of hormonal status and SNP, ANP, L-arginine and 8-bromo-cGMP on spontaneous myometrial contractions. Representative tracings illustrating the effects of SNP (5 mM), ANP (100 nM), L-arginine (1 mM) and 8-bromo-cGMP (100 μM) on spontaneous myometrial contractions from (A) ovariectomized, vehicle-treated (peanut oil), (B) ovariectomized, estrogen-treated, (C) ovariectomized, progesterone-treated, (D) ovariectomized, estrogen-primed, progesterone-treated, and (E) 18-day timed pregnant rats. Addition of drug is indicated by (↑) and results are typical of 6 experiments in groups A-D and 4 experiments in group E.
3.1.2 Effect of SNP, ANP, L-arginine and 8-bromo-cGMP on spontaneous myometrial contractions.

The effects of SNP (5 mM), ANP (100 nM), L-arginine (1 mM) and 8-bromo-cGMP (100 μM) on spontaneous myometrial activity are shown in Figure 2. In all five groups (A-E) there was no measurable effect observed with any of these agents. Although Figure 2 shows only the first five minutes following drug addition, in several instances, tissues were allowed to remain in the presence of the agent for longer periods but no detectable change was ever observed. In some strips from the estrogen-treated group (group B), 8-bromo-cGMP appeared to exert a relaxing effect as shown in Figure 2. However, this response was not seen consistently, and in many cases the control muscles in this group exhibited similar diminished spontaneous contractions during the same period of time. No responses to 8-bromo-cGMP were observed in any of the other groups of animals. In preliminary experiments, we could not demonstrate any relaxation of drug-induced (oxytocin-induced) contractions by any of these agents (data not shown).

3.1.3 Effect of SNP, ANP and L-arginine on cGMP levels in the rat myometrium.

Cyclic GMP levels were determined in experiments identical to those illustrated in Figure 2 and the results are presented in Table 1. In a comparison of the control levels of cGMP between the different hormone treatment groups it was found that basal levels of cGMP were significantly lower in the ovariectomized, untreated group than in all other groups. None of the remaining groups were found to have significantly different control cGMP levels. SNP produced a significant elevation of cGMP above control in all five treatment groups (A-E). The magnitude of cGMP elevation varied between groups,
Table 1. Effect of hormonal status, and SNP, ANP and L-arginine on cyclic GMP levels in spontaneously contracting rat myometrium.

<table>
<thead>
<tr>
<th></th>
<th>Ovariectomized, Non-Treated</th>
<th>Ovariectomized, Estrogen-Primed</th>
<th>Ovariectomized, Progesterone Treated</th>
<th>Ovariectomized, Estrogen-Primed Progesterone Treated</th>
<th>Pregnant</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(A)</td>
<td>(B)</td>
<td>(C)</td>
<td>(D)</td>
<td>(E)</td>
</tr>
<tr>
<td>Control</td>
<td>0.7 ± 0.3</td>
<td>2.3 ± 0.4</td>
<td>2.9 ± 0.4</td>
<td>2.3 ± 0.4</td>
<td>2.2 ± 0.6</td>
</tr>
<tr>
<td>SNP (5 mM)</td>
<td>4.6 ± 1.2 *</td>
<td>11.5 ± 1.1 *</td>
<td>24.8 ± 4.0 *</td>
<td>16.2 ± 1.3 *</td>
<td>7.6 ± 1.3 *</td>
</tr>
<tr>
<td>Control</td>
<td>0.6 ± 0.2</td>
<td>3.1 ± 0.2</td>
<td>2.9 ± 0.7</td>
<td>2.3 ± 0.4</td>
<td>2.2 ± 0.4</td>
</tr>
<tr>
<td>ANP (100 nM)</td>
<td>1.2 ± 0.2 *</td>
<td>4.6 ± 0.8 *</td>
<td>4.8 ± 0.6 *</td>
<td>3.7 ± 0.4 *</td>
<td>2.7 ± 0.4</td>
</tr>
<tr>
<td>Control</td>
<td>1.2 ± 0.4</td>
<td>4.7 ± 0.8</td>
<td>1.6 ± 0.5</td>
<td>2.9 ± 0.3</td>
<td>2.2 ± 0.4</td>
</tr>
<tr>
<td>L-arginine (1 mM)</td>
<td>3.0 ± 0.5 *</td>
<td>3.6 ± 0.5</td>
<td>2.1 ± 0.4</td>
<td>2.8 ± 1.0</td>
<td>2.1 ± 0.6</td>
</tr>
</tbody>
</table>

Note: All values were calculated as pmol cGMP/mg protein and results are expressed as mean ± SEM for n = 5 or 6. Treated tissues were compared to their respective controls by a paired Student's t-test and basal levels of cGMP among the different hormone groups were compared using a one-way ANOVA on ranks followed by Dunn's multiple comparison test. Groups A control levels of cGMP differed significantly from control levels in all other treatment groups (p<0.05). Asterisks indicate a significant drug-induced elevation of cGMP compared to the corresponding control (p<0.05).
ranging from 3.4-fold in the pregnant group to 8.8-fold in the ovariectomized progesterone-treated group. However, as shown in Figure 2, SNP did not inhibit spontaneous contractions in any of the groups. ANP significantly increased cGMP levels in groups A, B, C and D, whereas L-arginine elevated cGMP significantly only in group A. As was the case with SNP, no relaxant effects were seen with these agents in any of the groups. Thus, there did not appear to be a correlation between cGMP elevation and inhibition of tension development in rat myometria.

3.1.4 Effect of SNP on PKG activity in the rat myometrium.

The possibility was considered that the lack of relaxation seen with SNP-treated tissues, in spite of a marked elevation of cGMP, was due to a failure of cGMP to activate cGMP-dependent protein kinase (PKG). In order to test this possibility, the effect of SNP on PKG activity ratios was measured in myometrial preparations from ovariectomized, estrogen-treated animals. Because cGMP levels were significantly increased by SNP in all hormonal treatment groups, and because no relaxation was seen in any of the groups, only one representative group was used in the PKG study. Myometrial strips were suspended in tissue baths, allowed to equilibrate and either left untreated or exposed to 5 mM SNP for 5 minutes. As shown in Table 2, SNP produced a significant increase in the PKG activity ratio in rat myometrium. No significant change in total PKG activity (i.e. in the presence of added cGMP) was observed. Thus, the increase in the activity ratio demonstrated with SNP was due to activation of specific, cGMP-dependent protein kinase by increases in endogenous cGMP, and not to an increase in non-specific (cyclic nucleotide-independent) protein kinase activity.
Table 2. Effect of SNP on cGMP-dependent protein kinase activity in spontaneous contracting rat myometrium.

<table>
<thead>
<tr>
<th></th>
<th>PKG Activity (pmol PO₄/min/mg)</th>
<th>Activity Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(- cGMP)</td>
<td>(+ cGMP)</td>
</tr>
<tr>
<td>Control</td>
<td>3.9 ± 0.1</td>
<td>41.5 ± 0.6</td>
</tr>
<tr>
<td>SNP (5 mM)</td>
<td>10.5 ± 0.3 *</td>
<td>38.3 ± 2.1</td>
</tr>
</tbody>
</table>

Note: Values are mean ± SEM for n=3. Control and SNP-treated tissues were compared using Student's t-test. Asterisks indicate a significant difference from control (p<0.05).
3.1.5 Effect of SNAP on spontaneous myometrial contractions.

S-nitroso-N-acetylpenicillamine (SNAP) has been reported to be a better NO donor than SNP (Marks et al., 1995). In a separate set of experiments, preliminary results showed that SNAP (100 μM) induced consistent inhibitions of spontaneous contractions in uteri from hormonal treatment groups B, C and D. Because there was no difference in the responses with the different hormonal treatments, subsequent experiments were carried out in uteri from non-ovariectomized, estrogen-primed progesterone-treated animals which exhibit a high degree of strong, regular spontaneous activity. Figure 3 illustrates representative tracings from these experiments. As shown in panel C, 100 μM SNAP produced a consistent inhibition of spontaneous contractions (average of 5 experiments: 59.0 ± 9.7 % inhibition). DMSO, the vehicle used for SNAP, had no effect (Panel A). Because our earlier experiments had shown that cGMP elevation by SNP was not accompanied by relaxation, we used ODQ, a specific inhibitor of guanylyl cyclase (Schrammel et al., 1996), to determine the importance of cGMP in the relaxant responses to SNAP. Panel B illustrates an ODQ control preparation treated with DMSO and Panel D illustrates the effect of SNAP (100 μM) in the presence of ODQ. ODQ had no effect on the relaxant response produced by SNAP (average inhibition 67.6 ± 6.7 %). In many instances, ODQ appeared to potentiate inhibition of spontaneous contractions induced by 100 μM SNAP, but this was not statistically significant. In order to further demonstrate the lack of effect of ODQ on SNAP-induced inhibition of spontaneous contractions in rat myometrium, cumulative dose-response curves to SNAP were determined in the absence and presence of ODQ. As shown in Figure 4, 25 μM ODQ produced no measurable shift in the relaxant responses induced by SNAP over a concentration range of 1 μM to 440 μM.
Figure 3. Effect of SNAP on spontaneous myometrial contractions.

Representative tracings illustrating the effects of SNAP, in the presence and absence of ODQ, on spontaneous myometrial activity from estrogen-primed, progesterone-treated rats. (A) vehicle (DMSO), (B) ODQ (25 μM) for 20 min + vehicle, (C) SNAP (100 μM), (D) ODQ (25 μM) for 20 min + SNAP (100 μM). Drug additions are indicated by (⨯) and results are typical of 5 experiments.
Figure 4. Cumulative dose-response curves to SNAP in the absence and presence of ODQ. Effects of SNAP on spontaneous contractions from estrogen-primed, progesterone-treated rats in the absence and presence of 25 μM ODQ. Percent inhibition of spontaneous contractions was determined by measuring the change in the amplitudes of the contractions over a five minute period following the addition of drug. In control, untreated tissues myometrial spontaneous activity did not change over the dose-response period. Data are expressed as mean ± SEM for 6-7 myometrial strips from 3 different rats.
3.1.6 Effect of SNAP and ODQ on cGMP levels in spontaneously-contracting rat myometrium.

The effects of 10 μM, 30 μM, 100 μM and 1 mM SNAP on cGMP levels in myometrial strips from estrogen-primed, progesterone-treated rats are shown in Figure 5a. All four concentrations of SNAP generated a significant increase in the level of cGMP. In addition, 10 μM, 30 μM and 100 μM SNAP caused significant inhibition of spontaneous myometrial contractions (Figure 5b). The effects of SNAP at 10 μM, 30 μM, 100 μM and 1 mM were also measured in the presence of 25 μM ODQ. At each concentration of SNAP, ODQ produced a significant reduction in the ability of SNAP to increase cGMP levels. However, this decrease in cGMP elevation had no effect on the relaxant response elicited by SNAP. In the experiments with 10 μM SNAP, cGMP elevation was reduced such that cGMP levels were not significantly different from control values, yet an 18.7 ± 7.7 % inhibition of spontaneous contractions still occurred. With 30 μM, 100 μM and 1 mM SNAP, ODQ was unable to produce a complete blockade of cGMP elevation, although the levels were markedly reduced. The cGMP levels obtained with these concentrations of SNAP in the presence of ODQ were similar to the levels generated by 5 mM SNP which had no relaxant effect as shown in Figure 2 and Table 1. Once again, there appears to be a poor correlation between increases in cGMP and decreases in myometrial tension development. An interesting aspect of the SNAP relaxant response was the fact that the inhibition of spontaneous contractions lasted less than 10 minutes and a full recovery was common even in the continued presence of SNAP. To investigate the role of cGMP in the transient nature of this response, cGMP levels were measured at 12 minutes following the addition of 100 μM SNAP with and without a pretreatment with ODQ (25 μM). The cGMP levels found in
Figure 5. Effect of SNAP and ODQ on cGMP levels in spontaneously-contracting rat myometrium. Effect of ODQ on SNAP-induced elevation of cGMP and inhibition of spontaneous contractions in myometrial strips from estrogen-primed, progesterone-treated rats. 5a shows the effects of 10 μM, 30 μM, 100 μM and 1 mM concentrations of SNAP (S) on cGMP levels in the absence (open bars) and presence (shaded bars) of ODQ (O) (25 μM). ODQ had no significant effect on cGMP levels in untreated control muscles (C). 5b shows the contractions for the same muscles used in the cGMP studies shown in 5a. Percent inhibition of spontaneous contractions was determined by measuring the change in the amplitudes of the contractions following the addition of drug. Results are expressed as mean ± SEM for 5 experiments. Asterisks indicate a significant difference from control (p<0.05) and number signs indicate a significant difference from SNAP alone (p<0.05). All comparisons were made using a one-way ANOVA followed by Student-Newman-Keuls multiple comparison test.
these experiments (in pmol per mg protein) were as follows (n=4): control, 2.4 ± 0.2; ODQ control, 2.0 ± 0.4; SNAP, 17.0 ± 4.0; ODQ + SNAP, 3.0 ± 0.8. Although spontaneous contractions had completely recovered within 12 minutes, cGMP was still significantly elevated in the SNAP-treated tissues. In the ODQ + SNAP treated muscles, tissue levels of cGMP were not significantly different from control levels. The ability of myometrial strips to completely recover in the presence of elevated cGMP, and the lack of effect of ODQ on rate of recovery, provides further evidence against a role for cGMP in the relaxant effects induced by SNAP.

To ensure that the relaxant effects of SNAP were indeed due to a NO-mediated event, the compound acetyl penicillamine (AP) was used. At concentrations of 100 μM and 200 μM, this derivative of SNAP had no effect on spontaneous myometrial contractions (Figure 6). Therefore, it would appear that the S-nitroso, or NO donating portion, of SNAP is required for its relaxant effect.

3.1.7 Further investigation of the relaxant effects induced by SNAP.

Recently, several reports in the literature have suggested that NO may cause vascular smooth muscle relaxation by a direct effect on calcium-activated potassium channels in the sarcolemma (Bolotina et al., 1994; Plane et al., 1998). Because SNAP appears to induce uterine relaxation independently of cGMP, we investigated the possibility that NO may be acting directly on such a potassium channel to produce inhibition of spontaneous contractions in our preparations. Myometrial strips were relaxed with 100 μM SNAP, washed twice, allowed to re-equilibrate and pretreated with 50 nM charybdotoxin (CTX), a specific inhibitor of calcium-activated potassium channels. After a 20 minute incubation with the inhibitor, SNAP was once again added to the bath. As shown in Figure 7, CTX produced no measurable decrease in the
Figure 6. Effect of acetyl penicillamine on spontaneously-contracting rat myometrium. Representative tracing illustrating the effects of AP (200 μM) on spontaneous myometrial activity from estrogen-primed progesterone-treated rats. AP had no effect on spontaneous contractile activity. AP is a derivative of SNAP without the S-nitroso group. Addition of drug is indicated by (↑) and results are typical of 3 experiments.
Figure 7. Effect of charybdotoxin (CTX) on SNAP-induced inhibition of spontaneous myometrial contractions. Representative tracings illustrating the effect of vehicle (a) or SNAP (100 μM) in the presence and absence of CTX (50 nM) (b) in myometrial strips from estrogen-primed progesterone-treated rats. CTX had no effect on the relaxant response mediated by SNAP (relaxation before CTX was 41.8 ± 7.9%, relaxation after was 55.7 ± 8.6%). Vehicle for CTX was 150 mM NaCl and SNAP was dissolved in DMSO. Addition of drug is indicated by (↑) and results are typical of 4 experiments.
inhibition of spontaneous contractions induced by SNAP (relaxation before CTX was 41.8 ± 7.9 %, relaxation after CTX was 55.7 ± 8.6 %, n=4). In preliminary experiments, concentrations of CTX as high as 200 nM had no measurable effect on the relaxant responses to SNAP. Therefore, direct actions of NO on calcium-activated potassium channels cannot explain the relaxant effects of SNAP in the rat myometrium.

