STUDIES ON THE ROLE OF CYCLIC AMP IN THE
REGULATION OF VASCULAR SMOOTH MUSCLE TENSION

By

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A THESIS SUBMITTED IN PARTIAL FULFILMENT OF
THE REQUIREMENTS FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY
in
THE FACULTY OF GRADUATE STUDIES
Division of Pharmacology and Toxicology
of the Faculty of Pharmaceutical Sciences

We accept this thesis as conforming
to the required standard

THE UNIVERSITY OF BRITISH COLUMBIA
April 1986
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ABSTRACT

The precise role of cyclic AMP (cAMP) in the regulation of smooth muscle contraction has been a subject of controversy for the last two decades. It has been hypothesized that drug-induced elevation of cAMP levels is responsible for vascular smooth muscle relaxation. In the present studies this hypothesis was examined in vascular smooth muscle (1) by comparing the effects of prostacyclin (PGI₂) on cAMP levels and tension in two different vascular smooth muscles (bovine coronary arteries and rabbit aortic rings) and (2) by studying the effects of prostaglandin E₁ (PGE₁), isoproterenol and forskolin on cAMP levels, cyclic AMP-dependent protein kinase activity and tension in rabbit aortic rings.

In bovine coronary arteries, PGI₂ elevated cAMP levels and relaxed the potassium-depolarized muscles. The PGI₂-induced cAMP elevation preceded the relaxation and both parameters were altered in a dose-dependent manner by increasing concentrations of PGI₂ (0.3, 3, and 30 μM). These results are consistent with a role for cAMP as a mediator of vascular smooth muscle relaxation. Cyclic AMP levels were also elevated by PGI₂ in a concentration- and time-dependent manner in rabbit aortic rings. However, in direct contrast to the results in the bovine coronary arteries, PGI₂-induced elevation of cAMP in the aortic rings was accompanied by contraction rather than relaxation. Isoproterenol, a drug which is generally believed to relax smooth muscles by virtue of its ability to increase tissue cAMP levels, relaxed PGI₂-contracted
aortic rings with no further elevation of cAMP beyond that caused by the PG\textsubscript{1} alone. These results indicate that drug-induced elevation of cAMP in vascular smooth muscle is not always accompanied by relaxation.

Forskolin, a direct stimulant of adenylate cyclase, has been suggested to be a valuable tool for elucidating the role of cAMP in various physiological processes. We studied the effects of forskolin, PG\textsubscript{1} and isoproterenol on cAMP levels and tension in rabbit aortic rings to further examine the relationship between drug-induced elevation of cAMP levels and tension in vascular smooth muscle. PG\textsubscript{1}, isoproterenol and forskolin all increased cAMP levels in rabbit aortic rings. Isoproterenol and forskolin relaxed phenylephrine-contracted aortic rings, but PG\textsubscript{1} contracted the rings in the presence or absence of phenylephrine. Isoproterenol relaxed these PG\textsubscript{1}-contracted aortic rings without a further change in the total cAMP levels, which were already elevated by PG\textsubscript{1} alone. Pretreatment with forskolin potentiated the effects of PG\textsubscript{1} on cAMP levels. PG\textsubscript{1} contracted muscles partially relaxed by forskolin even though very large increases in cAMP levels (30 fold) were produced by PG\textsubscript{1} in the presence of forskolin. Isoproterenol was able to relax these forskolin-stimulated, PG\textsubscript{1}-contracted muscles with no further increase in cAMP levels. Thus, based on estimations of total tissue levels of cAMP, there does not appear to be a good correlation between changes in cAMP levels and tension in rabbit aortic rings under various conditions.

Physiological processes which are thought to be mediated by cAMP are assumed to be a consequence of selective activation of cyclic AMP-dependent protein kinase (cA kinase). As previously noted in cardiac
muscle, a differential activation of cA kinase in specific compartments (soluble vs particulate) by different drugs might possibly explain the differences in pharmacological responses observed in our experiments on rabbit aorta. In order to investigate this possibility, we studied the effects of isoproterenol, PGE₁ and forskolin on soluble and particulate cA kinase activity in rabbit aortic rings. A concentration of isoproterenol which produced a moderate increase in cAMP levels did not change the protein kinase activity in the soluble fraction. This could be partly due to the technical limitations of the assay. Both forskolin and PGE₁ significantly increased the kinase activity although they exerted opposite effects on the tension of the preparations. Isoproterenol relaxed the PGE₁-contracted muscles without any further activation of the kinase. Forskolin potentiated the effects of PGE₁ on protein kinase activity but PGE₁ still contracted the forskolin-relaxed aortic rings. Once again, isoproterenol was able to relax these preparations without further activating the kinase. Thus, as was the case with the cAMP data described above, activation of the kinase in the soluble fraction of aortic rings occurred whether the muscles were relaxed or contracted. It was anticipated that a difference in activation of cA kinase in the particulate fraction might possibly explain our results. However, the increase in cA kinase activity in the pellet appears to be the same with isoproterenol and PGE₁, alone or in combination. Significant increases in cA kinase activity were observed with forskolin and also with the combination of forskolin, PGE₁ and isoproterenol in the particulate fraction.
Our results thus demonstrate a clear dissociation between tension, elevation of cAMP and activation of cA kinase under various conditions in rabbit aortic rings. It can be suggested that elevation of cAMP and activation of cA kinase may not be directly responsible for the regulation of vascular smooth muscle tension or, alternatively, that some form of functional compartmentation of cA kinase might exist in this tissue.

Finally, an attempt was made to study the role of calcium in phenylephrine- and PGE$_1$-induced contractions and the effect of isoproterenol under these conditions. Phenylephrine produced a phasic contraction and PGE$_1$ produced a sustained contraction in the absence of extracellular calcium, suggesting that intracellular calcium is partially responsible for these contractions. Pretreatment with isoproterenol resulted in complete inhibition of the phenylephrine-induced phasic contraction and also relaxed the PGE$_1$-induced sustained contraction under similar conditions. These results suggest that at least part of isoproterenol's relaxant effect is mediated by an action at an intracellular site in rabbit aorta.