Nitric oxide has also been suggested to mediate some of its effects through changes in pH (Wyeth et al., 1996). Therefore, a preliminary study was carried out to investigate whether SNAP could alter pH levels following its addition to the tissue bath. In this study, 100 μM SNAP was added to a bath containing a myometrial strip and the pH was monitored with a pH probe. The pH did not vary by more than 0.02 pH units over a ten minute period following the addition of the drug.

3.1.8 Effect of SNAP on myometrial contractions and cGMP levels in 18-day timed pregnant rats.

In a preliminary experiment using pregnant myometrial strips it was found that SNAP (100 μM) did not cause inhibition of spontaneous contractions (Figure 8). This is in contrast to the results in non-pregnant animals shown in Figures 3 and 4 above. Cyclic GMP levels were found to be significantly elevated by SNAP in the pregnant myometria (Figure 8). However, the fold increase was much lower than that previously observed in non-pregnant animals (see Figure 5a). In the pregnant myometrial preparations, SNAP increased cGMP levels by only 5.7 fold, whereas in the non-pregnant, estrogen-primed progesterone-treated rats, tissue levels of cGMP increased 38.6 fold above control values. Thus, the hormonal state of the uterus during pregnancy appears to be affecting the generation of cGMP by SNAP, but the significance of this effect is not yet understood.
Figure 8. Effect of SNAP on myometrial contractions and cGMP levels in 18-day timed pregnant rats. Representative tracings illustrate the effect of (a) vehicle (DMSO) and (b) SNAP (100 µM). Cyclic GMP levels for the same muscles used in the contractility experiments are shown. No change in spontaneous contractile activity was found in the presence of SNAP, despite a significant increase in the tissue levels of cGMP. Results are presented as mean ± SEM for n=4 experiments. An asterisk indicates a significant difference from control (P<0.05). Cyclic GMP levels were compared by Student's t-test. Addition of drug is indicated by (▲) and results are typical of 4 experiments.
Representative Tracings

Cyclic GMP (pmol/mg protein) (n)

2.2 ± 0.5  
4

DMSO

12.6 ± 1.2 *  
4

SNAP

5 min
3.2 Contractile responses, cGMP levels and PKG activity in rat aorta.

3.2.1 Effect of SNP, ANP and 8-bromo-cGMP on PE-induced contraction and cGMP accumulation in rat aorta.

The effects of SNP (1 μM and 30 μM), ANP (100 nM) and 8-bromo-cGMP (100 μM) on PE-induced contractions (1 μM) and cGMP levels in rat aorta are shown in Figure 9. Representative tracings from these experiments are also illustrated. As shown, SNP, ANP and 8-bromo-cGMP all produced a complete relaxation of PE-induced contractions at the doses listed above. SNP (1 and 30 μM) and ANP (100 nM) generated significant increases in cGMP levels in association with their relaxant response. An additional experiment with 1 μM ANP produced complete relaxation of PE-induced contractions (105.7 ± 3.1%) and a further significant increase in the level of cGMP (4.3 ± 0.2 pmol/mg protein, n=3). To determine the importance of cGMP in the relaxations mediated by SNP, the specific inhibitor of sGC, ODQ (25 μM), was added to the bath 20 min prior to the addition of PE. ODQ completely reversed the relaxant response induced by 1 μM SNP and partially blocked the relaxation induced by 30 μM SNP (see Figure 9). An analysis of the cGMP levels in Figure 9 shows that ODQ completely blocked the elevation of cGMP at both doses of SNP, despite the fact that 30 μM SNP still produced a 25.4 ± 4.3 percent relaxation. These results indicate that at least a portion of the SNP-induced relaxation of rat aorta may be mediated through a cGMP-independent mechanism.

3.2.2 Effect of SNAP on PE-induced contraction and cGMP accumulation in rat aorta.

Figure 10 illustrates representative tracings showing the effects of SNAP (1 μM and 1 mM) on PE-induced contractions (1 μM) and presents corresponding cGMP levels
Figure 9. Effects of SNP in the absence and presence of ODQ, and ANP and 8-bromo-cGMP on PE-induced contractions and cGMP levels in rat aorta. After equilibration, aortic strips were contracted with 1 μM PE for 5 min and then washed 4 times for a further 40 min. Tension was continually adjusted to maintain a 2 g preload. Next, muscles were again contracted with 1.0 μM PE and then relaxed with SNP (1 and 30 μM), ANP (100 nM) or 8-bromo-cGMP (100 μM). In several experiments, ODQ (25 μM) was added to the bath 20 min prior to the addition of SNP. Representative tracings from these experiments as well as calculated percent relaxation values and corresponding cGMP levels are shown. Results are expressed as mean ± SEM and the number of experiments is indicated for each agent. Addition of drug is indicated by (↑). Asterisks indicate a significant difference from corresponding control and # indicates a significant difference between the absence and presence of ODQ. All comparisons were made by one-way ANOVA followed by Student-Newman-Keuls multiple comparison test.
Representative Tension Tracings

Percent Relaxation of PE-Induced Contraction (pmol/mg protein)

<table>
<thead>
<tr>
<th>Condition</th>
<th>Cyclic GMP</th>
<th>(n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>—</td>
<td>0.8 ± 0.4</td>
<td>11</td>
</tr>
<tr>
<td>ODQ PE</td>
<td>0.6 ± 0.3</td>
<td>8</td>
</tr>
<tr>
<td>PE SNP (1 μM)</td>
<td>104.4 ± 2.1 *</td>
<td>4</td>
</tr>
<tr>
<td>ODQ PE SNP (1 μM)</td>
<td>2.7 ± 1.8 #</td>
<td>4</td>
</tr>
<tr>
<td>PE SNP (30 μM)</td>
<td>98.4 ± 3.1 *</td>
<td>4</td>
</tr>
<tr>
<td>ODQ PE SNP (30 μM)</td>
<td>25.4 ± 4.3 *#</td>
<td>4</td>
</tr>
<tr>
<td>PE ANP (100 nM)</td>
<td>101.8 ± 4.0 *</td>
<td>3</td>
</tr>
<tr>
<td>PE 8-Br-cGMP (100 μM)</td>
<td>104.7 ± 3.2 *</td>
<td>3</td>
</tr>
</tbody>
</table>
Figure 10. Effect of SNAP in the absence and presence of ODQ on PE-induced contractions and cGMP levels in rat aorta. After equilibration, aortic strips were contracted with 1 μM PE for 5 min and then washed 4 times for a further 40 min. Tension was continually adjusted to maintain a 2 g preload. Next, muscles were again contracted with 1.0 μM PE and then relaxed with SNAP (1 μM and 1 mM). In several experiments, ODQ (25 μM) was added to the bath 20 min prior to the addition of SNAP. Representative tracings from these experiments as well as calculated percent relaxation values and corresponding cGMP levels are shown. Results are expressed as mean ± SEM and the number of experiments is indicated for each dose. Addition of drug is indicated by (†). Asterisks indicate a significant difference from corresponding control and † indicates a significant difference between the absence and presence of ODQ. All comparisons were made by one-way ANOVA followed by Student-Newman-Keuls multiple comparison test.
<table>
<thead>
<tr>
<th>Representative Tension Tracings</th>
<th>Percent Relaxation of PE-Induced Contraction</th>
<th>Cyclic GMP (pmol/mg protein)</th>
<th>(n)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0.6 ± 0.1</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.6 ± 0.2</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>!PE</td>
<td>104.5 ± 4.6 *</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>!PE, !SNAP (1 uM)</td>
<td>2.7 ± 0.3 *</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>!ODQ, !PE</td>
<td>23.2 ± 3.3 **</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>!ODQ, !PE</td>
<td>0.7 ± 0.1 #</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>!PE, !SNAP (1 μM)</td>
<td>100.0 ± 1.2 *</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>!PE, !SNAP (1 mM)</td>
<td>26.9 ± 4.2 *</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>!ODQ, !PE</td>
<td>94.1 ± 4.7 *</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>!ODQ, !PE</td>
<td>0.9 ± 0.1 #</td>
<td>3</td>
</tr>
</tbody>
</table>
measured in rat aorta. SNAP produced a complete relaxation of PE-induced contractions at both doses and significantly elevated cGMP levels. To investigate the role of cGMP in these relaxant responses, ODQ was used to inhibit sGC. As shown in Figure 10, ODQ (25 μM) produced a partial blockade of the relaxation induced by 1 μM SNAP, but had no effect on the relaxation produced by 1 mM SNAP. ODQ completely blocked the cGMP elevation caused by both concentrations of SNAP despite the continued presence of SNAP-induced relaxations. Once again, there appears to be a poor correlation between increases in cGMP and decreases in rat aorta tension. In comparison to SNP, the results with SNAP suggest that an even larger portion of the relaxant response to SNAP is mediated by cGMP-independent mechanisms.

3.2.3 Cumulative dose-responses to SNP and SNAP in the absence and presence of ODQ.

In order to further investigate the inability of an ODQ-mediated blockade of cGMP elevation to affect 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 Figures 11A and 11B, ODQ produced a significant shift in the relaxant response induced by these cGMP-elevating agents. In Figure 11A, ODQ markedly reduced the relaxation caused by concentrations of SNP from 10 nM to 1 μM, although as the concentration of SNP increased above that a large portion of the relaxant response continued to occur despite sGC inhibition by ODQ. In Figure 11B, ODQ only reduced SNAP-induced relaxation significantly at 100 nM. 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
Figure 11. Cumulative dose-responses to SNP (A) and SNAP (B) in the absence and presence of ODQ (25 μM) on PE-induced contractions in rat aorta. After equilibration, aortic strips were contracted with 1 μM PE for 5 min and then washed 4 times for a further 40 min. Tension was continually adjusted to maintain a 2 g preload. Next, muscles were again contracted with 1.0 μM PE and then relaxed with increasing concentrations of SNP and SNAP. To investigate the effect of ODQ on the cumulative dose-response, this sGC inhibitor was added to the bath 20 min prior to the final PE-contraction. Percent inhibition of contraction was determined by measuring the change in amplitude of the PE-induced contraction over time. Control, PE-contracted aortae were also monitored throughout the cumulative dose-response period to assess changes in the amplitude of contraction over time. In A, control, contracted muscles relaxed a total of 7.9 ± 4.2% (n=4) over the length of the dose-response period. In B, control, contracted muscles relaxed a total of 9.8 ± 1.7% (n=4) over the dose-response period. Data are expressed as means, with vertical lines showing SEM for 4 aortic strips from 4 different rats. Asterisks indicate a significant difference between the absence and presence of ODQ (P<0.05). All comparisons were made using a one-way ANOVA.
SNAP-induced relaxation has a greater cGMP-independent component making it less sensitive to sGC inhibition by ODQ.

### 3.2.4 Effect of SNP on PKG activity ratios in rat aorta.

PKG has been reported to exist in the rat aorta; however, total levels of the kinase and activity ratios have not yet been determined. To provide such data, the effect of SNP (30 μM) on PKG activity ratios in the rat aorta was measured. Strips of rat aorta were suspended in tissue baths as described in the Methods section, 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 3, 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 activity ratio with SNP was due to activation of specific PKG by increases in endogenous cGMP, and not to an increase in non-specific (cyclic nucleotide independent) protein kinase activity. The total levels of PKG are similar to those measured previously in rat myometria and are approximately double what has previously been found in rat vas deferens (Patel et al., 1997).

### 3.3 Two-dimensional gel electrophoresis results.

#### 3.3.1 Validation and optimization of technique.

A considerable amount of time was spent improving the sensitivity and reproducibility of the two-dimensional gel electrophoresis methodology used in this thesis. This was crucial for the kind of experiments proposed in this thesis. Before the two-dimensional gel electrophoresis technique could be utilized to determine protein phosphorylation patterns in drug-treated smooth muscles, it was necessary to ensure that the conditions in the protocol were stable and that tissue viability was maintained
Table 3. Effect of SNP on cGMP-dependent protein kinase activity in PE-contracted rat aorta.

<table>
<thead>
<tr>
<th>PKG Activity (pmol PO₄/min/mg)</th>
<th>Activity Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>(- cGMP)</td>
<td>(+ cGMP)</td>
</tr>
<tr>
<td>Control</td>
<td>4.4 ± 0.5</td>
</tr>
<tr>
<td>SNP (30 μM)</td>
<td>12.1 ± 1.4 *</td>
</tr>
</tbody>
</table>

Note: Values are mean ± SEM for n=4. Control and SNP-treated tissues were compared using Student's t-test. Asterisks indicate a significant difference from control (p<0.05).
throughout the labeling and treatment process. Figure 12 illustrates a preliminary experiment in which the degree of radiolabeled phosphate incorporation into two soluble proteins identified as PKG substrates (proteins A and B of Table 4), as well as two non-stimulated spots (proteins X and Y) representing endogenous phosphorylation was determined over time. Tissue strips were incubated in 500 μCi/ml $^{32}$P at 37°C for 30 minutes, 2 hours, 3 hours and 5 hours. Prior to freezing, tissues were contracted with 1 μM phenylephrine for 7 minutes, with 30 μM SNP being added for the last 3 minutes. A protein sample was prepared as described in the Methods section and separated using two dimensional gel electrophoresis. Analysis of the autoradiographs showed that the level of phosphate incorporation increased gradually from 30 minutes to 3 hours when it reached a plateau. For this figure, we did not apply the normalization procedure described in the Methods section. The values shown are background-corrected optical densities taken directly from the autoradiographs. Because $^{32}$P incorporation reached an apparent saturation point at approximately 3 hours, we concluded that all subsequent experiments would be carried out with a 3 hour radiolabelled phosphate incubation.

To ensure that no changes in the specific radioactivity of the extracellular phosphate pool were occurring over the 5 hour incubation period, samples of incubation buffer were assayed for total (chemical) phosphate content and radioactivity. No changes in specific radioactivity of the extracellular phosphate pool were detected at any of the time points. Therefore, our labeling conditions appear stable and the plateau in $^{32}$P incorporation observed at 3 hours is most likely due to a saturation of intracellular ATP pools with $^{32}$P and was not influenced by changes in specific radioactivity of the incubation buffer. Smooth muscle viability was assessed in a tissue bath.
Figure 12. Time course examining the degree of radiolabeled phosphate incorporation over time into two PKG substrates and two endogenously phosphorylated proteins from intact rat aortic smooth muscle. Tissue strips were incubated with 500 μCi/ml of $^{32}$P at 37°C for the times indicated. Prior to freezing, tissues were contracted with 1 μM phenylephrine for 7 mins and relaxed with the addition of 30 μM SNP for the last 3 mins. The soluble protein fraction from each tissue was separated using two dimensional gel electrophoresis. Second dimension gels were silver stained, dried and exposed to x-ray film for a period of 24 hours. The integrated intensity values determined from the autoradiographs are a quantitative assessment of the degree of phosphorylation of a particular substrate or protein.
Protein B
Protein C
Protein X
Protein Y

Incubation time with radiolabeled phosphate

Background corrected integrated intensity
Muscles exhibited good responses to contraction with 1 μM phenylephrine, and to relaxation with 30 μM SNP, following the 3 hour incubation period at 37°C. The pH was also found to remain steady at 7.4 throughout the incubation period.

3.3.2 Studies in rat aorta.

3.3.2.1 Effect of SNP on intact rat aorta smooth muscle phosphorylation.

Figure 13 shows a typical silver stain of our 11% (total monomer concentration) two-dimensional gels. This particular gel illustrates the soluble protein pattern generated from a control, untreated intact aorta segment following labeling of the intracellular ATP pools. The sensitivity of protein detection for this silver stain is rated at approximately 0.25 ng. The molecular weight (MW) markers on the left hand side show that the MW of the proteins in this gel range from approximately 10 kDa to 140 kDa. The isoelectric point (pI) ranges from approximately 4.0 to 7.8, moving from left to right, respectively. In all of the 11% autoradiographs that follow, similar MW and isoelectric point ranges were found.

Figures 14-17 are representative autoradiographs of a single experiment showing soluble smooth muscle protein phosphorylation from untreated control tissues (Figure 14) and from tissues treated with 1 μM PE for 7 min (Figure 15), 1 μM PE for 7 min plus 30 μM SNP for the last 3 min (Figure 16) and 25 μM ODQ for 20 min plus 1 μM PE for 7 min plus 30 μM SNP for the last 3 min (Figure 17). Ten proteins whose phosphorylation levels are altered in the presence of SNP have been identified on the gels as A through J. The reproducibility of the technique is demonstrated by comparing the four images in Figures 14 through 17. The protein patterns are nearly identical, thus making detection of new drug-induced protein phosphorylation relatively easy. In the autoradiographs
Figure 13. Representative 11%T silver stained gel of a soluble protein sample following two dimensional gel electrophoresis. This particular protein sample was generated from an untreated intact aortic segment. In order to determine the molecular weights of the proteins resolved by two dimensional gel electrophoresis, the molecular weight standards must be shifted up by the depth of the well seen at the top left hand corner.
Figure 14. Representative autoradiograph of soluble protein phosphorylation in control, untreated rat aortic smooth muscle. Intact tissue strips were incubated with $^{32}$P for three hours at 37°C and frozen. The soluble protein fraction from each tissue was separated using two dimensional gel electrophoresis. Second dimension SDS-PAGE gels of 11%T were silver stained, dried and exposed to x-ray film for 24 hours. Ten proteins of interest to our study have been identified on the autoradiograph as A through J.
Figure 15. Representative autoradiograph of soluble protein phosphorylation in rat aortic smooth muscle treated with 1 μM PE for 7 min. Intact tissue strips were incubated with $^{32}$P for three hours at 37°C, treated with PE and frozen. The soluble protein fraction from each tissue was separated using two dimensional gel electrophoresis. Second dimension SDS-PAGE gels of 11%T were silver stained, dried and exposed to x-ray film for 24 hours. Ten proteins of interest to our study have been identified on the autoradiograph as A through J. The phosphorylation of proteins I and J is significantly increased above control.
Figure 16. Representative autoradiograph of soluble protein phosphorylation in rat aortic smooth muscle treated with 1 μM PE for a total of 7 min with 30 μM SNP added for the last 3 min. Intact tissue strips were incubated with $^{32}$P for three hours at 37°C, treated with PE+SNP and frozen. The soluble protein fraction from each tissue was separated using two dimensional gel electrophoresis. Second dimension SDS-PAGE gels of 11%T were silver stained, dried and exposed to x-ray film for 24 hours. Ten proteins of interest to our study have been identified on the autoradiograph as A through J. SNP resulted in a significant increase in the phosphorylation of proteins A through G and a significant decrease in the phosphorylation of proteins H through J compared to control- and PE-treated muscles.
Figure 17. Representative autoradiograph of soluble protein phosphorylation in rat aortic smooth muscle pretreated with 25 μM ODQ for 20 min followed by 1 μM PE for 7 min plus 30 μM SNP for the last 3 min. Intact tissue strips were incubated with $^{32}$P for three hours at 37°C, treated with ODQ+PE+SNP and frozen. The soluble protein fraction from each tissue was separated using two dimensional gel electrophoresis. Second dimension SDS-PAGE gels of 11%T were silver stained, dried and exposed to x-ray film for 24 hours. Ten proteins of interest to our study have been identified on the autoradiograph as A through J. The seven proteins that exhibited increased phosphorylation in the presence of SNP alone are significantly decreased during a blockade of cGMP elevation by ODQ. In addition, the decreased phosphorylation of proteins H through J has been significantly reversed by ODQ.
from the 11% gels described above, a few of the proteins identified required further resolution for densitometry analysis. As a result, the protein samples from the above treated tissues were also separated on 7.5% and 15% gels. Figures 18 and 19 show typical silver stains of 7.5% and 15% gels. Both gels illustrate the soluble protein pattern generated from an intact aorta segment treated with 1 μM PE for 7 min. The molecular weight markers on the left-hand side indicate that the MW of the proteins in the 7.5% gel range from approximately 20 kDa to 200 kDa, whereas the MW ranges in the 15% gel extend from 5 kDa to 100 kDa. The isoelectric points in both gels range from approximately 4.0 to 7.8, moving from left to right. Since the purpose of these gels was to further resolve specific proteins of interest, only a section of the original autoradiograph has been included in Figures 20 and 21. These sections are identified by MW markers and pI markers, and the proteins of interest have been labeled with the same corresponding letters as shown in the 11% gels above. In Figure 20, the three 56 kDa proteins D, E and F have been further resolved and in Figure 21, several of the low MW proteins have been more clearly identified.

Quantitative assessments of the degree of phosphorylation in the autoradiographs described above are shown in Table 4. SNP resulted in a significant increase in $^{32}$P incorporation into proteins A through G, whereas it produced a significant decrease in $^{32}$P incorporation into proteins H through J. By comparing the pattern of phosphorylation in an untreated aorta (Figure 14) with that of a PE control (Figure 15), it was found that the level of phosphorylation of proteins I and J increased significantly in the presence of PE, presumably in a calcium-dependent manner (Table 4). Thus, when muscles were relaxed with SNP, presumably accompanied by a decrease in cytoplasmic calcium, proteins I and J were dephosphorylated (see Table 4). From this comparison
Figure 18. Representative 7.5%T silver stained gel of a soluble protein sample following two dimensional gel electrophoresis. This particular protein sample was generated from an intact aortic segment treated with 1 μM PE for 7 min. In order to determine the molecular weights of the proteins resolved by two dimensional gel electrophoresis, the molecular weight standards must be shifted up by the depth of the well seen at the top left hand corner.
Figure 19. Representative 15%T silver stained gel of a soluble protein sample following two dimensional gel electrophoresis. This particular protein sample was generated from an intact aortic segment treated with 1 μM PE for 7 min. In order to determine the molecular weights of the proteins resolved by two dimensional gel electrophoresis, the molecular weight standards must be shifted up by the depth of the well seen at the top left hand corner.
Figure 20. Representative autoradiographs of soluble protein phosphorylation in rat aortic smooth muscle separated on 7.5%T second dimension SDS-PAGE gels. Intact tissue strips were incubated with $^{32}$P for three hours 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 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. 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 two dimensional gel electrophoresis. Second dimension SDS-PAGE gels of 7.5%T were silver stained, dried and exposed to x-ray film for 24 hours. A specific section of the autoradiographs for each treatment has been chosen to illustrate an increased resolution of the proteins D, E and F. These sections are identified by MW markers and pi markers, and the proteins have been labeled with the same corresponding letters as shown in Figure 16 above. SNP resulted in a significant increase in the phosphorylation of proteins D, E and F compared to control- and PE-treated muscles and this effect was significantly reversed by a blockade of cGMP elevation with ODQ.
Figure 21. Representative autoradiographs of soluble protein phosphorylation in rat aortic smooth muscle separated on 15%T second dimension SDS-PAGE gels. Intact tissue strips were incubated with $^{32}$P for three hours 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 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. 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 two dimensional gel electrophoresis. Second dimension SDS-PAGE gels of 15%T were silver stained, dried and exposed to x-ray film for 24 hours. A specific section of the autoradiographs for each treatment has been chosen to illustrate an increased resolution of several of the lower molecular weight proteins of interest identified in Figure 16. These sections are identified by MW markers and pi markers, and the proteins have been labeled with the same corresponding letters as shown in our 11%T autoradiographs above. SNP resulted in a significant increase in the phosphorylation of proteins A through C and G and a significant decrease in the phosphorylation of proteins H through J compared to control and PE-treated muscles. The effects of SNP were significantly reversed by blockade of cGMP elevation with ODQ.
Table 4. Effect of SNP in the absence and presence of ODQ, on $^{32}$P incorporation into intact rat aortic smooth muscle proteins.

<table>
<thead>
<tr>
<th>Protein</th>
<th>MW</th>
<th>$P_i$</th>
<th>Control</th>
<th>PE (1 μM) 7 min</th>
<th>PE (1 μM) 4 min + SNP (30 μM) 3 min</th>
<th>ODQ (25 μM) 20 min + PE + SNP</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>22</td>
<td>5.8</td>
<td>2.1 ± 0.9</td>
<td>1.7 ± 0.6</td>
<td>14.7 ± 4.8 **</td>
<td>3.32 ± 1.4 *</td>
</tr>
<tr>
<td>B</td>
<td>21</td>
<td>6.3</td>
<td>0.6 ± 0.2</td>
<td>0.5 ± 0.1</td>
<td>10.1 ± 2.9 **</td>
<td>1.5 ± 0.5 *</td>
</tr>
<tr>
<td>C</td>
<td>26</td>
<td>6.6</td>
<td>0.3 ± 0.1</td>
<td>0.3 ± 0.05</td>
<td>1.6 ± 0.4 **</td>
<td>0.3 ± 0.09 *</td>
</tr>
<tr>
<td>D</td>
<td>56</td>
<td>7.5</td>
<td>0.7 ± 0.3</td>
<td>0.6 ± 0.2</td>
<td>9.0 ± 3.7 **</td>
<td>1.7 ± 0.7 *</td>
</tr>
<tr>
<td>E</td>
<td>56</td>
<td>7.4</td>
<td>1.1 ± 0.5</td>
<td>1.2 ± 0.6</td>
<td>6.2 ± 1.9 **</td>
<td>1.5 ± 0.4 *</td>
</tr>
<tr>
<td>F</td>
<td>56</td>
<td>7.3</td>
<td>1.1 ± 0.5</td>
<td>0.6 ± 0.2</td>
<td>4.0 ± 1.0 **</td>
<td>1.6 ± 0.6 *</td>
</tr>
<tr>
<td>G</td>
<td>16</td>
<td>6.9</td>
<td>0.6 ± 0.3</td>
<td>0.8 ± 0.3</td>
<td>3.9 ± 0.8 **</td>
<td>1.0 ± 0.3 *</td>
</tr>
<tr>
<td>H</td>
<td>22</td>
<td>6.4</td>
<td>7.4 ± 1.7</td>
<td>9.4 ± 1.8</td>
<td>3.4 ± 1.0 **</td>
<td>6.4 ± 0.9 *</td>
</tr>
<tr>
<td>I</td>
<td>21</td>
<td>5.2</td>
<td>5.1 ± 1.7</td>
<td>11.7 ± 2.1 *</td>
<td>3.8 ± 1.6 *</td>
<td>6.8 ± 1.8</td>
</tr>
<tr>
<td>J</td>
<td>21</td>
<td>5.3</td>
<td>5.2 ± 0.8</td>
<td>11.6 ± 1.5 *</td>
<td>3.3 ± 0.8 *</td>
<td>6.7 ± 0.9 **</td>
</tr>
</tbody>
</table>

Values are presented as background-corrected optical density, integrated over all pixels in the spot. The data shown are mean ± SEM from n=5 or 6 experiments, with each experiment including a control, PE, PE+SNP, and ODQ+PE+SNP. Densitometry values are converted to percent of corresponding control for statistical analysis. This conversion eliminates some of the variations in densitometry that occur from one experiment to the next (see Methods). Groups were compared by one-way ANOVA followed by Student-Newman-Keuls multiple comparison test. Molecular weight (MW) in kDa and isoelectric point ($P_i$) were calculated from standards run concurrently. Asterisks indicate a significant difference from control (p<0.05), plus signs indicate a significant difference from PE-treated (p<0.05), and number signs indicate a significant difference between the absence and presence of ODQ in SNP-treated muscles.
we can conclude that proteins I and J are not PKG substrates. 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 may be somehow directly mediated by PKG.

In order to determine if the changes in phosphorylation induced by SNP in proteins A through H were, in fact, PKG-mediated, we compared phosphorylation patterns in control untreated aortae (Figure 14), SNP treated aortae (Figure 16) and in aortae pretreated with the soluble guanylyl cyclase inhibitor, ODQ (Figure 17). ODQ effectively inhibited both the increase in phosphorylation of proteins A-G and the decrease in phosphorylation of proteins H through J (see Table 4). This is consistent with the conclusion that proteins A through 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 protein as H. PKG-mediated phosphorylation of protein H appears to induce a shift in the isoelectric point giving rise to protein B. The fact that protein H is already phosphorylated in our control gels suggests that this protein undergoes additional phosphorylation by PKG at a separate site leading to the isoelectric point shift observed in our gels. Similar shifts in isoelectric point have been described during smooth muscle myosin light chain phosphorylation (Silver and Stull, 1982). In that study, the isoelectric point of myosin light chain changed from approximately 5.1 to 5.0 during a myosin light chain kinase-mediated addition of phosphate (Silver and Stull, 1982).
3.3.2.2 Effect of ANP, 8-bromo-cGMP and SNAP on intact rat aorta smooth muscle phosphorylation.

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 other cGMP-elevating agents (ANP and SNAP) and a PKG activator (8-bromo-cGMP). Because ANP and 8-bromo-cGMP do not rely on sGC to increase cGMP and activate PKG, ODQ was not used in this group of experiments. Figures 22 and 23 are typical autoradiographs from 11% gels showing soluble smooth muscle protein phosphorylation in tissues treated with 1 μM PE for 9 min plus ANP (10 μM) for the last 5 min (Figure 22) and 1 mM 8-bromo-cGMP for 15 min followed by 1 μM PE-induced contraction for 5 min. The same ten proteins that were identified during SNP-induced relaxation also showed altered phosphorylation in the presence of ANP and 8-bromo-cGMP. To further resolve the proteins A through J, protein samples from ANP- and 8-bromo-cGMP-treated muscles were also separated on 7.5% and 15% gels. Figures 24 and 25 show specific sections of these autoradiographs, which are indicated by MW markers and pI values. The proteins of interest have been labeled with the same corresponding letters as indicated above. Figure 24 increases the resolution of proteins D, E and F, whereas Figure 25 more clearly identifies several of the lower molecular weight proteins.