Jack Diamond, Ph.D.
Thesis Supervisor
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<tr>
<td>ACS</td>
<td>aqueous counting scintillant</td>
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<tr>
<td>ADP</td>
<td>adenosine 5'-diphosphate</td>
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<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
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<tr>
<td>Ca++</td>
<td>calcium</td>
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<tr>
<td>CaM</td>
<td>calmodulin</td>
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<tr>
<td>cAMP</td>
<td>cyclic adenosine 3'-5'-monophosphate</td>
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<tr>
<td>cA kinase</td>
<td>cyclic AMP-dependent protein kinase</td>
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<tr>
<td>cGMP</td>
<td>cyclic guanosine monophosphate</td>
<td></td>
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<tr>
<td>cpm</td>
<td>counts per minute</td>
<td></td>
</tr>
<tr>
<td>DEAE</td>
<td>diethylaminoethyl</td>
<td></td>
</tr>
<tr>
<td>DOC</td>
<td>sodium deoxycholate</td>
<td></td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediamine tetraacetate, disodium salt</td>
<td></td>
</tr>
<tr>
<td>EGTA</td>
<td>ethyleneglycol-bis-(β-aminoethyl ether) N,N'-tetraacetic acid</td>
<td></td>
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<tr>
<td>FORSK</td>
<td>forskolin</td>
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<td>g</td>
<td>grams</td>
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<td>GTP</td>
<td>guanosine triphosphate</td>
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<tr>
<td>5-HT</td>
<td>5-hydroxy tryptamine</td>
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<tr>
<td>ISO</td>
<td>isoproterenol</td>
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<tr>
<td>KCl</td>
<td>potassium chloride</td>
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<td>μl</td>
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<td>mm</td>
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<td>mM</td>
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µM  micromolar concentration
MLCK myosin light chain kinase
min minute
MIX methylisobutylxanthine
N  number of experiments
NE  norepinephrine
P  probability
PE phenylephrine
PGE₁ prostaglandin E₁
PGI₂ prostacyclin
pmole picomole
R₁, R₁₁ regulatory subunits of cyclic AMP-dependent protein kinase
ROC receptor operated channels
sec second
SEM standard error of the mean
VIP vasoactive intestinal polypeptide
VOC voltage operated channels
ACKNOWLEDGEMENTS

No words can substitute to express my heartfelt gratitude to my advisor Dr. Jack Diamond, Ph.D. for his excellent guidance and encouragement throughout this work.

I would like to express sincere thanks to the members of my supervisory committee, Drs. V. Palaty, S. Katz, B.D. Roufogalis, K. MacLeod and J.H. McNeill for their encouraging criticism and the scientific input during the course of this work.

I am grateful to the Canadian Heart Foundation for financial aid.

Last but never the least, I wish to thank all the members of the faculty, colleagues and friends for making my stay at the University of British Columbia a memorable event in my life.

Raju V.K. Vegesna
DEDICATION

To my parents,
brothers and sisters
INTRODUCTION

A. Role of Calcium in the Contraction of Vascular Smooth Muscle

Unlike cardiac and skeletal muscles, different smooth muscle tissues present important variations in their characteristics of excitation-contraction coupling, in their responses to hormones and pharmacological agents and also in the properties of their cellular calcium (Ca\(^{++}\) stores. To describe the regulatory role of Ca\(^{++}\) in different smooth muscles in detail is beyond the scope of this thesis. However, these topics have been reviewed in recent years (Webb and Bohr, 1981; Kuriyama et al., 1982; Casteels and Droogmans, 1984; and Somlyo, 1985). The present analysis of the role of Ca\(^{++}\) is limited to vascular smooth muscle.

In vascular smooth muscle, the ultimate factor that regulates the tone of the smooth muscle appears to be the concentration of intracellular free Ca\(^{++}\) often referred to as activator Ca\(^{++}\). Evidence from physiological and pharmacological studies indicates that there are two different sources of calcium ions in smooth muscle. One source of Ca\(^{++}\) is that present in the extracellular fluid, the other is tightly bound calcium associated with intracellular binding sites such as plasma membrane, sarcoplasmic reticulum and mitochondria (for review, see Daniel et al., 1983). The main components in the regulation of Ca\(^{++}\) in vascular smooth muscle are the plasma membrane and the intracellular stores.
By using contractile force measurements and studies utilizing measurements of Ca\(^{++}\) fluxes (Meisheri et al., 1981) in vascular smooth muscle, evidence has been documented for the existence of two sets of different and independent Ca\(^{++}\) channels on the plasma membrane controlling the permeability of Ca\(^{++}\) (Fig. 1). One set of channels, referred to as voltage-operated channels (VOC), respond principally to changes in the voltage across the membrane. For example, application of excess extracellular potassium depolarizes the membrane, reduces the membrane resistance and produces contraction. This contraction can be completely eliminated in Ca\(^{++}\)-free EGTA-containing solution, indicating that the contraction is solely due to the influx of Ca\(^{++}\) into the cell. The contraction-induced by Ca\(^{++}\) entering through VOC has been referred to as electromechanical coupling (Somlyo and Somlyo, 1968). Another set of channels respond principally to hormonal agents, including muscarinic agonists, histamine, angiotensin, serotonin and norepinephrine which act through receptors on the plasma membrane. These channels are referred to as receptor operated channels (ROC). For example, norepinephrine (NE) produces a maintained force development in a Ca\(^{++}\)-containing medium by opening the ROC. If the smooth muscle cells are exposed to a Ca\(^{++}\)-free EGTA containing medium, NE will induce only a transient phasic contraction. The latter finding indicates that this agonist not only opens ROC but also releases Ca\(^{++}\) from the intracellular stores. The initial phasic contraction induced by NE is dependent upon the release of intracellular Ca\(^{++}\), whereas the maintained tonic contraction is due to the continuous influx of external Ca\(^{++}\) through ROC into the
FIGURE 1: Schematic illustration of mechanisms proposed for calcium to regulate vascular smooth muscle contraction. Abbreviations are as follows: R, receptor; ROC, receptor operated channels; VOC, voltage operated channels; SR, sarcoplasmic reticulum, CaM, calmodulin; MLCK, myosin light chain kinase.
cytoplasm. In rabbit aorta, it has recently been suggested that the phosphatidylinositol turnover mechanism may account for the rapid phase of NE-induced contraction and phosphatidic acid or its immediate precursor diacylglycerol (possibly activating phospholipid dependent protein kinase C) may account for receptor-induced Ca\(^{++}\) influx (Campbell et al., 1985). The contraction induced without polarization of the smooth muscle and involving entry of Ca\(^{++}\) through ROC or release of Ca\(^{++}\) from sequestered stores or both is known as pharmaco-mechanical coupling.