Table 5 quantitates the changes in phosphorylation induced by ANP and 8-bromo-cGMP in the autoradiographs described above. ANP resulted in a significant increase in phosphorylation of proteins A through F and produced a significant decrease in phosphorylation of proteins H through J. Only protein G failed to show a significant change, although the level of phosphorylation does appear to have increased.
Figure 22. Representative autoradiograph of soluble protein phosphorylation in rat aortic smooth muscle treated with 1 μM PE for a total of 9 min with 10 μM ANP added for the last 5 min. Intact tissue strips were incubated with $^{32}$P for three hours at 37°C, treated with PE+ANP and frozen. The soluble protein fraction from each tissue was separated using two dimensional gel electrophoresis. Second dimension SDS-PAGE gels of 11%T were silver stained, dried and exposed to x-ray film for 24 hours. Ten proteins of interest to our study have been identified on the autoradiograph as A through J. ANP resulted in a significant increase in the phosphorylation of proteins A through F and a significant decrease in the phosphorylation of proteins H through J compared to PE-treated muscles.
Figure 23. Representative autoradiograph of soluble protein phosphorylation in rat aortic smooth muscle treated with 1 mM 8-bromo-cGMP for 15 min followed by 1 µM PE for 5 min. Intact tissue strips were incubated with $^{32}$P for three hours at 37°C, treated with 8-bromo-cGMP+PE and frozen. The soluble protein fraction from each tissue was separated using two dimensional gel electrophoresis. Second dimension SDS-PAGE gels of 11%T were silver stained, dried and exposed to x-ray film for 24 hours. Ten proteins of interest to our study have been identified on the autoradiograph as A through J. 8-bromo-cGMP resulted in a significant increase in phosphorylation of proteins B, D and F and a significant decrease in the phosphorylation of proteins I and J compared to PE-treated controls.
Figure 24. Representative autoradiographs of soluble protein phosphorylation in rat aortic smooth muscle separated on 7.5%T second dimension SDS-PAGE gels. Intact tissue strips were incubated with $^{32}$P for three hours at 37°C, and then left untreated to act as control or treated with 1 µM PE for 9 min, 1 µM PE for 9 min plus 10 µM ANP for the last 5 min and 1 mM 8-bromo-cGMP for 15 min plus 1 µM PE for 5 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 two dimensional gel electrophoresis. Second dimension SDS-PAGE gels of 7.5%T were silver stained, dried and exposed to x-ray film for 24 hours. A specific section of the autoradiographs for each treatment has been chosen to illustrate an increased resolution of the proteins D, E and F. These sections are identified by MW markers and pl markers, and the proteins have been labeled with the same corresponding letters as described above. ANP resulted in a significant increase in the phosphorylation of proteins D, E and F, whereas 8-bromo-cGMP only significantly increased the phosphorylation of protein D and F.
Figure 25. Representative autoradiographs of soluble protein phosphorylation in rat aortic smooth muscle separated on 15%T second dimension SDS-PAGE gels. Intact tissue strips were incubated with $^{32}$P for three hours at 37°C, and then left untreated to act as control or treated with 1 μM PE for 9 min, 1 μM PE for 9 min plus 10 μM ANP for the last 5 min and 1 mM 8-bromo-cGMP for 15 min plus 1 μM PE for 5 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 two dimensional gel electrophoresis. Second dimension SDS-PAGE gels of 15%T were silver stained, dried and exposed to x-ray film for 24 hours. A specific section of the autoradiographs for each treatment has been chosen to illustrate an increased resolution of several of the lower molecular weight proteins of interest identified above with SNP. These sections are identified by MW markers and pI markers, and the proteins have been labeled with the same corresponding letters as shown in our 11%T autoradiographs above. ANP resulted in a significant increase in the phosphorylation of proteins A through C, whereas 8-bromo-cGMP only significantly increased the phosphorylation of protein B compared to control- and PE-treated muscles. Both agents resulted in a significant reduction in the phosphorylation of proteins I and J.
<table>
<thead>
<tr>
<th>Protein</th>
<th>MW</th>
<th>P_i</th>
<th>Control</th>
<th>PE (1 μM) 9 min</th>
<th>PE (1 μM) 4 min + ANP (10 μM) 5 min</th>
<th>8-Br-cGMP (1mM) 15 min + PE (1 μM) 5 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>22</td>
<td>5.8</td>
<td>1.1 ± 0.5</td>
<td>1.3 ± 0.3</td>
<td>4.6 ± 1.9 **</td>
<td>3.6 ± 1.5</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>
<td>2.9 ± 1.0 **</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>
<td>1.2 ± 0.6</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>
<td>14.8 ± 8.2 **</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>
<td>5.4 ± 1.9</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>
<td>7.1 ± 2.7 **</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>
<td>1.8 ± 0.5</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>
<td>5.6 ± 1.5</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>
<td>6.6 ± 2.4</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>
<td>8.4 ± 2.4*</td>
</tr>
</tbody>
</table>

Values are presented as background-corrected optical density, integrated over all pixels in the spot. The data shown are mean ± SEM from n=5 or 6 experiments, with each experiment including a control, PE, PE+ANP, and 8-Br-cGMP+PE. Densitometry values are converted to percent of corresponding control for statistical analysis. This conversion eliminates some of the variations in densitometry that occur from one experiment to the next (see Methods). Molecular weight (MW) in kDa and isoelectric point (P_i) were calculated from standards run concurrently. Groups were compared by one-way ANOVA followed by Student-Newman-Keuls multiple comparison test. Asterisks indicate a significant difference from control (p<0.05), and plus signs indicate a significant difference from PE-treated (p<0.05).
8-bromo-cGMP induced a significant increase in $^{32}$P incorporation into proteins B, D and F and significantly decreased $^{32}$P incorporation into proteins I and J (Table 5). All the remaining proteins identified in Table 5 do show a change in the level of phosphorylation during 8-bromo-cGMP-induced relaxation, but the changes are not statistically significant. In general, the levels of phosphorylation induced by ANP and 8-bromo-cGMP were noticeably lower than those observed with SNP. Because ANP is not as good an elevator of cGMP (see Figure 9), this may explain the lesser degree of PKG-mediated phosphorylation with this agent. Problems associated with the ability of 8-bromo-cGMP 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 phosphorylation described above, the results with ANP and 8-bromo-cGMP do confirm that the phosphorylations of proteins A through G are PKG-mediated. Since no new PKG-substrates were identified from these autoradiographs, it is likely that ANP and 8-bromo-cGMP are causing relaxation similarly to SNP in rat aorta, assuming that phosphorylation of these proteins is, in fact, responsible for relaxation.

Figures 26 and 27 are representative autoradiographs showing soluble protein phosphorylation from aortic segments treated with 1 μM PE for 7 min plus 1 mM SNAP for the last 3 min (Figure 26) and 25 μM ODQ for 20 min plus 1 μM PE for 7 min plus 1 mM SNAP for the last 3 min (Figure 27). SNAP altered the phosphorylation of the same ten proteins (A through J) identified during SNP-induced relaxation in the aorta. Pretreatment with ODQ resulted in a blockade of SNAP-induced changes in protein phosphorylation (see Figure 27). Table 6 shows the quantitative assessments of the degree of PKG-mediated phosphorylation during SNAP-induced relaxation. Since 7.5% and 15% gels were not analyzed in this study, densitometry analyses were limited to
Figure 26. Representative autoradiograph of soluble protein phosphorylation in rat aortic smooth muscle treated with 1 µM PE for a total of 7 min with 1 mM SNAP added for the last 3 min. Intact tissue strips were incubated with $^{32}$P for three hours at 37°C, treated with PE+SNAP and frozen. The soluble protein fraction from each tissue was separated using two dimensional gel electrophoresis. Second dimension SDS-PAGE gels of 11%T were silver stained, dried and exposed to x-ray film for 24 hours. Ten proteins of interest to our study have been identified on the autoradiograph as A through J. SNAP resulted in a significant increase in the phosphorylation of proteins A through C and a significant decrease in phosphorylation of proteins H and I compared to PE-treated controls.
Figure 27. Representative autoradiograph of soluble protein phosphorylation in rat aortic smooth muscle pretreated with 25 µM ODQ for 20 min followed by 1 µM PE for 7 min plus 1 mM SNAP for the last 3 min. Intact tissue strips were incubated with $^{32}$P for three hours at 37°C, treated with ODQ+PE+SNAP and frozen. The soluble protein fraction from each tissue was separated using two dimensional gel electrophoresis. Second dimension SDS-PAGE gels of 11%T were silver stained, dried and exposed to x-ray film for 24 hours. Ten proteins of interest to our study have been identified on the autoradiograph as A through J. ODQ resulted in significant changes in SNAP-induced protein phosphorylation of proteins A through H and J.
Table 6. Effect of SNAP, in the absence and presence of ODQ, on \(^{32}\)P incorporation into intact rat aortic smooth muscle proteins.

<table>
<thead>
<tr>
<th>Protein</th>
<th>MW</th>
<th>(P_i)</th>
<th>Control</th>
<th>PE (1 (\mu)M) 7 min</th>
<th>PE (1 (\mu)M) 4 min + SNAP (1 mM) 3 min</th>
<th>ODQ (25 (\mu)M) 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>
<td>4.8 ± 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>
<td>1.9 ± 0.4 **</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>
<td>3.9 ± 1.9 #</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>
<td>2.9 ± 0.9 #</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>
<td>16.3 ± 2.6 *</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>
<td>17.9 ± 1.2 **</td>
</tr>
</tbody>
</table>

Values are presented as background-corrected optical density, integrated over all pixels in the spot. The data shown are mean ± SEM from n=5 or 6 experiments, with each experiment including a control, PE, PE+SNAP, and ODQ+PE+SNAP. Densitometry values are converted to percent of corresponding control for statistical analysis. This conversion eliminates some of the variations in densitometry that occur from one experiment to the next (see Methods). Molecular weight (MW) in kDa and isoelectric point (\(P_i\)) were calculated from standards run concurrently.

Groups were compared by one-way ANOVA followed by Student-Newman-Keuls multiple comparison test. Asterisks indicate a significant difference from control (\(p<0.05\)), plus signs indicate a significant difference from PE-treated (\(p<0.05\)), and number signs indicate a significant difference between the absence and presence of ODQ in SNAP-treated muscles.
proteins A through C and H through J. SNAP induced a significant increase in 32P incorporation into proteins A through 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 due to 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 through C, H and J. It also appeared to reverse the effect of SNAP on protein I, although because of the variability ODQ+SNAP is not significantly different from SNAP alone. Note that ODQ+PE+SNAP is not significant from PE alone. The results of Table 6 and Figures 26 and 27 clearly demonstrate that SNAP mimics SNP-induced and 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.

3.3.2.3 Protein phosphorylation in the particulate fraction of rat aorta.

In all of 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. A representative silver stain from these experiments is shown in Figure 28. This particular 11% gel illustrates the particulate protein pattern generated from a control, untreated intact aorta segment. The MW and isoelectric point ranges are similar to those described in our soluble protein separations above. A typical particulate fraction autoradiograph is shown in Figure 29. Several in vitro investigations have demonstrated
Figure 28. Representative 11%T silver stained gel of a particulate protein sample following two dimensional gel electrophoresis. This particular protein sample was generated from an untreated intact aortic segment. In order to determine the molecular weights of the proteins resolved by two dimensional gel electrophoresis, the molecular weight standards must be shifted up by the depth of the well seen at the top left hand corner.
Figure 29. Representative autoradiograph of particulate protein phosphorylation in rat aortic smooth muscle treated with 1 μM PE for 7 min. Intact tissue strips were incubated with $^{32}$P for three hours at 37°C, treated with PE and frozen. The particulate protein fraction from each tissue was separated using two dimensional gel electrophoresis. Second dimension SDS-PAGE gels of 11%T were silver stained, dried and exposed to x-ray film for 24 hours.
PKG-mediated phosphorylation of numerous membrane bound proteins in smooth muscle (Casnellie et al., 1980; Parks et al., 1987; Li et al., 1996). However, we were unable to detect these phosphorylation events in the intact tissue setting.

3.3.3 Non-vascular smooth muscles.

3.3.3.1 Studies in rat myometrium.

3.3.3.1.1 Effect of SNAP on intact myometrial smooth muscle phosphorylation.

The rat myometrium can be classified as a "non-responsive" smooth muscle in that it does not relax in response to an elevation of cGMP and activation of PKG by agents such as SNP. However, as shown earlier (Figure 5), it does relax in the presence of SNAP through an unknown mechanism that may involve a cGMP-independent component. 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 this NO donor for our initial investigations into the patterns of PKG-mediated phosphorylation in the myometrium. In the research objectives of this thesis, 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(s) may be of particular importance in cGMP-mediated smooth muscle relaxation. Figure 30 shows a typical silver stain of the soluble protein pattern generated from an untreated intact preparation of myometrium. The MW and isoelectric point ranges are similar to earlier gels and are indicated on the figure. Autoradiographs showing intact myometrial phosphorylation in a control tissue and from tissues treated with 1 mM SNAP for 5 min, and 25 μM ODQ for 20 min plus 1 mM SNAP for 5 min are shown in Figures 31 through 33, respectively.
Figure 30. Representative 11%T silver stained gel of a soluble protein sample from rat myometrium following two dimensional gel electrophoresis. This particular protein sample was generated from an untreated intact myometrial preparation. In order to determine the molecular weights of the proteins resolved by two dimensional gel electrophoresis, the molecular weight standards must be shifted up by the depth of the well seen at the top left hand corner.
Figure 31. Representative autoradiograph of soluble protein phosphorylation in control, untreated rat myometrial smooth muscle. Intact tissue strips were incubated with $^{32}$P for three hours at 37°C and frozen. The soluble protein fraction from each tissue was separated using two dimensional gel electrophoresis. Second dimension SDS-PAGE gels of 11%T were silver stained, dried and exposed to x-ray film for 48 hours. Four proteins of interest to our study have been identified on the autoradiograph as K through N.
Figure 32. Representative autoradiograph of soluble protein phosphorylation in rat myometrial smooth muscle treated with 1 mM SNAP for 5 min. Intact tissue strips were incubated with $^{32}$P for three hours at 37°C, treated with SNAP and frozen. The soluble protein fraction from each tissue was separated using two dimensional gel electrophoresis. Second dimension SDS-PAGE gels of 11%T were silver stained, dried and exposed to x-ray film for 48 hours. Four proteins of interest to our study have been identified on the autoradiograph as K through N. SNAP resulted in a significant increase in the phosphorylation of proteins K through M.
Figure 33. Representative autoradiograph of soluble protein phosphorylation in rat myometrial smooth muscle pretreated with 25 μM ODQ for 20 min followed by 1 mM SNAP for 5 min. Intact tissue strips were incubated with $^{32}$P for three hours at 37°C, treated with ODQ+SNAP and frozen. The soluble protein fraction from each tissue was separated using two dimensional gel electrophoresis. Second dimension SDS-PAGE gels of 11%T were silver stained, dried and exposed to x-ray film for 48 hours. Four proteins of interest to our study have been identified on the autoradiograph as K through N. ODQ had no effect on the SNAP-induced increases in protein phosphorylation demonstrated in Figure 32.
Table 7. Effect of SNAP in the presence and absence of ODQ, on $^{32}$P incorporation into intact rat myometrial smooth muscle proteins.

<table>
<thead>
<tr>
<th>Protein</th>
<th>MW</th>
<th>$P_i$</th>
<th>Control</th>
<th>SNAP (1 mM) 5 min</th>
<th>ODQ (25 μM) 20 min + SNAP (1 mM) 5 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>K</td>
<td>43</td>
<td>7.8</td>
<td>0.5 ± 0.1</td>
<td>10.9 ± 3.8 *</td>
<td>16.8 ± 2.0 *#</td>
</tr>
<tr>
<td>L</td>
<td>43</td>
<td>7.6</td>
<td>0.8 ± 0.2</td>
<td>2.7 ± 0.7 *</td>
<td>1.4 ± 0.3 *</td>
</tr>
<tr>
<td>M</td>
<td>43</td>
<td>7.5</td>
<td>0.5 ± 0.2</td>
<td>2.1 ± 0.7 *</td>
<td>1.7 ± 0.3 *</td>
</tr>
<tr>
<td>N</td>
<td>28</td>
<td>5.8</td>
<td>3.3 ± 1.0</td>
<td>8.6 ± 2.2</td>
<td>8.3 ± 0.3 *</td>
</tr>
</tbody>
</table>

Values are presented as background-corrected optical density, integrated over all pixels in the spot. The data shown are mean ± SEM from n=4 or 5 experiments, with each experiment including a control, SNAP and ODQ+SNAP. Densitometry values are converted to percent of corresponding control for statistical analysis. This conversion eliminates some of the variations in densitometry that occur from one experiment to the next (see Methods). Molecular weight (MW) in kDa and isoelectric point (Pi) were calculated from standards run concurrently. Groups were compared by one-way ANOVA followed by Student-Newman-Keuls multiple comparison test. Asterisks indicate significant difference from control (p<0.05) and number signs indicate a significant difference between the absence and presence of ODQ in SNAP-treated muscles (p<0.05).
From these autoradiographs we were unable to detect any SNAP-induced changes in phosphorylation of the seven proteins identified as 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 whose phosphorylation levels were altered in the presence of SNAP are identified and labeled K through N (see Figure 32). Interestingly, these increases in phosphorylation induced by SNAP (1 mM) were unaffected by the inhibition of sGC with ODQ (Figure 33). Quantitative assessments of the degree of phosphorylation of proteins K through N are shown in Table 7. SNAP resulted in a significant increase in $^{32}$P incorporation into proteins K through M and also increased the phosphorylation of protein N, but because of the variability the change in phosphorylation of protein N was not statistically significant. ODQ had no effect on SNAP-induced phosphorylation of proteins K through N, which remained significantly phosphorylated in the presence of the sGC inhibitor (see Table 7). We have previously shown that a blockade of cGMP elevation with ODQ does not block SNAP's ability to induce relaxation (see Figure 5). The apparent cGMP-independent phosphorylation of proteins K through N described above would suggest that the cGMP-independent component of SNAP-induced myometrial relaxation in Figure 5 could involve activation of another kinase.

3.3.3.1.2 Effect of SNP and 8-bromo-cGMP on intact myometrial smooth muscle phosphorylation.

In the rat myometrium it has been shown that SNP (5 mM) can induce a significant 8-fold increase in cGMP levels and a significant 3-fold activation of PKG without relaxing the smooth muscle (see Figure 2 and Table 1). It is possible that the lack of relaxation in the presence of SNP resides in the ensuing patterns of PKG-
mediated phosphorylation. Figures 34 and 35 are representative autoradiographs of soluble protein phosphorylation induced by 5 mM SNP (5 min) in the absence and presence of 25 μM ODQ (20 min), respectively. Similar to the results with SNAP, we were unable to detect any SNP-induced changes in protein phosphorylation in any of the PKG substrates identified in the aorta. Moreover, it is difficult to determine whether or not these substrates even exist in the myometrium. In view of the fact that SNAP increased the phosphorylation of four new proteins in the rat myometrium, we also investigated whether these proteins were phosphorylated in the presence of SNP. As shown in Figure 34, SNP did not produce any change in phosphorylation of proteins K through N. The quantitative assessments of phosphorylation confirm that no measurable change in phosphorylation occurred in the presence of SNP (Table 8). We also investigated the phosphorylation patterns induced by 8-bromo-cGMP in the rat myometrium and again found no presence of the PKG substrates identified in the aorta or changes in $^{32}\text{P}$ incorporation compared to controls (see Figure 36). Table 8 also shows that 8-bromo-cGMP did not increase the phosphorylation of proteins K through N.

3.3.3.2 Studies in rat vas deferens.

3.3.3.2.1 Effect of SNP and 8-bromo-cGMP on intact vas deferens smooth muscle phosphorylation.

The rat vas deferens can also be classified as a "non-responsive" smooth muscle in that it does not relax in response to an elevation of cGMP and activation of PKG induced by SNP. In the research objectives of this thesis, it was proposed that the non-responsiveness of this smooth muscle may be due to either differing abilities of PKG to phosphorylate proteins or to the absence of crucial PKG substrates in this tissue. If one or more of the PKG substrates identified in the aorta is absent or not phosphorylated...
Figure 34. Representative autoradiograph of soluble protein phosphorylation in rat myometrial smooth muscle treated with 5 mM SNP for 5 min. Intact tissue strips were incubated with $^{32}$P for three hours at 37°C, treated with SNP and frozen. The soluble protein fraction from each tissue was separated using two dimensional gel electrophoresis. Second dimension SDS-PAGE gels of 11%T were silver stained, dried and exposed to x-ray film for 48 hours. Four proteins of interest to our study have been identified on the autoradiograph as K through N. No change in the phosphorylation of proteins K through N was detected compared to untreated control muscles.
Figure 35. Representative autoradiograph of soluble protein phosphorylation in rat myometrial smooth muscle pretreated with 25 μM ODQ for 20 min followed by 5 mM SNP for 5 min. Intact tissue strips were incubated with $^{32}$P for three hours at 37°C, treated with ODQ+SNP and frozen. The soluble protein fraction from each tissue was separated using two dimensional gel electrophoresis. Second dimension SDS-PAGE gels of 11%T were silver stained, dried and exposed to x-ray film for 48 hours. Four proteins of interest to our study have been identified on the autoradiograph as K through N. Since SNP did not produce any significant changes in protein phosphorylation, we were unable to show any effect of ODQ.
Figure 36. Representative autoradiograph of soluble protein phosphorylation in rat myometrial smooth muscle treated with 1 mM 8-bromo-cGMP for 10 min. Intact tissue strips were incubated with $^{32}$P for three hours at 37°C, treated with 8-bromo-cGMP and frozen. The soluble protein fraction from each tissue was separated using two dimensional gel electrophoresis. Second dimension SDS-PAGE gels of 11%T were silver stained, dried and exposed to x-ray film for 48 hours. Four proteins of interest to our study have been identified on the autoradiograph as K through N. 8-bromo-cGMP did not result in any changes in the phosphorylation of proteins K through N.
Table 8. Effect of SNP (in the absence and presence of ODQ) and of 8-Br-cGMP on $^{32}$P incorporation into intact rat myometria smooth muscle proteins.

<table>
<thead>
<tr>
<th>Protein</th>
<th>MW</th>
<th>$P_i$</th>
<th>Control</th>
<th>SNP (5 mM) 5 min</th>
<th>ODQ (25 µM) 20 min + SNP (5 mM) 5 min</th>
<th>8-Br-cGMP (1 mM) 10 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>K</td>
<td>43</td>
<td>7.8</td>
<td>0.8 ± 0.2</td>
<td>0.7 ± 0.1</td>
<td>1.4 ± 0.5</td>
<td>1.1 ± 0.3</td>
</tr>
<tr>
<td>L</td>
<td>43</td>
<td>7.6</td>
<td>0.5 ± 0.1</td>
<td>1.1 ± 0.2</td>
<td>1.4 ± 0.5</td>
<td>2.0 ± 0.5</td>
</tr>
<tr>
<td>M</td>
<td>43</td>
<td>7.5</td>
<td>0.5 ± 0.2</td>
<td>0.5 ± 0.1</td>
<td>0.3 ± 0.05</td>
<td>0.6 ± 0.2</td>
</tr>
<tr>
<td>N</td>
<td>28</td>
<td>5.8</td>
<td>3.3 ± 1.0</td>
<td>5.0 ± 1.2</td>
<td>3.4 ± 1.7</td>
<td>6.7 ± 2.5</td>
</tr>
</tbody>
</table>

Values are presented as background-corrected optical density, integrated over all pixels in the spot. The data shown are mean ± SEM from n=4 or 5 experiments with SNP and n=3 for 8-bromo-cGMP. Each experiment included a control, SNP, ODQ+SNP and 8-bromo-cGMP. Densitometry values are converted to percent of corresponding control for statistical analysis. This conversion eliminates some of the variations in densitometry that occur from one experiment to the next (see Methods). Molecular weight (MW) in kDa and isoelectric point ($P_i$) were calculated from standards run concurrently. Groups were compared by one-way ANOVA followed by Student-Newman-Keuls multiple comparison test. No statistically significant changes were found throughout the treatment groups.
during an elevation of cGMP and activation of PKG in the rat vas deferens, that protein(s) may be of particular importance in cGMP-mediated smooth muscle relaxation. Figure 37 shows a typical silver stain of the soluble protein pattern generated from an untreated intact segment of rat vas deferens. The MW and isoelectric point values are indicated on the figure. Autoradiographs showing intact vas deferens phosphorylation in a control, untreated tissue and from tissues treated with 3 μM PE for 30 seconds, 1 mM SNP for 2 min followed by 3 μM PE for 30 seconds and 1 mM 8-bromo-cGMP for 15 min followed by 3 μM PE for 30 seconds are shown in Figures 38 through 41, respectively. Surprisingly, we were unable to detect any SNP-induced changes in phosphorylation of the eight proteins identified as PKG-substrates in the aorta. Similarly, SNP did not increase the phosphorylation of the four new proteins identified in rat myometrium during SNAP-induced relaxation. Furthermore, we were unable to detect any change in $^{32}$P incorporation between control and SNP-treated tissues. The failure of SNP to induce increases or decreases in phosphorylation was confirmed by densitometric quantitation as shown in Table 9. In this table, only four of the previously identified PKG substrates are quantitated (A, B, C and H) as it is difficult to determine whether or not the proteins are actually present in the gels. Because 8-bromo-cGMP (1 mM) is an effective smooth muscle relaxant in vas deferens we next investigated the phosphorylation patterns produced by this cGMP analog. Once again, none of the eight PKG substrates identified in the aorta, or the four proteins identified in the myometrium, exhibited increased or decreased phosphorylation. As described in the Introduction, an interesting comparison in protein phosphorylation was expected between 8-bromo-cGMP-induced relaxation and SNP "non-responsiveness". In a comparison of these autoradiographs, no differences in PKG-mediated phosphorylation were observed.
Figure 37. Representative 11%T silver stained gel of a soluble protein sample from rat vas deferens following two dimensional gel electrophoresis. This particular protein sample was generated from an untreated intact vas deferens. In order to determine the molecular weights of the proteins resolved by two dimensional gel electrophoresis, the molecular weight standards must be shifted up by the depth of the well seen at the top left hand corner.
Figure 38. Representative autoradiograph of soluble protein phosphorylation in control, untreated rat vas deferens smooth muscle. Intact tissue strips were incubated with $^{32}$P for three hours at 37°C and frozen. The soluble protein fraction from each tissue was separated using two dimensional gel electrophoresis. Second dimension SDS-PAGE gels of 11%T were silver stained, dried and exposed to x-ray film for 48 hours. Ten proteins of interest to our study have been identified on the autoradiograph as A-C and H-N.
Figure 39. Representative autoradiograph of soluble protein phosphorylation in rat vas deferens smooth muscle treated with 3 μM PE for 30 seconds. Intact tissue strips were incubated with $^{32}$P for three hours at 37°C, treated with PE and frozen. The soluble protein fraction from each tissue was separated using two dimensional gel electrophoresis. Second dimension SDS-PAGE gels of 11%T were silver stained, dried and exposed to x-ray film for 48 hours. Ten proteins of interest to our study have been identified on the autoradiograph as A-C and H-N. PE had no effect on the phosphorylation of any of these proteins.
Figure 40. Representative autoradiograph of soluble protein phosphorylation in rat vas deferens smooth muscle treated with 1 mM SNP for 2 min followed by 3 μM PE for 30 seconds. Intact tissue strips were incubated with $^{32}$P for three hours at 37°C, treated with SNP+PE and frozen. The soluble protein fraction from each tissue was separated using two dimensional gel electrophoresis. Second dimension SDS-PAGE gels of 11%T were silver stained, dried and exposed to x-ray film for 48 hours. Ten proteins of interest to our study have been identified on the autoradiograph as A-C and H-N. SNP had no effect on the phosphorylation of any of these proteins.
Figure 41. Representative autoradiograph of soluble protein phosphorylation in rat vas deferens smooth muscle treated with 1 mM 8-bromo-cGMP for 15 min followed by 3 µM PE for 30 seconds. Intact tissue strips were incubated with $^{32}$P for three hours at 37°C, treated with 8-bromo-cGMP+PE and frozen. The soluble protein fraction from each tissue was separated using two dimensional gel electrophoresis. Second dimension SDS-PAGE gels of 11%T were silver stained, dried and exposed to x-ray film for 48 hours. Ten proteins of interest to our study have been identified on the autoradiograph as A-C and H-N. 8-bromo-cGMP resulted in a significant decrease in the phosphorylation of protein J, but no other changes in protein phosphorylation were detected.
Table 9. Effect of SNP and 8-Br-cGMP on $^{32}$P incorporation into intact rat vas deferens smooth muscle proteins.

<table>
<thead>
<tr>
<th>Protein</th>
<th>MW</th>
<th>$P_i$</th>
<th>Control</th>
<th>PE (3 μM) 30 seconds</th>
<th>SNP (1 mM) 2 min + PE (3 μM) 30 seconds</th>
<th>8-Br-cGMP (1 mM) 15 min + PE (3μM) 30 seconds</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>
<td>0.3 ± 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>
<td>0.2 ± 0.01</td>
</tr>
<tr>
<td>C</td>
<td>26</td>
<td>6.6</td>
<td>0.3 ± 0.1</td>
<td>0.3 ± 0.04</td>
<td>0.3 ± 0.05</td>
<td>0.2 ± 0.01</td>
</tr>
<tr>
<td>H</td>
<td>22</td>
<td>6.4</td>
<td>5.1 ± 1.8</td>
<td>7.3 ± 2.6</td>
<td>5.8 ± 2.0</td>
<td>6.1 ± 1.3</td>
</tr>
<tr>
<td>I</td>
<td>21</td>
<td>5.2</td>
<td>3.6 ± 2.2</td>
<td>4.6 ± 2.1</td>
<td>3.6 ± 1.9</td>
<td>3.3 ± 1.7</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>
<td>2.3 ± 0.9</td>
</tr>
<tr>
<td>K</td>
<td>43</td>
<td>7.8</td>
<td>0.2 ± 0.02</td>
<td>0.3 ± 0.04</td>
<td>0.4 ± 0.1</td>
<td>0.3 ± 0.1</td>
</tr>
<tr>
<td>L</td>
<td>43</td>
<td>7.6</td>
<td>0.3 ± 0.1</td>
<td>0.3 ± 0.03</td>
<td>0.2 ± 0.05</td>
<td>0.3 ± 0.04</td>
</tr>
<tr>
<td>M</td>
<td>43</td>
<td>7.5</td>
<td>0.3 ± 0.01</td>
<td>0.3 ± 0.04</td>
<td>0.3 ± 0.05</td>
<td>0.2 ± 0.01</td>
</tr>
<tr>
<td>N</td>
<td>28</td>
<td>5.8</td>
<td>4.1 ± 0.6</td>
<td>5.2 ± 1.7</td>
<td>3.7 ± 1.2</td>
<td>4.8 ± 2.1</td>
</tr>
</tbody>
</table>

Values are presented as background-corrected optical density, integrated over all pixels in the spot. The data shown are mean ± SEM from n=4 or 5 experiments, with each experiment including a control, PE, SNP+PE, and 8-Br-cGMP+PE. Densitometry values are converted to percent of corresponding control for statistical analysis. This conversion eliminates some of the variations in densitometry that occur from one experiment to the next (see Methods). Molecular weight (MW) in kDa and isoelectric point ($P_i$) were calculated from standards run concurrently. Groups were compared by one-way ANOVA followed by Student-Newman-Keuls multiple comparison test. A plus sign indicates a significant difference from PE-treated muscles ($p<0.05$).
However, quantitation of the changes in phosphorylation induced by 8-bromo-cGMP revealed that the calcium-dependent protein, J, was significantly dephosphorylated (Table 9). Since 8-bromo-cGMP is capable of relaxing the rat vas deferens and SNP is not, it is conceivable that a calcium-dependent phosphorylation is decreased in the presence of 8-bromo-cGMP.

3.3.4 Dissociation between dose and detection limits.

Early in our analysis of PKG-mediated phosphorylation in the rat aorta, we discovered that there was a dissociation between the concentration of SNP required to cause relaxation and the concentration of SNP required to show PKG-mediated phosphorylation of the proteins identified in Table 4. At 100 nM SNP we were unable to detect significant changes in protein phosphorylation although the smooth muscle was nearly 100% relaxed (see Figure 11A). 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-bromo-cGMP 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 non-specific kinase activation. It is possible that our 2-D technique is not sensitive enough to measure the levels of phosphorylation required to induce relaxation in smooth muscle. In an attempt to increase the sensitivity of detection, several 2-D experiments were performed with higher concentrations of \(^{32}\text{P}\)
(1 mCi/ml) in the incubation buffer to increase $^{32}$P incorporation and increase the detection limits of the technique. Endogenous phosphorylation increased significantly in these experiments. However, we were still unable to detect any changes in phosphorylation between control, contracted smooth muscles and low dose 100% relaxed smooth muscles.