There is a great deal of uncertainty regarding the location of the intracellular Ca\(^{++}\) pool in vascular smooth muscle. Although present in small amounts, sarcoplasmic reticulum in the smooth muscle has been implicated as the important source for recycling of Ca\(^{++}\) (Somlyo, 1985) or in the Ca\(^{++}\)-induced Ca\(^{++}\) release mechanisms (Saida and Van Breemen, 1984) in some vascular smooth muscles. Alternatively, the inner surface of the plasma membrane appears to be a major storage site for release of calcium intracellularly (Daniel et al., 1983). Experimental evidence also suggests that these intracellular storage sites are functionally at close proximity to the receptor-operated channels in the plasma membrane (Ito et al., 1982).

During the prolonged contraction of vascular smooth muscle by agonists, Ca\(^{++}\) continuously flows into the cell and is also extruded continuously to preserve the integrity of the cell. Two possible mechanisms have been suggested to operate for the extrusion of calcium in vascular smooth muscle. The first is transmembrane Na\(^{+}\)–Ca\(^{++}\)
exchange, whereby cytoplasmic Ca\(^{++}\) is extruded against its
electrochemical gradient by Na\(^{+}\) ions moving down their gradient
(Blaustein et al., 1977). However, under physiological conditions the
extent of its participation is not certain (Daniel et al., 1983).
Alternatively, an ATP-dependent Ca\(^{++}\) pump depending on the function of
Ca\(^{++}\)-Mg\(^{++}\)-ATPase has been shown to be present on the plasma membrane.
The presence of this pump and its function has been well documented
using subcellular fractionation techniques (see for review, Daniel et al., 1983).

From the aforementioned information, it can be understood that
activation of the receptor or depolarization of the membrane results in
the increased permeability of plasma membrane to Ca\(^{++}\). Additionally,
the release of Ca\(^{++}\) from the intracellular stores increases the
cytoplasmic Ca\(^{++}\) concentration. Now the final step in the contraction
of vascular smooth muscle is the development of force produced by
interaction of actin and myosin filaments. Various regulatory
mechanisms have been proposed that regulate actin-myosin interaction by
Ca\(^{++}\) in smooth muscle (Marston, 1982).

One of the most well studied mechanisms in recent years in vascular
smooth muscle appears to be the Ca\(^{++}\)/calmodulin-induced myosin
phosphorylation (Adelstein and Hathaway, 1979) (see Fig. 1). According
to this theory, when the intracellular Ca\(^{++}\) concentration rises above
the threshold value (> 10\(^{-6}\) M), Ca\(^{++}\) binds to calmodulin (an
intracellular Ca\(^{++}\) receptor), which then forms an active complex with
myosin light chain kinase (MLCK). The activated MLCK catalyzes the
transfer of phosphate from ATP to the 20,000 dalton light chains of
myosin. The phosphorylated myosin then combines with actin and undergoes a complex intracellular arrangement. As a result, actin and myosin slide past each other leading to contraction of smooth muscle. MLC is dephosphorylated by a Ca\({\text{++}}\)-independent specific phosphatase present in the smooth muscle (Pato and Adelstein, 1980). Although the phosphatase also appears to be a potential site for regulation of vascular smooth muscle tension, less attention has been paid to its role.

In direct contrast to the Ca\({\text{++}}\)-calmodulin theory, Ebashi and coworkers (1977, 1982) suggested the leiotonin hypothesis. According to this theory, Ca\({\text{++}}\) binds to leiotonin C, a Ca\({\text{++}}\)-binding protein (not calmodulin), which in turn binds to leiotonin A (actin-binding protein) and tropomyosin. This results in an actin-activated myosin ATPase accompanied by contraction. A dual regulatory system involving phosphorylation of proteins associated with both actin and myosin filaments also has been proposed by others (Walters and Marston, 1981).

At the present state of knowledge, all these mechanisms appear to be potentially valid. However, most of the evidence reported to date suggests that phosphorylation of myosin is an important event mediating smooth muscle contraction (Walsh et al., 1983).

For a long time it has been known that certain drugs and hormones produce relaxation of smooth muscle by virtue of their ability to release certain intracellular second messengers. These second messengers in turn participate in a cascade of events which results in
the final response. Cyclic AMP has been implicated as one of the most important second messengers in the regulation of smooth muscle tension in general and vascular smooth muscle tension in particular (Kramer and Hardman, 1980).

B. CYCLIC AMP: General View

After the discovery of cyclic AMP (cAMP) as a second messenger of various cellular functions (Robison, Butcher and Sutherland, 1971), tremendous progress has been made in recent years not only in understanding the mechanisms whereby many drugs, neurotransmitters and hormones produce their well known effects on the target tissue, but also in unraveling the biosynthetic mechanisms involved at the molecular level in the production of cAMP.

Hormones such as catecholamines bind to specific receptors on the surface of the plasma membrane of the cell resulting in activation of the enzyme adenylate cyclase. Recent developments in the study of these drug-receptor interactions have been reviewed (Vegesna and Roufogalis, 1985). The activation of adenylate cyclase leads to increased conversion of ATP to cAMP. Recent advances in radioligand binding techniques and β-receptor isolation techniques (Lefkowitz et al., 1984) have opened new lines in understanding the adenylate cyclase coupling mechanisms. As shown in Fig. 2, molecular components of the catecholamine-sensitive adenylate cyclase system are composed of at least three distinct protein entities: hormone receptor binding component (e.g., the β-adrenergic receptor), the catalytic moiety of
FIGURE 2: Hypothetical model showing the possible sites of action of cyclic AMP-dependent protein kinase (PK) in the regulation of vascular smooth muscle tension. The abbreviations are as follows: Rs and Ri, stimulatory and inhibitory hormone receptors coupled to Ns and Ni guanine nucleotide regulatory components; C, catalytic unit of adenylate cyclase; cAMP, cyclic AMP; PDE, phosphodiesterase inhibitor; SR, sarcoplasmic reticulum, MLCK, myosin light chain kinase; CaM, calmodulin.
\[ \text{MLCK} \rightarrow \text{Ca-CaM} \rightarrow \text{ADP} \rightarrow \text{MYOSIN-P} \rightarrow \text{ACTIN} \rightarrow \text{CONTRACTION} \]

\[ \text{MLCK-P} \rightarrow \text{ATP} \rightarrow \text{MYOSIN} \rightarrow \text{RELAXATION} \]
the enzyme (C) which converts ATP to cyclic AMP, and a coupling protein (N) which is regulated by guanine nucleotides such as guanosine triphosphate (GTP). These regulatory mechanisms have been reviewed recently (Minocherhomjee and Roufogalis, 1982). Experimental evidence suggests that two structurally related forms of this coupling protein exist, termed Ns and Ni, and these proteins might serve to couple the stimulatory or inhibitory receptors, respectively, to the catalytic moiety. Hormones and drugs such as epinephrine and isoproterenol activate adenylate cyclase and elevate cAMP, via activation through the Ns unit of the coupling protein. On the other hand, agents such as opiates and some α adrenergic agents inhibit adenylate cyclase and reduce cAMP levels, via an effect through the Ni unit. Other agents may bypass hormone-receptor interaction and alter cAMP generation. For example, cholera toxin elevates cAMP levels by irreversibly activating the Ns subunit whereas pertussis toxin (by ribosylation of the Ni subunit) reduces the inhibitory input of Ni on the enzyme. Recently, another interesting drug, forskolin, has been shown to activate the adenylate cyclase enzyme by a direct interaction with the catalytic moiety (C) (Seamon and Daly, 1981a) or a closely associated protein (Seamon and Wetzel, 1984).