Due to the dissociation described above and the high dose of drug required to demonstrate PKG-mediated phosphorylation, we have not investigated the patterns of protein phosphorylation in the proximal and distal rat colon. The distal rat colon is non-responsive to a 100 nM ANP-induced elevation of cGMP and activation of PKG. However, as the dose of ANP is increased, the distal colon does exhibit relaxation similar to that observed in the proximal segment. Since our 2-D protocol requires higher doses, this comparison is not likely to produce any significant differences in protein phosphorylation.
4.0 DISCUSSION

Numerous agents are capable of activating guanylyl cyclases and increasing tissue levels of cGMP, including nitrovasodilators such as SNP and SNAP, particulate guanylyl cyclase activators such as ANP, and endogenous NO generated within the endothelium. 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. However, there are two key points made in the last statement that require an explanation and further investigation. The first point is that the cGMP hypothesis may only apply in vascular smooth muscles. Experiments in the non-vascular smooth muscle of the rat uterus, described in the Results section of this thesis, demonstrate that cGMP is poorly correlated with decreases in smooth muscle tone and its role in mediating relaxation of this tissue remains unclear. Results in other non-vascular smooth muscles, including the rat vas deferens and distal colon, have supported similar conclusions regarding the importance of cGMP in mediating relaxation in these tissues (Suthamnatpong et al., 1993a,b; Patel et al., 1997). The second key point in the statement above is that cGMP may be involved in vascular smooth muscle relaxation, but not entirely responsible for it. In the present study, we demonstrate that vascular smooth muscle relaxation may be mediated in part by cGMP, but also by other undefined mechanisms that could be cGMP-independent.

In vascular smooth muscle, it is generally well accepted that the cGMP-mediated component of relaxation involves activation of a specific PKG. This protein kinase presumably elicits an effect 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. In the present study, we identify seven cGMP-dependent intact tissue substrates of PKG in the rat aorta. We demonstrate that these substrates are absent or not phosphorylated in non-responsive, non-vascular smooth muscles and also identify four separate proteins whose phosphorylation occurs independent of cGMP in the rat myometrium. The following is a detailed discussion of these and other results described in this thesis.

4.1 Effect of hormonal status and role of cGMP in regulation of uterine contractions.

In the early 1980's, the smooth muscle of the myometrium was characterized as non-responsive with respect to increases in tissue levels of cGMP and relaxation (Diamond, 1983). In contrast, several recent reports have described cGMP-mediated relaxations in the rat uterus (Potvin and Varma, 1990; Potvin et al., 1991; Yallampalli et al., 1993a,b; Izumi et al., 1993; Buhimschi et al., 1995). The main objective of the uterine studies proposed in this thesis was to repeat some of the recent experiments published in the literature describing a role for the NO/cGMP pathway in mediating uterine smooth muscle relaxation. Since these studies suggested that changes in hormonal status affected the responsiveness of the uterus to cGMP, we also investigated the control of uterine motility by cGMP in several different hormonally-influenced animal models. By carefully repeating these experiments, our goal was to resolve some of the apparent inconsistencies in the literature. In many of the present experiments, an effort was made to duplicate the experimental conditions and protocols used in the original studies.
In the studies of Potvin and Varma (1990) and Potvin et al. (1991), it was found that the particulate guanylyl cyclase activator, ANP, relaxed myometrial strips from ovariectomized, estrogen-treated rats and that this relaxant effect was abolished by progesterone treatment. It was suggested that the relaxant effect was due to cGMP elevation and that the absence of relaxation in the progesterone-treated muscles was due to the failure of ANP to elevate cGMP under conditions of progesterone dominance. In a more recent study, ANP receptor expression was shown to be downregulated during pregnancy, providing some support for the above finding that progesterone dominance affects ANP-induced cGMP accumulation (Vaillancourt et al., 1998). In the present study, we were unable to demonstrate any relaxant effect of ANP in myometrial strips from ovariectomized estrogen-treated rats or from any of the other hormonal treatment groups studied. Cyclic GMP levels were significantly elevated by ANP in our studies, but there was no significant difference in the degree of elevation seen in the various treatment groups irrespective of whether they were under the influence of estrogen or progesterone. At the present time, we have no explanation for the difference between our results and those of Potvin and Varma. Although obtained from different sources, the same type of ANP (rat, amino acids 1-28) was used in both studies. One difference in the experimental approach was that in the studies of Potvin and Varma, estrogen and progesterone were dissolved in sesame oil and injected intraperitoneally, whereas in the present study these hormones were dissolved in peanut oil and injected subcutaneously. In preliminary experiments, no differences were found in responses to drugs in animals treated by either method. Therefore, subcutaneous hormone injections were utilized in all subsequent experiments. With subcutaneous injections in peanut oil, the hormones will gradually enter the blood stream and be
delivered to the uterus continuously over a period of several days. This approach is the same as that used previously to demonstrate the effects of ovarian hormones on the response of rat myometrium to \( \alpha \)- and \( \beta \)-adrenoceptor agonists, and marked differences were found between hormonal treatment groups in that study (Diamond and Brody, 1966). In the present experiments, estrogen administration had the expected dramatic effects on the size and vascularity of the uterus in ovariectomized animals, indicating that they were under the influence of estrogen. It is interesting to note that Potvin and Varma, while concluding that ANP could relax uterine preparations by elevating cGMP, also reported that a soluble guanylyl cyclase activator, SNP, did not relax any of their myometrial preparations. This agrees with our results, which showed that concentrations of SNP as high as 5 mM, which elevated myometrial cGMP levels by as much as 9-fold, had no measurable effect on spontaneous contractions in any of our preparations.

In contrast to our results, and those of Potvin and Varma, Yallampalli et al. (1993a & b) and Izumi et al. (1993) reported that several cGMP-elevating agents, including SNP, were able to inhibit spontaneous contractions of myometrial strips from 18-day pregnant rats. Although no cGMP measurements were made in these studies, it was concluded that a NO/cGMP pathway is present in the uterus and is responsible for regulation of uterine contractions. As shown in Yallampalli et al. (1993a & b), spontaneous contractions of 18-day pregnant myometria were not inhibited by 5 mM SNP until 15-20 minutes of exposure, at which time the contractions ceased abruptly. Because cGMP levels are maximally elevated by SNP in smooth muscle preparations in less than one minute (see e.g. Janis and Diamond, 1979), this does not appear to be a cGMP-mediated process. It is possible that long exposure to high concentrations of
SNP might have a non-specific toxic effect due, for example, to release of cyanide from the breakdown of SNP. Surprisingly, L-arginine, a substrate for nitric oxide synthase and therefore a precursor of nitric oxide, was shown in the same study to cause an instantaneous and complete inhibition of spontaneous contractions (Yallampalli et al., 1993a & b). Because no cGMP levels were reported in these studies, it is difficult to decide what role the cyclic nucleotide plays in this process. As noted above, L-arginine, at the same concentration as that used by Yallampalli (1 mM), produced little or no cGMP elevation and failed to relax any of our myometrial preparations. Thus, our results fail to provide support for a role for cGMP in the control of uterine motility.

4.2 PKG activity in the rat myometrium.

In the present experiments, the possibility was considered that the lack of relaxation seen in SNP-treated tissues, in spite of a marked elevation of cGMP, was due to a failure of cGMP to activate PKG. As shown in the Results, SNP produced a significant elevation in PKG activity compared to a non-treated control. Therefore, a failure to activate PKG cannot be used to explain the inability of cGMP-elevating agents, such as SNP, to inhibit spontaneous contractions of rat myometrium. To our knowledge, these results are the first demonstration of activation of PKG, by cGMP-elevating agents, in the rat myometrium. Because elevation of cGMP and activation of PKG by SNP were not accompanied by inhibition of spontaneous contractions in these studies, the results provide strong evidence that cGMP does not play a direct role in the regulation of myometrial contractions.

In other non-responsive, non-vascular smooth muscles such as the rat vas deferens and distal colon, failure to activate PKG has also been ruled out as the possible reason for a lack of relaxation in the face of significant elevations of cGMP in
these tissues (Patel et al., 1997). If it is assumed that PKG activation is responsible for cGMP-mediated relaxation in vascular smooth muscle, then an explanation for the lack of relaxation in these non-responsive smooth muscles may reside in some aspect of PKG's downstream effects. This could include translocation and co-localization of PKG with its substrates as suggested by Cornwell et al. (1991) and Pryzwansky et al. (1995) or a complete absence of the necessary substrates for PKG. Preliminary studies failed to demonstrate PKG translocation in the rabbit aorta and rat vas deferens (Patel, 1996). Thus, a failure of translocation and co-localization to occur in a non-responsive smooth muscle compared to a responsive smooth muscle cannot be used to explain a lack of relaxation during cGMP elevation and PKG activation in rat vas deferens (Patel, 1996). An analysis of PKG-mediated phosphorylation in responsive and non-responsive smooth muscles forms a major component of this thesis, the results of which will be discussed in later sections.

In our opinion, the PKG assay used in our experiments is a more sensitive and reliable technique than those used previously. The key differences between our method and those used previously are incorporation of a short tissue processing time and use of a novel PKG substrate, BPDEtide. The specificity of our assay for PKG has been confirmed by experiments using MonoQ column chromatography and immunoblotting techniques (MacDonell and Diamond, 1997; Patel and Diamond, 1997). These studies provide evidence that the phosphotransferase activity measured in our PKG assay is, in fact, due to PKG. Despite the increased sensitivity, PKG assays in general are compromised by the dissociation of cGMP from the kinase during sample preparation and kinase activity measurements. The most common problem associated with cGMP dissociation is an underestimation of actual kinase activity. To limit the dissociation, our
assay is performed at 0°C, which increases the affinity of cGMP for PKG 100-fold (Fiscus et al., 1984; Francis et al., 1988). A novel, rapid homogenization procedure is also used that decreases the amount of time for sample preparation and maintains relatively low temperatures. Although we are confident that the assay conditions provide as accurate an estimate of PKG activity as possible, we cannot rule out the possibility that PKG activity changes during homogenization. In intact tissues, cGMP may be compartmentalized and unable to access the kinase, but during tissue disruption this cGMP pool may come into contact with PKG resulting in increased activity. Fiscus et al. (1984) used charcoal in a homogenization buffer to absorb free cGMP following homogenization. However, other studies revealed that charcoal may bind and inactivate PKG and promote dissociation of cGMP bound to PKG (Lincoln, 1983). When considering all of the possible sources of error in our PKG assay, it is still likely that we are underestimating the actual PKG activity.

4.3 Cyclic GMP-independent, NO-mediated relaxation of the rat myometrium.

In our myometrial experiments, we also investigated the effects of SNAP, which has been reported to be a better NO donor than SNP (Marks et al., 1995). Unlike SNP, SNAP produced significant inhibition of spontaneous contractions in myometrial strips from estrogen-primed, progesterone-treated rats. Initially, it appeared that SNAP might be capable of relaxing the uterine preparations simply because it produced much larger elevations in cGMP than did SNP, a result which would be consistent with a role for cGMP in the control of uterine motility. However, the ability of SNAP to inhibit spontaneous contractions was unaffected by a blockade of soluble guanylyl cyclase with ODQ. The cGMP elevation caused by 10 µM SNAP was completely blocked by ODQ, and the cGMP elevations caused by 30 and 100 µM SNAP were markedly
decreased, but there was no change in the inhibition of spontaneous contractions produced by any concentration of SNAP. The possibility was considered that the levels of cGMP generated by higher concentrations of SNAP in the presence of ODQ were still sufficiently elevated to contribute to the relaxant response of SNAP. However, the levels of cGMP obtained with SNAP in the presence of ODQ are similar to the levels generated by SNP, which had no relaxant effect. These results suggest that the relaxant effect of SNAP is exerted by a mechanism independent of cGMP elevation. The finding that acetylpenicillamine (a derivative of SNAP without the S-nitroso group) did not have a relaxant effect further suggests that the effect of SNAP may be due to the direct action of high concentrations of NO released from this compound. These results are in agreement with those of Kuenzli et al. (1996) who found that cGMP elevation was not required for the relaxation of drug-induced contractions of guinea pig myometrium caused by another NO donor, S-nitroso-L-cysteine (CysNO). An additional study in human myometrium also found that CysNO-induced relaxations were unaffected by a blockade of cGMP accumulation with methylene blue and LY83583. Possible roles of NO in control of uterine function have been thoroughly reviewed by Sladek et al. (1997).

It has recently been shown that NO may exert a direct relaxant effect in vascular smooth muscle preparations through the activation of a calcium-activated potassium channel (Bolotina et al., 1994; Plane et al., 1998). However, the administration of an inhibitor of this channel, charybdotoxin, to our smooth muscles had no effect on the relaxant response to SNAP. In subsequent studies of the human myometrium, charybdotoxin has been shown to be a potent inhibitor of cGMP-independent CysNO-induced relaxations (Bradley et al., 1998). It is possible that species differences in myometrial responses to NO are responsible for these findings (Bradley et al., 1998).
As noted in the results, a change in pH was ruled out as a possible mechanism of SNAP-induced relaxation. Thus, at the present time, the underlying mechanism of the relaxation produced by SNAP is still unclear. The fact that SNAP did not cause relaxation in preliminary experiments in pregnant myometrial tissues warrants further investigation. In any case, it is unlikely that a NO/cGMP relaxation pathway is responsible for maintaining a quiescent uterus during pregnancy because none of the cGMP-elevating agents used in this study, including SNAP, caused any inhibition of spontaneous contractions during late-stage pregnancy.

The above experiments confirm the previous suggestion (Diamond, 1983) that the rat myometrium constitutes another cGMP non-responsive smooth muscle. These results provide strong support for the conclusion that cGMP does not play an important role in the control of contractile responses in the rat myometrium. It is possible that high concentrations of NO itself may directly influence contractions in this tissue. However, the physiological significance of this remains unclear.

4.4 Smooth muscle relaxation in the rat aorta.

As discussed above, the criteria necessary to conclude that cGMP plays a role in vascular smooth muscle relaxation have been satisfied. However, more recent advances in the development of more selective inhibitors and activators of specific mediators in this pathway have lead to numerous publications describing results that do not exactly fit with the cGMP hypothesis. A common conclusion in these reports appears to be the presence of cGMP-independent components in NO-mediated smooth muscle relaxation (Weisbrod et al., 1998; Plane et al., 1996, 1998). One of the most useful agents for studying the NO/cGMP system is the selective sGC inhibitor, ODQ. As mentioned in the Introduction, some previous studies have reported poor correlations
between cGMP levels and percent relaxation (Axelsson et al., 1979; Gruetter et al., 1981; Keith et al., 1982, 1983). However, it was only in the last five years that cGMP-independent components of smooth muscle relaxation have become accepted as part of NO-mediated responses. Before beginning our analysis of PKG-mediated phosphorylation in the rat aorta, we performed a number of experiments to investigate the role of cGMP in NO-mediated relaxation of this tissue. Although results of similar experiments can be found in the literature (Gruetter et al., 1981; Keith et al., 1982; Lincoln, 1983), we were interested in confirming their conclusions about the effect of NO and the role of cGMP in the presence of ODQ.