The increased concentration of cAMP in the cell leads to activation of the enzyme cAMP-dependent protein kinase. Phosphorylation of specific proteins by the activated kinase is believed to be responsible for the general biological responses. Cyclic AMP in the cell is hydrolyzed to 5'-AMP, a reaction catalyzed by one or more cyclic
nucleotide phosphodiesterases that exist in all mammalian cells (Robison et al., 1971).

For the past twenty years, regulation of cellular responses by cyclic nucleotides has been a subject of intense investigation. Cyclic AMP has been suggested to be a mediator of many hormonal functions such as glucagon stimulation of hepatic glycogenolysis, lipolysis, several processes involved in the contraction-relaxation cycle of the heart, steroidogenesis, thyroglobulin secretion and salivary amylase secretion (for detailed references, see Kebabian and Nathanson 1982). Although cAMP is thought to regulate many cellular events, it has proven difficult to firmly establish the relationship of cAMP levels to physiological function in intact cells, tissues and organisms. Smooth muscle appears to be one of the most controversial areas in this regard.

C. Role of cAMP in the Regulation of Smooth Muscle Contraction

The precise role of cAMP in the regulation of smooth muscle contraction has been a subject of controversy for the last two decades. A substantial amount of data has accumulated in the literature exploring the role of cAMP in the regulation of smooth muscle tension and this topic has been excellently reviewed in recent years (Namm and Leader, 1976; Diamond, 1978; Namm, 1982; Kukovetz et al., 1981; Kroeger, 1983; Hardman, 1981, 1984).

Sutherland and Rall (1960) first observed that epinephrine-induced relaxation of smooth muscle might be related to elevation of cAMP levels in that tissue, and proposed the general hypothesis of cAMP-mediated
relaxation of different smooth muscles. They also proposed four criteria to be satisfied in order to demonstrate that a hormone or drug-induced elevation of cAMP plays a mediator role in the physiological response (Robison et al., 1971). Briefly, in smooth muscle:

1. The hormone must be able to stimulate adenylate cyclase in a cell free system of the target smooth muscle.
2. Hormone-induced elevation of cAMP levels should be correlated time-dependently and dose-dependently to the relaxation of smooth muscle.
3. Cyclic AMP or its derivatives should be able to reproduce the relaxation of the hormone when applied to the target tissue.
4. Phosphodiesterase inhibitors should be able to potentiate hormone-induced relaxation of the smooth muscle.

From that time onwards, many researchers attempted to test the general hypothesis of cAMP-mediated relaxation by using a variety of drugs in different tissues. There is a substantial amount of evidence supporting the concept that there is a causal relationship between increases in cAMP levels produced by β-adrenergic drugs and other compounds and their relaxant effects on smooth muscle in general and vascular smooth muscle in particular. However, there is also evidence which is inconsistent with this hypothesis (see later sections).

For example, studies done by Marshall and Kroeger (1973) and Honeyman et al. (1978) have demonstrated a temporal and quantitative correlation between the elevation of cAMP levels and the relaxing effect
of isoproterenol and other drugs. Evidence has also been provided for a role of cAMP by studying a series of phosphodiesterase inhibitors (Kramer and Wells, 1979) and cAMP derivatives (Webb and Bohr, 1980) in different vascular smooth muscle preparations. Extensive evidence for a mediator role of cAMP in smooth muscle relaxation produced by β-adrenergic drugs and other stimulants of adenylate cyclase was also provided by Kukovetz and his colleagues (see Kukovetz et al., 1981 for review). In isolated coronary arteries, isoproterenol, prostacyclin and adenosine have been shown to produce concentration-dependent increases in cAMP in close association with their relaxant effects on potassium contracted tissues. Kukovetz et al. have shown that the extent of changes in cAMP is consistent with a mediator role in the relaxant response.

As discussed earlier, a novel drug, forskolin, has been shown to increase cAMP levels in a variety of tissues by acting directly on the catalytic subunit of adenylate cyclase enzyme. Several forskolin induced cellular responses have been correlated to increased cAMP synthesis and it has been suggested that forskolin may be a valuable tool in elucidating the role of cAMP in the physiological responses to various hormones (see for review, Seamon and Daly, 1981b). Forskolin has been shown to relax a variety of smooth muscles (Dubey et al., 1981; Muller and Baer, 1983; Burka, 1983a; Vegesna and Diamond, 1983). In some vascular preparations, forskolin-induced elevation of cAMP levels was correlated to relaxation (Lincoln and Simpson, 1983; Jones et al., 1984; Nickols, 1985). Another striking aspect of forskolin effects observed was its potentiation of hormonally-induced effects on
cAMP generation. It has been shown that low concentrations of forskolin, which alone produce very small increases in cAMP levels, can greatly potentiate responses to certain hormones such as norepinephrine, isoproterenol, histamine, PGE₂, and VIP in a number of hormonally-responsive cells (for review, see Seamon and Daly, 1981b). Forskolin has also been shown to potentiate hormonal-induced cAMP levels in vascular smooth muscle (VegeSna and Diamond, 1983, 1984; Nickols, 1985).

Thus, many researchers have studied the ability of a variety of agents to elevate cAMP levels and produce relaxation of various vascular smooth muscles isolated from different species (see also Table 1). The majority of the studies support a role of cAMP in the relaxation of smooth muscle.

The only known mechanism by which cAMP mediates various physiological functions is through activation of cAMP-dependent protein kinases as described previously. Because of the technical limitations in measurement of cAMP levels in certain studies (Sala et al., 1979) estimation of cAMP-dependent protein kinase appears to be the most meaningful parameter in demonstrating a role of cAMP in physiological responses such as smooth muscle relaxation.

D. Cyclic AMP-Dependent Protein Kinase (cA Kinase)

The initial study of the cA kinase (Walsh et al., 1968) demonstrated that this enzyme might be a primary site of action of the cyclic nucleotide. Kuo and Greengard (1969) extended this concept by identifying this enzyme in a wide range of mammalian tissues. The
TABLE 1
DRUGS THAT PRODUCE ELEVATION OF CYCLIC AMP AND RELAXATION IN DIFFERENT VASCULAR TISSUES

<table>
<thead>
<tr>
<th>Animal</th>
<th>Tissue</th>
<th>Drug</th>
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<tbody>
<tr>
<td>Rat</td>
<td>Aorta</td>
<td>Catecholamines</td>
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<td></td>
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<td>Forskolin</td>
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<td></td>
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<td>Halothane</td>
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<td></td>
<td>Tail Artery</td>
<td>Theophylline</td>
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<td></td>
<td>Portal Vein</td>
<td>Mepivacaine</td>
</tr>
<tr>
<td>Rabbit</td>
<td>Aorta</td>
<td>Isoproterenol</td>
</tr>
<tr>
<td></td>
<td>Mesenteric Vein</td>
<td>PGE₁, PGI₂</td>
</tr>
<tr>
<td></td>
<td>Pulmonary Artery</td>
<td>Dipyridamole</td>
</tr>
<tr>
<td>Canine</td>
<td>Coronary Artery</td>
<td>PGE₁, PGI₂</td>
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<tr>
<td></td>
<td>Mesenteric Artery</td>
<td>PGE₁, PGI₂</td>
</tr>
<tr>
<td></td>
<td>Lobar Artery</td>
<td>PGE₁</td>
</tr>
<tr>
<td></td>
<td>Lobar Vein</td>
<td>Isoproterenol</td>
</tr>
<tr>
<td></td>
<td>Sephaneous Vein</td>
<td>PGE₁</td>
</tr>
<tr>
<td></td>
<td>Pulmonary Vein</td>
<td>PGE₂</td>
</tr>
<tr>
<td>Bovine</td>
<td>Coronary Artery</td>
<td>Forskolin</td>
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<td></td>
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<td>Isoproterenol</td>
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<td></td>
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<tr>
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<td>Adenosine</td>
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<td>Papaverine</td>
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<td>Diazoxide</td>
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<td>Hydralazine</td>
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<td></td>
<td>Mesenteric Artery</td>
<td>Isoproterenol</td>
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<tr>
<td></td>
<td>Digital Vein</td>
<td>PGE₂</td>
</tr>
<tr>
<td></td>
<td>Facial Artery</td>
<td>KCl</td>
</tr>
<tr>
<td>Human</td>
<td>Umbilical Artery</td>
<td>PGE₁</td>
</tr>
</tbody>
</table>

Modified from Kramer and Hardman (1980).
ubiquitous occurrence of cA kinase in different tissues in which cAMP is the presumed second messenger led to the proposal that a majority of the effects produced by cAMP are mediated by activation of the protein kinase. It appears that activation of cA kinase may be an obligatory step in the expression of many hormone and drug-induced responses. This enzyme has been well characterized and the regulatory mechanisms controlled by this protein kinase have been extensively reviewed by several authors in recent years (Walsh and Cooper, 1979; Glass, 1980; Flockhart and Corbin, 1982). This enzyme has two principal classes of isozymes (Corbin et al., 1975) named type I and type II on the basis of their elution from DEAE cellulose. It has been confirmed that the relative distribution of these two isozymes varies from species to species and from tissue to tissue. The two isozymes appear to share a common catalytic subunit but have different regulatory subunits which might be responsible for the differences in their properties (Corbin and Keely, 1977). Another striking difference between the two regulatory subunits (R₁ and R₁₁) is the susceptibility of R₁₁ to phosphorylation by the catalytic subunit (autophosphorylation). It was also proposed that the type II holoenzyme exists in vivo primarily in the autophosphorylated form. The ability of certain drugs such as isoproterenol to decrease this auto phosphorylation of the R₁₁ subunit has been suggested to be a mechanism by which smooth muscle relaxation can take place (Scott and Mumby, 1985). It was also implied that specific activation of either of the isozymes might be responsible for the cAMP-mediated cellular processes in different tissues (Schwoch,
1978; Corbin et al., 1977; Mednieks and Hand, 1982) including smooth muscle (Guinovart and Larner, 1980; Silver et al., 1982). However, further studies are necessary to elucidate selective activation of isozymes by drugs and hormones.

Agents which stimulate the accumulation of cAMP activate cA kinase by the following mechanism.

\[
R_2C_2 + 4 \text{cAMP} \rightleftharpoons R_2 \text{cAMP}_4 + 2C
\]

(Inactive) \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \ Quad
Although it is widely accepted that cAMP mediates its physiological responses through activation of cA kinase, very few studies have been done relating changes in the activation of cA kinase and tension in vascular smooth muscle. In bovine coronary arteries, isoproterenol (Silver et al., 1982; Vegesna and Diamond, 1984) and adenosine (Silver et al., 1984) have been shown to activate cA kinase accompanied by relaxation of potassium-contracted arteries. Similarly in rat aorta, forskolin produced a dose-dependent activation of the kinase and relaxed the NE-contracted strips (Lincoln and Simpson, 1983). These results are consistent with a role of cAMP in the regulation of smooth muscle tension.

It has been suggested that protein phosphorylation may be a final common pathway for many biological regulatory agents (Greengard, 1978) and cA kinase is known to phosphorylate certain intracellular substrates which might be functionally important in the regulation of smooth muscle tension as discussed in the following section.

E. Possible Mechanisms by which cAMP Mediates Vascular Smooth Muscle Tension

It is widely believed that many drugs and hormones produce relaxation of smooth muscle by altering the free cytoplasmic Ca\(^{++}\) concentration. This can be achieved in several ways such as preventing the influx of Ca\(^{++}\) or promoting the efflux and/or uptake of Ca\(^{++}\). It may also be possible to inhibit the effect of Ca\(^{++}\) on the contractile machinery without actually lowering cytoplasmic Ca\(^{++}\) levels. As shown
in Fig. 2, several possible cellular sites of action for cAMP and protein kinase in the regulation of tension in vascular smooth muscle have been considered. Experimental evidence for some of these possibilities has been provided in the last few years.