4.4.1 Role of cGMP in the contractile responses of rat aorta preparations.

SNP, SNAP, ANP and 8-bromo-cGMP all 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 doses of SNP and SNAP despite a complete blockade of cGMP elevation, indicating that at least a portion of the relaxant responses of 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 percent relaxation in the rat aorta. For both agents, the dissociation becomes more apparent as the concentration of drug is increased. Because very low levels of cGMP have been shown to accompany ANP-mediated
relaxation in the rat aorta (Figure 9), we considered the possibility that despite ODQ's complete reversal of cGMP elevation in the presence of SNP and SNAP, slight increases in the tissue levels of cGMP may be sufficient to mediate relaxation. In the rat myometrium, we were able to eliminate the possibility that SNAP-induced relaxations in the presence of ODQ might still be mediated by cGMP because SNP-induced increases in cGMP occurred at similar levels to those obtained with SNAP in the presence of ODQ, but SNP did not induce relaxation. Because the rat aorta appears to be responsive to any elevation in cGMP, it is more difficult to conclude that the relaxations observed with SNP and SNAP in the presence of ODQ are completely cGMP-independent. In a detailed review of the literature, Nakatsu and Diamond (1989) attempted to resolve this issue. Their conclusions suggest that smooth muscle relaxation cannot be simply explained by the elevation of cGMP and that more complex models are likely involved. One possible model could be the existence of multiple pools of cGMP in smooth muscle, with only one being involved in the mediation of relaxation. However, investigations addressing this particular question have not found evidence for the existence of more than one cGMP pool in the rabbit aorta (Nakatsu and Diamond, 1989). Goldberg et al. (1983) suggested that cGMP turnover may be more important than cGMP concentration, thus explaining the lack of correlation between relaxation and tissue levels of cGMP, but this has yet to be proven. The most probable model to explain poor correlations between relaxation and cGMP elevation is one in which multiple mechanisms mediate smooth muscle relaxation, including cGMP-dependent and possibly cGMP-independent mechanisms (Nakatsu and Diamond, 1989). Taking into account this analysis, we have concluded that the partial relaxant responses induced by SNP and SNAP in our
experiments in the presence of a complete blockade of cGMP elevation by ODQ occur via mechanisms independent of cGMP.

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 (Marks et al., 1995), this may be the reason for its larger NO-mediated cGMP-independent component. It is also possible that differences in the mechanisms of NO release from SNP and SNAP account for the contrasting magnitudes of the cGMP-independent components in their relaxant responses. S-nitrosothiol-mediated transnitrosation reactions that are characteristic of SNAP certainly distinguish this NO donor from SNP (Feelisch, 1998).

Although it is difficult to determine what concentration of NO was actually affecting the smooth muscle strips in our experiments, the 1 µM doses of SNP and SNAP used are likely close to physiological, as NO concentrations above 0.1 µM have been demonstrated in situ following acetylcholine stimulation of the vascular endothelium in rabbit carotid artery (Malinski et al., 1993; Cohen et al., 1997). Similar to our results, most, but not all, of the relaxation induced by acetylcholine-stimulated endothelium-derived NO production is blocked by ODQ in rabbit carotid artery (Plane et al., 1998). This indicates that cGMP-independent components may exist in both endogenous NO-mediated smooth muscle relaxation as well as in nitrovasodilator-induced relaxation. It is important to note that in other vascular smooth muscles, such as the rabbit aorta, LY83583 completely blocked cGMP elevation by acetylcholine and completely blocked the relaxation (Diamond, 1987).
Because the focus of our studies was to investigate the larger cGMP-dependent component of smooth muscle relaxation, we have not attempted to elucidate the mechanisms of the NO-mediated cGMP-independent relaxations observed in our rat aorta experiments described above. In our myometrial studies, we investigated calcium-activated potassium channel activation as one possible mechanism of cGMP-independent SNAP-induced relaxation, but found no evidence that channel activation was responsible for the relaxant response. However, evidence in the literature does not necessarily support these findings in the rat myometrium. Several studies have shown that direct stimulation of calcium-activated potassium channels in several different smooth muscles is an important component of NO-mediated cGMP-independent relaxation. The resulting efflux of potassium ions following channel stimulation causes hyperpolarization of smooth muscle cell membranes which indirectly affects the activity of voltage-gated calcium channels leading to a decrease in intracellular calcium and smooth muscle relaxation. Although this mechanism was first demonstrated in patch clamp experiments of vascular smooth muscle cells (Bolotina et al., 1994), it has now been demonstrated in intact human myometrium (Anwer et al., 1993; Bradley et al., 1998), rat pulmonary artery (Zhao et al., 1997), rabbit carotid artery (Plane et al., 1998) and in vascular and tracheal smooth muscle of the guinea pig (Bialecki and Stinson-Fisher, 1995). In the studies above, the selective inhibitor of calcium-activated potassium channels, charybdotoxin, inhibits a portion of NO-mediated relaxation, while the remaining segment of the relaxation is assumed to be mediated via cGMP-dependent mechanisms. In the original study of Bolotina et al. (1994), it was proposed that NO-mediated nitrosylation interactions with sulfhydryl groups on calcium-activated potassium channels were responsible for increased channel activity. This was later
confirmed by Stamler (1994) who showed that a thiol-containing domain in calcium-activated potassium channels interacts with NO to form disulfide bonds that modulate channel opening.

Despite the presence of an apparent cGMP-independent component in SNP- and SNAP-induced relaxation of rat aorta, cGMP is still responsible for a large part of the relaxant responses in this tissue. The complete relaxations induced by ANP and 8-bromo-cGMP, which occur without the generation of NO, certainly confirm this point.

4.4.2 PKG activity ratios and total levels of PKG in rat aorta.

The activation of PKG by cGMP in rat aorta has been assessed previously. Lincoln and Fisher-Simpson (1983) and Fiscus et al. (1984) both demonstrated an increase in PKG activity during nitrovasodilator-induced increases in cGMP. The PKG assay utilized in these studies incorporated a non-specific PKG substrate, histone H2B. As described in the Introduction, this substrate can be phosphorylated independent of cGMP leading to inaccurate kinase activity assessments. To confirm that PKG activity is increased in the rat aorta following SNP-induced elevations in cGMP, we measured PKG activity ratios with an updated PKG assay described above. In these experiments, we demonstrated significant elevations in PKG activity compared to controls and in our opinion these results are more sensitive and reliable than those reported earlier. A comparison of the activity ratios reported by Lincoln and Fisher-Simpson (1983) and Fiscus et al. (1984) indicates that our activity ratios are considerably lower. However, the activity ratios reported by Jiang et al. (1992) using BPDEtide in pig coronary arteries are similar. Because increases in PKG activity are reasonably well correlated with the elevation of cGMP in rabbit aorta (Patel and Diamond, 1997), and we have demonstrated increased PKG activity during cGMP elevation in rat aorta, a natural
progression would be to investigate cGMP-activated PKG-mediated phosphorylation to further elucidate the downstream mechanisms that lead to smooth muscle relaxation.

4.5 Two-dimensional gel electrophoresis.

4.5.1 Validation and optimization of technique.

Two dimensional gel electrophoresis has gained widespread use in many aspects of scientific research. The demand for greater resolution and more sensitive protein detection by this technique have resulted in a steadily changing methodology. A considerable amount of time was spent improving the sensitivity and reproducibility of the two-dimensional gel electrophoresis methodology during these studies. This was necessary if the technique was to be useful for the kind of experiments proposed in this thesis. As a result, the two-dimensional gel electrophoresis method described in the Methods of this thesis has been published (Hennan and Diamond, in press). The method incorporates several novel changes developed in our own laboratory as well as numerous modifications compiled from several published sources. The outcome is a high resolution, reproducible two dimensional gel electrophoresis technique useful for measuring protein phosphorylation in intact tissues.

The majority of two dimensional gel electrophoresis literature is made up of studies utilizing in vitro, broken cell preparations. As noted in the Introduction, these studies provide useful information, but it is often difficult to extrapolate the results to an intact tissue setting. The two-dimensional gel electrophoresis technique described in the Methods section of this thesis details the necessary steps to investigate drug-induced protein phosphorylation in intact smooth muscle preparations. Thus, the importance of these phosphorylations may be related back to physiological events taking place in the whole tissue. In some cases, proteins that are phosphorylated by
kinases under in vitro conditions may be unavailable or fail to be phosphorylated by these kinases in intact muscle preparations (Bergh et al., 1995). In our experiments, we utilize a small volume homogenization procedure that allows for the maintenance of low temperatures and relatively quick sample preparation times. This helps to maintain protein integrity and eliminates protein precipitation techniques required to reduce large homogenization volumes.

The methodology for the first dimension isoelectric focusing tube gels is based on the original work of O'Farrell (1975). However, protocol modifications and availability of improved electrophoresis equipment have significantly altered the application of this technique today. The methods of Hochstrasser et al., 1988 as well as Dunn and Corbett, 1996 are well described, but in our hands a combination of their methodologies, with some of our own modifications produces greater resolution. Carbamylyte calibration IEF standards were an effective tool to monitor and optimize first dimension separation conditions and facilitated the incorporation of a 10°C running temperature and a 17,500 volt-hour running time. These standards have been utilized in the past for IEF (Anderson and Hickman, 1979); however, published methods utilizing them do not appear in more current literature. The addition of a tube gel freezing step was crucial in completing this two dimensional gel electrophoresis method. This approach helps to prevent urea from interfering with the running of the second dimension, and decreases the need for a long equilibration period in transfer buffer prior to the movement of the proteins into the SDS-PAGE gel (Dunn and Burghes, 1983). The incorporation of a 0.2% SDS concentration in the second dimension running buffer further supported a reduction in equilibration time by increasing the likelihood that proteins would be saturated in SDS as they moved out of the tube gel and into the slab gel. In our opinion,
these modifications result in more reproducible protein transfers and an increase in protein resolution throughout the molecular weight range of our gels. Because low resolution in higher molecular weight proteins is a common pitfall associated with the use of two dimensional gel electrophoresis, this modification provides a definite advantage over the older methods. In addition to saturating proteins with SDS in the tube gel prior to transfer into the second dimension, we also incorporated a low current electrophoresis to begin the transfer (4 mA for 12 hours). This allows for a slow transfer of proteins from the tube gel and gives greater resolution when the electrophoresis is complete. Other more simplistic efforts to enhance reproducibility included casting and running eight SDS-PAGE gels simultaneously, using a faster more sensitive silver stain, gel drying between cellophane to eliminate uneven shrinkage and multiple concurrent autoradiograph exposure to avoid variability between control and treated groups. The reproducibility of our protein patterns and the use of highly sensitive detection methods have allowed us to statistically compare phosphorylation levels, as integrated densities of spots, between treated and control smooth muscles. Although such quantitative comparisons are rarely reported in the literature, they can strongly support any conclusions that are made based on the pharmacological manipulation of a tissue. Phosphoimaging can also be used as a detection technique for radiolabeled phosphoproteins in two dimensional gels. This technique utilizes phosphor storing screens and is more sensitive than x-ray film. However, due to the large number of gels (8-16 per week), cost of phosphor screens and availability of the instrumentation to analyze the gels, it was not feasible to use this method of detection in our studies.

In a preliminary study, we investigated the level of phosphate incorporation into our intact smooth muscle preparations, over a five hour period, to evaluate whether or
not endogenous phosphate pools had become saturated with radiolabeled phosphate. If phosphate pools are not saturated with radiolabel, the reproducibility of the phosphorylation patterns can be compromised. Detected changes in phosphorylation may reflect differences in the incorporation of $^{32}$P into intracellular ATP pools rather than kinase-mediated changes in phosphorylation. In the literature, studies using intact tissue labeling have used incubation periods of 1 to 2 hours with no indication of the degree of radiolabel incorporation (Garrison and Borland, 1978; Rapoport et al., 1982). In the present study, we found that $^{32}$P incorporation reached saturation at 3 hours and maintained this level at a 5 hour time point. All subsequent experiments were carried out with a three hour incubation. Total chemical phosphate content was also measured in the extracellular buffer throughout the incubation period to ensure that the specific radioactivity of the phosphate precursor pool was not changing with time. This measurement does not indicate the exact specific radioactivity of the intracellular phosphate pool, but the extracellular phosphate content provides a reasonable surrogate. When we were confident that our technique maintained viable tissues during incubation and produced reproducible protein patterns, we began comparing the phosphorylation patterns of control and treated intact smooth muscles in an attempt to identify substrates of PKG.

4.5.2 PKG-mediated phosphorylation in rat aorta.

4.5.2.1 Soluble fraction.

The results of our two-dimensional gel electrophoresis studies provide the first 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 assess statistical significance of differences between different treatment groups. We identified ten proteins whose phosphorylation levels are significantly altered in the presence of SNP (A through J, Table 4). Through analysis of different treatments, two of these phosphorylation events were discovered to be calcium-dependent (I and J) and seven others were confirmed as cGMP-dependent using ODQ and concluded to be PKG substrates (A through G). This sGC inhibitor has been used extensively to decrease cGMP levels in smooth muscle. However, its potential ability to inhibit PKG-mediated phosphorylation has never been studied. As described in the results, the phosphorylation of protein H does not necessarily decrease in the presence of PKG-mediated phosphorylation, but instead results in a shift in the isoelectric point of the protein giving rise to protein B. The fact that protein H is endogenously phosphorylated in our control gels suggests that this protein is phosphorylated by PKG at a separate site which leads to the isoelectric point shift observed in our gels. Similar shifts in isoelectric point have been described during smooth muscle myosin light chain phosphorylation (Silver and Stull, 1982). In this study, the isoelectric point of myosin light chain changes from approximately 5.1 to 5.0 during a myosin light chain kinase-mediated addition of phosphate (Silver and Stull, 1982). 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-bromo-cGMP). Each of these PKG-activating agents resulted in patterns of PKG-mediated phosphorylation similar to those found with SNP.
4.5.2.2 Possible identities of PKG substrates.

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, I and J, have molecular weights of approximately 21 kDa and isoelectric points of approximately 5.2 and 5.3, respectively. Numerous reports investigating \textit{in vivo} smooth muscle phosphorylation have identified proteins with similar molecular weights and isoelectric points that exhibit a decrease in phosphorylation during relaxation (Johnson and Lincoln, 1985; Paglin et al., 1988; McDaniel et al., 1992; Lincoln and Cornwell, 1993; Ishibashi et al., 1995; Lee et al., 1997; Surks et al., 1999). In the experiments of Paglin et al. (1988) and Ishibashi et al. (1995), these proteins were identified as myosin light chains by their co-migration with appropriate standard proteins extracted from rabbit aorta and bovine trachea, respectively. In other experiments, these two proteins were identified as myosin light chains based on their MW and \textit{pl} as described by Silver and Stull (1982). Given the results of the studies above, we have concluded that proteins H and I in our studies are most likely myosin light chains.

Although the exact mechanisms of smooth muscle relaxation are unknown, there is good evidence to suggest that myosin light chain dephosphorylation is involved in the final relaxant response. Just how this dephosphorylation event might occur, if it were directly mediated by PKG, remains to be determined as PKG would lead to increased phosphorylation rather than a decrease. Because myosin light chain phosphorylation is increased during PE-induced contraction, presumably in a calcium-dependent manner, and decreased during relaxation and calcium re-uptake, it appears as though this phosphorylation event is not directly mediated by PKG, but controlled by intracellular
calcium levels in our experiments. Investigations into myosin light chain dephosphorylation during smooth muscle relaxation have implicated myosin phosphatase as a possible mediator of the decrease in phosphorylation and several reports have shown PKG-mediated increases in myosin phosphatase activities (Paglin et al., 1988; Lincoln and Cornwell, 1993; Lee et al., 1997; Wu et al., 1999). Intermediate filament phosphorylation has also been suggested as a mechanism for PKG-mediated relaxation as this may block cross bridge attachments normally created by myosin light chain phosphorylation and lead to a decrease in tone (Ishibashi et al., 1995). From our results, it is difficult to conclude which mechanisms are responsible for myosin light chain dephosphorylation. However, given that the level of myosin light chain phosphorylation increases during contraction, it is more likely that calcium sequestering mechanisms activated by PKG to decrease intracellular calcium are responsible for the decrease in phosphorylation of these proteins following cGMP elevation.