For example, Adelstein and co-workers (1982) suggested from their studies on cell free systems isolated from turkey gizzard smooth muscle that phosphorylation of myosin light chain kinase (MLCK) may inhibit actin-myosin interaction. As noted earlier (see role of Ca$^{++}$ in smooth muscle contraction), Ca$^{++}$ causes contraction of smooth muscle by binding to calmodulin and thus activating MLCK which in turn phosphorylates myosin. According to Adelstein et al. (1982), cA kinase phosphorylates MLCK. This phosphorylation weakens the interaction of Ca$^{++}$ and calmodulin with MLCK, resulting in an inhibition of MLCK, accompanied by relaxation. Consistent with this hypothesis, similar findings were reported with enzymes isolated from vascular smooth muscle (Silver and Disalvo, 1979; Bhalla et al., 1982). The ability of the catalytic subunit of protein kinase to inhibit Ca$^{++}$-induced tension in skinned smooth muscle preparations (Kerrick and Hoar, 1981; Rugg et al., 1983) further documents evidence in favour of this hypothesis. These results also suggest that protein kinase could cause relaxation without reducing cytoplasmic Ca$^{++}$ concentration. Similar to these observations, using aequorin as an intracellular Ca$^{++}$ marker, Morgan and Morgan (1984) noted that isoproterenol, forskolin and dibutyryl cAMP can cause relaxation of ferret portal vein without apparently reducing cytoplasmic calcium.
Although in vitro experiments and indirect evidence does support the phosphorylation of MLCK-relaxation hypothesis, results obtained in intact preparations appear to be contradictory. For example, in canine tracheal smooth muscle, forskolin has been shown to phosphorylate MLCK accompanied by relaxation of the methacholine-contracted tissue (deLanerolle et al., 1984). However, the results obtained in bovine tracheal smooth muscle (Miller et al., 1983) and in vascular smooth muscle (Gerthoffer and Murphy, 1984) do not appear to be consistent with this phosphorylation hypothesis. For example, in bovine carotid artery, Gerthoffer and Murphy (1984) have shown that forskolin elevates cAMP levels and relaxes swine carotid arteries without changing the phosphorylation state of myosin. Further experiments in intact tissue are necessary to clarify this issue.

In contrast to the Ca\(^{++}\)-calmodulin hypothesis, other investigators have emphasized the control of membrane transport processes by protein kinase, indicating the involvement of more sites for control than MLCK alone (for review, see Kuriyama et al., 1982). Scheid et al. (1979) postulated that \(\beta\)-adrenergic agents stimulate protein kinase, activate Na\(^{+}\)-K\(^{+}\) ATPase and enhance Na\(^{+}\)-K\(^{+}\) transport at the plasma membrane of smooth muscle (see Fig. 2). This in turn leads to a change in the Na\(^{+}\)-Ca\(^{++}\) exchange process due to an increase in the sodium gradient. Similar conclusions were reached from studies done on sarcolemmal vesicles prepared from rat aorta (Brockbank and England, 1980). Using forskolin as a tool to study the membrane fluxes of rat aorta, Jones et al. (1984) postulated that cAMP-dependent regulation of membrane
transport is a primary locus for relaxation. Webb and Bohr (1980), in their studies on different vascular smooth muscle preparations using cAMP elevating agents, suggested that participation of an electrogenic sodium pump in the relaxation of vascular smooth muscle does seem to be important. However, this depends upon experimental conditions and the species from which vascular tissues were obtained.

Some investigators considered extrusion of Ca\(^{++}\) by a calmodulin-dependent Ca\(^{++}\) pump as a potential site for regulation by protein kinase (Bhalla et al., 1978). For example, Suematsu et al. (1984) using purified sarcolemmal fractions of vascular smooth muscle from porcine aorta, demonstrated enhanced Ca\(^{++}\) transport by a Ca\(^{++}\)-calmodulin-dependent Ca\(^{++}\) pump in the presence of the catalytic subunit of protein kinase. However, there are some studies inconsistent with these results (Kreye and Schlicker, 1980). Intracellular sequestration of Ca\(^{++}\) (Mueller and Van Breemen, 1979) or inhibition of the influx of Ca\(^{++}\) (Meisher and Van Breemen, 1982) in some smooth muscles are also suggested to be possible sites of action of β-adrenergic drugs and other cAMP-elevating agents.

Thus, the mechanism by which cAMP mediates relaxation is not simple, and as suggested by Hardman (1984), multiple sites of regulation might exist in vascular smooth muscle.

F. Dissociations Between Elevation of cAMP and Tension in Smooth Muscle

As presented in the previous sections, although several lines of evidence exist in support of a role of cAMP in the regulation of smooth
muscle tension, there is also evidence that is not consistent with this concept (for reviews, see Diamond, 1978; Kramer and Hardman, 1980).

In some smooth muscles, it has been demonstrated that relaxation does not always occur when cAMP levels are increased. For example, in rat myometrium, Diamond and Holmes (1975) observed that KCl-induced depolarization increased cAMP levels. Instead of relaxation, these elevated cAMP levels were accompanied by contraction of the rat myometrium. They further observed that papaverine and nitroglycerin relaxed these depolarized muscles without any detectable change in cAMP levels. These authors suggested that increases in cAMP levels might not be responsible for relaxation in that preparation. In support of these observations, Verma and McNeill (1976) have shown a dose-related relaxation of depolarized rat uteri with isoproterenol. However, no alteration in cAMP levels was observed at any dose tested. Similar to these findings Harbon and associates (see Harbon et al., 1978 for review) found increases in cAMP levels in rat myometrium treated with isoproterenol or PGE₁. However, isoproterenol produced relaxation whereas PGE₁ produced contraction in this tissue. These authors thus questioned the exclusive role of cAMP in the relaxation of smooth muscle.

We have recently reported a dissociation between elevation of cAMP and tension in vascular smooth muscle as well (Vegesna and Diamond, 1983, 1984). For example, the effects of isoproterenol and forskolin on tension, cAMP levels and cA kinase activity were compared in helical strips of bovine coronary arteries. Isoproterenol and forskolin
produced time-dependent and dose-dependent increases in cAMP levels, activated the kinase and both compounds relaxed potassium-contracted arteries. Relaxation, elevation of cAMP and activation of the kinase appeared to be well correlated in the isoproterenol experiments. However, a similar correlation was not evident at lower concentrations of forskolin. For example, 0.1 μM forskolin increased cAMP levels in the arteries by approximately 5.5 fold and activated the kinase but did not relax the muscles. A smaller elevation of cAMP and activation of the kinase produced by 1 μM isoproterenol, on the other hand, was accompanied by an almost complete relaxation of the arteries. Similarly, in dog basilar artery, Fujioka (1984) studied the effect of isoproterenol and papaverine on tension and cAMP levels. Both compounds elevated cAMP. However, papaverine but not isoproterenol, was able to relax high potassium- or 5-HT- induced contractions in this tissue.

The apparent discrepancy existing in this area can partly be explained on the basis of cellular heterogeneity of some tissues. It should be noted that smooth muscle preparations, in addition to nerve cells and blood vessels, contain other cell types such as fibroblasts, interstitial cells, mast cells and endothelial cells (Gabella, 1981). Because of this cellular heterogeneity of the smooth muscle preparations, drug treatment may lead to elevation of cAMP levels in other cell types in addition to smooth muscle cells (Buonassisi and Venter, 1976). This situation might result in erroneous conclusions because of the apparent dissociation between estimated total tissue cAMP and contractile responses observed in such studies.
Some authors tried to explain their results by suggesting compartmentalization of cAMP in the smooth muscle (for example, see Vesin and Harbon, 1974). Elucidation of a role of cA kinase in several cellular processes led to the suggestion that not only cAMP but also cA kinase might be compartmentalized by selective activation of hormones in some tissues (Corbin et al., 1977; Brunton et al., 1981). For example, in their experiments in cardiac tissue, Hayes, Brunton and Mayer (1980) observed that isoproterenol and prostaglandin E₁ (PGE₁) increased cAMP levels and activated the soluble protein kinase. However, isoproterenol, but not PGE₁, produced the anticipated activation of phosphorylase followed by an inotropic action on the heart. Also, isoproterenol, but not PGE₁, activated the particulate protein kinase, suggesting specific pools of cAMP and its protein kinase might be important in the elucidation of hormonal action. The possibility that multiple cell types account for the difference in cA kinase activation was also addressed and rejected in their subsequent experiments using isolated cardiomyocytes (Buxton and Brunton, 1983). Based on these results and also on extensive evidence existing in the literature, Hayes and Brunton suggested compartmentalization of cyclic nucleotide action (see Hayes and Brunton, 1982, for review). Recently, Harper et al. (1985), documented several interesting possibilities for the compartmentalization of cyclic nucleotides in different tissues based on biochemical and immunocytochemical studies. It is logical to assume that such a possibility does exist in smooth muscle as well, and at least some of the discrepancies existing in the smooth muscle area with
respect to cyclic nucleotides can be explained based on the compartmentalization theory.

G. Controversy Regarding Prostaglandin-induced cAMP Levels and Tension in Vascular Smooth Muscle

Prostaglandins are known to elevate cAMP levels in various tissues (Kadowitz, et al., 1975; Tateson et al., 1977). In some vascular smooth muscle preparations, prostaglandins E₁, E₂ and I₂ have been shown to increase cAMP levels under some conditions, but a role for cAMP in the vascular relaxant effects of these drugs including prostacyclin (PGI₂) has not been definitely established (Burka, 1983b).

Several contradictory reports have appeared in the literature regarding PGI₂-induced relaxation of coronary arteries and its correlation to increased levels of cAMP. In 1979, Schrör and Rösen reported that PGI₂ (0.003, 0.030 and 0.300 μM) caused a dose-dependent decrease in cAMP levels in isolated bovine coronary arteries which was accompanied by relaxation of the arteries. It was suggested that the two effects were not causally related. Dembinska-Kiec et al. (1979) reported that, in the presence of the phosphodiesterase inhibitor, methylisobutylxanthine (MIX), PGI₂ (0.03, 0.30 and 3.00 μM) caused a dose-dependent increase in cAMP content in bovine coronary artery. No changes in cAMP were seen in the absence of MIX. Miller et al. (1979) observed that in the presence of MIX, the basal level of cAMP was elevated in bovine arterial strips and PGI₂ caused a further dose-dependent increase in cAMP levels. However, in the absence of MIX,
a low dose of PGI₂ (0.28 μM) produced a decrease in cAMP content, and higher doses (0.84 and 2.80 μM) had no effect on the levels of the cyclic nucleotide. In still another report, Kukovetz et al. (1979) found that even in the absence of MIX, PGI₂ (0.30 and 26.70 μM) significantly elevated cAMP levels in bovine coronary artery strips which had been partially depolarized by 27 mM KCl. These authors reported good correlations between PGI₂-induced vascular relaxation and elevation of cAMP, and it was suggested that the two effects were causally related (Kukovetz et al., 1981). Thus, some studies suggest that elevation of cAMP and relaxation of coronary arteries by PGI₂ may be correlated, while others do not appear to be consistent with this hypothesis.

Recently, prostaglandin E₁ (PGE₁) and PGI₂ (0.1 and 20.0 μM) have been shown to activate adenylate cyclase dose-dependently in isolated smooth muscle cells from rabbit aorta (Nicosia et al., 1984). However, earlier reports had indicated that PGE₁ caused contractions of isolated rabbit aortic strips rather than relaxation (Chandler and Strong, 1972; Pfaffman and Chu-Sun, 1979). Similarly, PGI₂ has been reported to produce either no change (Omini et al., 1977; Furchgott and Zawadzki, 1980; Forsterrmann et al., 1984) or an increase (Hadhazy et al., 1984) in the tone of intact rabbit aortic strips. If elevation of cAMP is responsible for vascular smooth muscle relaxation, PGI₂- and PGE₁-induced elevation of cAMP should be correlated to relaxation in this tissue. However, no studies have been reported to date in which the effects of PGI₂ and PGE₁ on both cAMP levels and tension were monitored in intact strips of rabbit aorta.
H. Summary and Rationale for Proposed Experiments

From the aforementioned information, it is clear that, although elevation of cAMP does not appear to be the primary mechanism of action of some smooth muscle relaxants, there is a great deal of evidence consistent with such a mechanism for relaxation of vascular smooth muscle by β-adrenergic agonists. However, very few systematic time-dependent and dose-dependent studies have been done relating changes in cAMP levels to relaxation of vascular smooth muscle (see Kramer and Hardman, 1980, for review). The role of cAMP in the prostacyclin-induced relaxation of bovine coronary arteries appears to be controversial. The biochemical data obtained in the isolated smooth muscle cells of rabbit aorta with PGE₁ and PGI₂ appear to be in conflict with the pharmacological data obtained in the intact rabbit aortic strips. Further experiments such as estimation of both parameters in the intact preparation might clarify this issue. The ability of forskolin to potentiate hormonally-induced increases in cAMP levels may be a helpful way to explore the cAMP-mediated effects in smooth muscle. Finally, estimation of cA kinase might further promote our understanding of a role of cAMP in the relaxation of vascular smooth muscle.