The proteins identified as A and B in our experiments with MW's of 22 and 21 kDa and pl's of 5.8 and 6.3, respectively, may have been identified as PKG substrates previously (Rapoport et al., 1982; Bergh et al., 1995). Bergh et al. (1995) identified two proteins with molecular weights of 20 kDa that showed similar mobilities in two dimensions as proteins A and B above. In subsequent experiments, these proteins were identified as heat shock-related proteins (HSP20). Rapoport et al. (1982) also identified two proteins with MW's of 24 kDa and pl's of 6.5 and 6.2 that showed increased phosphorylation following SNP-induced relaxation. Although the molecular weights and pl's are slightly different in this study, an analysis of the autoradiographs in the paper by Rapoport et al. (1982) indicates that these proteins may be the same as those described above in our experiments and by Bergh et al. (1995). Considering
these results, it is possible that proteins A and B in our studies are heat shock-related proteins. If PKG does mediate the phosphorylation of HSP's during intact smooth muscle relaxation, it is unknown what function these proteins may have. It has been suggested that they 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 (Beall et al., 1997). Other heat shock proteins such as αβ-crystallin are co-localized in the Z-band of cardiac muscle whose counterpart in smooth muscle is dense bodies (Bennardini et al., 1992). Phosphorylation of heat shock proteins in dense bodies of smooth muscle may lead to a blockade of cross-bridge attachments and, as a consequence, relaxation (Hai and Murphy, 1988).

The 26 kDa protein, C, identified in our experiments with a pI of 6.6 may be a non-dissociated form of phospholamban (Jones et al., 1995). In cardiac cell extracts, phospholamban was shown to exhibit several distinct mobilities, including MW's of 25 kDa and 5 to 6 kDa. When phosphorylated, the pI of phospholamban was approximately 6.7 (Jones et al., 1995). Although cardiac tissues differ from smooth muscles in many ways, it is possible that protein C in the rat aorta is, in fact, phospholamban. However, the similarities between this study and ours are worth noting.

The remaining proteins identified as PKG substrates in our experiments have not been identified. Although a few of them may have been previously detected as showing increased phosphorylation during an elevation in cGMP in the intact aorta studies of Rapoport et al. (1982). However, there was no attempt to quantify or statistically confirm the changes in phosphorylation in that report. Thus, our statistical analysis of quantitated protein phosphorylation in the absence and presence of ODQ, and with
other PKG-activating agents, provides a more accurate evaluation of the intact smooth muscle substrates of PKG.

In the Introduction, several soluble and particulate targets of PKG were discussed as possible mediators of smooth muscle relaxation. In our intact tissue assessments of PKG-mediated phosphorylation, we were unable to detect changes in $^{32}$P incorporation into a number of these suggested substrates. Lincoln and colleagues reported that PKG phosphorylates, and is anchored to, the soluble protein vimentin in vascular smooth muscle cells (Pryzwansky et al., 1995). 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 the intact tissue setting. The remaining proposed PKG targets undetected in our studies are discussed below as they are found in the particulate fraction of smooth muscles.

4.5.2.3 PKG-mediated phosphorylation in the particulate fraction of rat aorta.

After testing several membrane extraction methods, we found that the detergent mixture described by Baltensberger et al. (1990) produced effective solubilization of our particulate fractions with the least amount of interference during the running of the first and second dimension gels. Despite achieving good solubilization of our particulate fractions, we were unable to detect any significant changes in phosphorylation of membrane-bound proteins, including those described in the Introduction as substrates of PKG. Casnellie et al. (1980) demonstrated particulate fraction PKG-mediated phosphorylation of four proteins in vascular smooth muscle cell fractions treated with cGMP (250 kDa, 130 kDa, 85 kDa, 75 kDa). In rat aorta smooth muscle cells, Sarcevic
et al. (1989) showed similar in vitro particulate fraction PKG-mediated phosphorylation of three proteins (225 kDa, 132 kDa, 11 kDa) in the presence of ANP. A few of these particulate fraction proteins were subsequently identified as a splice variant of the IP₃ receptor, a phosphatidylinositol kinase closely associated with calcium ATPase activation, and phospholamban (Furukawa and Nakamura, 1987; Vrolix et al., 1988; Yoshida et al., 1991; Sarcevic et al., 1989; Huggins et al., 1989). However, we did not detect phosphorylation of these proteins in our studies, which suggests that they are not active substrates of PKG in intact smooth muscle.

The IP₃ receptor was subsequently shown to be phosphorylated in intact vascular smooth muscle cells (Komalavilas and Lincoln, 1994). Due to the high molecular weight 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. Cornwell et al. (1991) demonstrated phosphorylation of the 11 kDa protein, phospholamban, in intact vascular smooth muscle cells stimulated with SNP and ANP, but in our intact tissue measurements of PKG-mediated phosphorylation, we were unable to detect the phosphorylation of this protein. Since phospholamban phosphorylation has also been shown to exist in in vitro preparations (Sarcevic et al., 1989; Huggins et al., 1989), it is possible that vascular smooth muscle cells in culture more closely resemble a broken cell preparation than a true intact tissue setting. The final membrane-bound PKG target described in the Introduction that was not detected in our studies is the calcium-activated potassium channel. Because the in vivo studies describing PKG-mediated phosphorylation of the 125 kDa α-subunit of this channel were performed in genetically altered Xenopus oocytes (Alioua et al., 1998), it is not surprising that we were unable to detect this.
phosphorylation event in segments of rat aorta. Because we have not clearly identified all of the proteins identified as PKG substrates in our experiments, it is possible that some of the remaining PKG targets that were discussed as possible mediators of smooth muscle relaxation in the Introduction could exist in the proteins we have resolved. 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.

4.5.3 PKG-mediated phosphorylation in the rat myometrium.

As described above, the non-vascular smooth muscle of the rat myometrium does 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 or not downstream PKG-mediated phosphorylation (or lack of phosphorylation) can be used to explain the lack of relaxation in this type of smooth muscle. In all of our analyses of PKG-mediated phosphorylation 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 entirely absent from the myometrium, as no traces of them could be detected from our gels. In addition, we were unable to detect any changes in phosphorylation of any other proteins. Endogenous protein phosphorylation was slightly lower in certain areas of the autoradiographs from myometrial preparations. Therefore, we performed preliminary experiments in which 150 µg of protein from a myometrial extract were separated using two-dimensional gel electrophoresis. Normally, only 50 µg of protein was 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 prior to the addition of the cGMP-elevating agents above, we were also unable to show any 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 (see Tables 2 and 5). If one or more of the proteins we identified in the rat aorta is responsible for relaxation, then the lack of relaxation in the myometrium could be attributed to the absence of these protein(s).

In our analysis of PKG-mediated 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 levels of cGMP are not completely reversed by ODQ in the presence of SNAP, they are significantly reduced, which should correspond to some level of a reduction in PKG-mediated phosphorylation. Because the levels of phosphorylation of these four proteins are similar or even elevated in the presence of ODQ, it seems likely that these phosphorylation events are occurring independent of cGMP and PKG. It has already been shown that SNAP-induced relaxation in the myometrium is unaffected by significant reductions in cGMP by ODQ. 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. If such a pathway does exist where NO mediates the activity of another kinase independent of cGMP, it has not been previously identified or even suggested. NO has been shown to increase the activity of p38 mitogen-activated protein kinase (Browning et al., 2000). However, cGMP and PKG were shown to be required for this effect (Browning et al., 2000). It is possible that the low level of cGMP still present in the myometrium during sGC inhibition with ODQ could allow enough PKG activation to permit activation of this kinase. However, further investigation is required to implicate such a pathway in smooth muscle.

4.5.4 PKG-mediated phosphorylation in the rat vas deferens.

Rat vas deferens has been demonstrated as a non-responsive smooth muscle with respect to increases in the tissue levels of cGMP and PKG activity induced by SNP (Patel et al., 1997). Interestingly, the vas deferens does relax in the presence of 8-bromo-cGMP. Whether or not the relaxant response induced by 8-bromo-cGMP involves PKG was one of the objectives of our analysis of PKG-mediated phosphorylation in the rat vas deferens. As shown in the Results, no changes in PKG-mediated phosphorylation were observed in any of our autoradiographs of rat vas deferens smooth muscle. None of the seven proteins previously identified in the rat aorta as PKG substrates were identified during 8-bromo-cGMP-induced relaxation or during cGMP elevations induced by SNP. One calcium-dependent protein, possibly myosin light chain, did show a significant decrease in the presence of 8-bromo-cGMP, which would correlate well 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. The levels of phosphorylation for those proteins that appear to exist have been quantitated in Table 9. 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, further investigations are required to substantiate such a hypothesis.

Similar to our results in the rat myometrium, the overall levels of phosphorylation seem to be lower in the rat vas deferens compared to 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 were more clearly resolved. A comparison of the total levels of PKG in rat vas deferens, rat aorta and rat myometrium reveals that the total levels of PKG in the rat vas deferens are approximately half those found in these other smooth muscles (Patel et al., 1997). The degree of activation of PKG caused by 5 mM SNP in the rat vas deferens was similar to that observed in the rat aorta and myometrium (Patel et al., 1997). It is unlikely that the decrease in total kinase level described above is responsible for our inability to measure PKG-mediated phosphorylation in the rat vas deferens. It is possible that some of the PKG substrates found in the rat aorta are absent from the rat vas deferens resulting in a lack of relaxation, in spite of an activation of PKG. However, from our analysis of the patterns of protein phosphorylation in the rat
vas deferens, it appears that the lack of relaxation in this smooth muscle may be a result of a failure of PKG to phosphorylate its substrates.

4.5.5 Dissociation between dose and detection limits.

As mentioned in the 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 through 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 well 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 an accurate evaluation 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 relaxation, the short duration of incubation with these agents used in our studies, and the confirmation of the PKG substrates as cGMP-dependent using ODQ, eliminates the likelihood of such events contributing to the patterns of phosphorylation observed in our studies. Further investigation into the four proteins phosphorylated in the rat myometrium in the presence of SNAP will be required to determine whether or not these proteins are involved in the relaxant response to SNAP.
5.0 SUMMARY AND CONCLUSIONS

The principal observations of this study were as follows:

1.) In rat myometrium, changes in hormonal status produced slight differences in the elevation of cGMP induced by agents such as SNP, ANP and L-arginine. However, none of the agents used (SNP, ANP, L-arginine and 8-bromo-cGMP) were able to inhibit spontaneous myometrial contractions under any of the hormonal conditions studied. The above experiments confirm the previous suggestion (Diamond, 1983) that the rat myometrium can be classified as another "non-responsive" smooth muscle. These results provide strong support for the conclusion that cGMP does not play an important role in the control of relaxation in the rat myometrium.

2.) SNP was shown to significantly activate PKG in our myometrial preparations. Therefore, a lack of PKG activation cannot be used to explain the failure of cGMP-elevating agents such as SNP to cause relaxation of the rat myometrium.

3.) Another NO-donor, SNAP, did produce significant inhibition of spontaneous contractions in myometrial preparations. However, the ability of SNAP to inhibit spontaneous contractions was unaffected by a blockade of sGC with ODQ. These results suggest that the relaxant effect of SNAP is exerted by a mechanism independent of cGMP elevation. The possibility was considered that direct activation of calcium-activated potassium channels could be responsible for the relaxant effect of SNAP. However, the administration of an inhibitor of this channel, charybdotoxin, to our smooth muscles had no effect on the relaxant response to SNAP. Therefore, a direct effect of SNAP
on calcium-activated potassium channels cannot be used to explain the relaxant response induced by this agent. The underlying mechanism of SNAP-induced relaxation in the rat myometrium remains unclear.

4.) SNP, SNAP, ANP and 8-bromo-cGMP all produced complete relaxation of PE-induced contractions in the rat aorta. 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. It is important to note that partial relaxation still occurred at the higher doses of SNP and SNAP despite a complete blockade of cGMP elevation, indicating that at least a portion of the relaxant responses of these agents may be mediated via mechanisms independent of cGMP.

5.) In intact rat aorta, nine proteins showed altered levels of phosphorylation during SNP-, SNAP-, ANP- and 8-bromo-cGMP-induced smooth muscle relaxation. Seven of these proteins were identified as PKG substrates while the remaining two proteins appear to be calcium-dependent phosphorylation events. Two of the PKG substrates have been previously identified and may be heat shock-related proteins. The two proteins phosphorylated in a calcium-dependent manner are most likely myosin light chains. Because the PKG substrates identified in our experiments reflect phosphorylation during smooth muscle relaxation in the intact tissue setting, they are more likely to
be involved in the mechanisms of PKG-mediated relaxation than those previously described in in vitro and cell culture experiments.

6.) None of the PKG substrates identified in the rat aorta could be identified in the myometrium following administration of high concentrations of SNP. In addition, we were unable to detect changes in phosphorylation of any other proteins. Thus, the failure of this smooth muscle to relax in the face of cGMP elevation and PKG activation may be due to a lack of PKG substrate phosphorylation. Whether or not the PKG substrates present in the aorta are even present in the uterus requires further investigation.

7.) Four proteins were found to be phosphorylated in an apparently cGMP-independent manner during SNAP-induced rat myometrial relaxation. This is consistent with our observation that SNAP-induced inhibition of spontaneous contractions in the rat myometrium also occur independently of cGMP elevation. Thus, the mechanism of SNAP-induced, cGMP-independent relaxation may include activation of another unknown kinase.

8.) None of the seven PKG substrates identified in the rat aorta could be identified in the rat vas deferens following administration of high concentrations of SNP. In addition, we were unable to detect changes in phosphorylation of any other proteins. Thus, as was the case with the myometrium, the failure of this smooth muscle to relax during elevations in cGMP and increases in PKG activity may be due to a lack of PKG substrate phosphorylation. Whether or not the PKG substrates present in the aorta are actually present in the vas deferens remains unknown.
The experiments described above provide valuable information concerning the roles of cGMP, PKG and protein phosphorylation in the control of smooth muscle tension. A unifying hypothesis that can be drawn from these results is that the PKG-mediated phosphorylation identified in our studies of the rat aorta may play an important role in smooth muscle relaxation as it does not appear to exist in other smooth muscles that fail to relax in the face of elevated cGMP and increased PKG activity. However, further investigations are required to substantiate such a conclusion.

5.1 Future Directions.

The two dimensional gel electrophoresis experiments in this thesis have demonstrated the importance of measuring PKG-mediated phosphorylation in the intact tissue setting. Numerous previously identified *in vitro* and cell culture PKG-substrates thought to play a role in smooth muscle relaxation do not appear to be involved in the intact tissue setting. Although we have tentatively identified a few of our PKG substrates, establishing the exact identity of these proteins could provide useful information concerning the mechanisms of PKG-mediated smooth muscle relaxation. If the exact identities of these proteins were known, detailed experiments to determine their absence or presence in non-responsive smooth muscles could be performed. If additional two dimensional gel electrophoresis experiments could have been done, it would have been interesting to investigate the presence of our PKG substrates in another cGMP-responsive smooth muscle such as the trachea or pulmonary artery. Because our investigation into the patterns of phosphorylation in the rat myometrium has implicated a novel pathway in SNAP-induced relaxation, it would be of interest to confirm that these phosphorylation events are a result of NO by analyzing the patterns of phosphorylation in the presence of acetylpenicillamine. It would also be of interest to
measure the relaxant responses to SNAP in the rat vas deferens and subsequently investigate whether or not these cGMP-independent phosphorylation events occur in another non-responsive smooth muscle.
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