The present study has been undertaken to address some of these problems. Attempts were made to examine the cAMP-relaxation hypothesis in vascular smooth muscle in two ways: (1) Comparing the effects of PGI₂ on cAMP levels and tension in two different vascular smooth muscle preparations (bovine coronary artery strips and rabbit aortic rings).
(2) Studying the effects of PGE\textsubscript{1} on cAMP levels, cA kinase activity and tension in isolated rabbit aortic rings. The effects of PGE\textsubscript{1} were compared with those of isoproterenol, a \(\beta\)-adrenergic agonist believed to relax vascular smooth muscle by virtue of its ability to increase cAMP levels, and with those of forskolin, a direct stimulant of adenylate cyclase which can mimic the actions of \(\beta\)-adrenergic drugs and prostaglandins in some tissues.
SPECIFIC GOALS OF THE PRESENT INVESTIGATION

Specific goals of the present study were:

1. To study time-dependent and dose-dependent effects of prostacyclin on cAMP levels and tension in isolated bovine coronary arteries and to compare these with the effects of the drug on the same parameters in rabbit aortic rings.

2. To compare the effects of isoproterenol and PGE₁ on cAMP levels and tension in rabbit aortic rings to determine whether a correlation exists between elevation of cAMP and relaxation of the vascular smooth muscle.

3. To investigate possible interactions between forskolin, PGE₁ and isoproterenol on cAMP levels and tension in vascular smooth muscle.

4. To study the effects of isoproterenol, PGE₁ and forskolin on cA kinase activity and tension in rabbit aortic rings.

5. To study the role of different pools of calcium in the effects of isoproterenol on phenylephrine- and PGE₁-induced contractions of rabbit aortic rings.
MATERIALS AND METHODS

A. Materials

The following chemicals were purchased from Sigma Chemical Co.: sodium chloride, potassium chloride, magnesium chloride, magnesium sulphate, calcium chloride, sodium bicarbonate, sodium phosphate monobasic, glucose, trichloroacetic acid, Triton X-100, Trizma adenosine triphosphate (Tris ATP), potassium phosphate, ethylenediamine tetra acetic acid (EDTA), methyl isobutylxanthine (MIX), dithiothreitol, histone II-A, sodium fluoride, cyclic AMP, bovine serum albumin, (-)-isoproterenol, L-phenylephrine, prostaglandin E1 (PGE1), tetrascodium pyrophosphate, sodium deoxycholate (DOC), sodium carbonate, sodium potassium tartrate and copper sulphate. Forskolin was obtained from Calbiochem and prostacyclin (PGI2) was a generous gift from the Upjohn Company. Folin phenol reagent was obtained from Fisher Scientific Company.

Whatman 3MM (GF/A) filter papers (2.3 cm diameter) and 5 ml scintillation vials were obtained from Western Scientific Co.

ACS scintillation fluid and [γ-32P] ATP (20 Ci/mMole) were purchased from Amersham.

Stock solutions (1 mM) of forskolin and PGE1 were prepared in 95% ethanol and stored at -30°C. These solutions were diluted with water and added to the muscle baths to give the desired final concentrations.
Control rings were treated with the appropriate concentration of ethanol. The ethanol alone had no effect on tension or cAMP levels in these preparations.

A 3 mM stock solution of PGI$_2$ was prepared in either ethanol or Tris buffer (50 mM, pH 9.7). These solutions were diluted in Krebs-bicarbonate solution and added to the muscle baths to give the desired final concentration of PGI$_2$. The response to the PGI$_2$ was the same in either diluent, and neither diluent alone had any significant effect on cAMP levels or tension in our experiments.

Cyclic AMP radioimmunoassay kits were purchased from Beckton Dickinson Canada Ltd.

Cyclic GMP radioimmunoassay kits were obtained from New England Nuclear, Canada.

Fresh bovine hearts were obtained from a local slaughter house (Inter Continental Packers, Vancouver, B.C.) and white New Zealand rabbits were purchased from Animal Care Unit, University of British Columbia.

B. Methods

1. Preparation and Handling of Bovine Coronary Arteries

Bovine hearts were obtained immediately after slaughter, immersed in ice cold regular Krebs solution (composition given below) and transported to the laboratory. Branches of the left anterior descending and circumflex coronary arteries were dissected out and helical strips approximately 4 mm wide and 15 mm long were prepared. The strips were
suspended at 37°C under 2 g tension in modified Krebs-bicarbonate solution with the following composition (mM): NaCl, 118; KCl, 5.7; MgSO₄, 2.33; CaCl₂, 1.26; NaHCO₃, 25; NaH₂PO₄, 1.17 and glucose, 11. Solutions were aerated with 95% O₂ and 5% CO₂, which maintained the pH at approximately 7.4. Tension was monitored isometrically. After a 2 hr equilibration period, the strips were initially contracted by replacing the bathing solution with one containing 124 mM KCl. After repeated washings with normal buffer, the strips were recontracted submaximally with 30 mM KCl as described by Napoli et al. (1980). When the strips had attained a steady state level of tension in 30 mM KCl, test drugs were added directly to the muscle baths, and the muscle strips were quick-frozen at predetermined times by clamping them with a pair of tongs precooled in liquid nitrogen. The frozen samples were stored at -80°C until used for cAMP assay. Tension and cAMP levels were thus determined in the same muscle strips.

2. Preparation and Handling of Rabbit Aortic Rings

Descending thoracic aortas were carefully excised from white New Zealand rabbits of either sex (2-3 kg) and trimmed free of adhering fat and connective tissue. In preliminary experiments tension responses were qualitatively the same whether helical strips or transverse rings were used. All results reported below were obtained using transverse rings. Rings approximately 5-7 mm wide were prepared and suspended in isolated organ baths between L-shaped stainless steel hooks for recording of isometric tension. Rings were equilibrated for 2 hr at
37°C under 2 g tension in Krebs-bicarbonate solution as described above for bovine coronary arteries. However, unlike the coronary arteries, the aortic rings were not precontracted with KCl. Drugs were added directly to the muscle baths to give the final concentrations indicated in the results. Aortic rings were frozen at appropriate times after drug addition, and samples were stored at -80°C until used for cAMP and cA kinase assays. Tension and cAMP or cA kinase were thus determined in the same muscle strips.

Endothelium was removed from both bovine coronary arteries and rabbit aortic rings by rubbing the lumen against a glass rod. The success of the removal of endothelium was verified in some experiments by the inability of muscarinic agonists to relax these preparations (Diamond and Chu, 1983) and by histological examination using freezing microtome and standard staining techniques (Stevens, 1977).

3. **Measurement of Cyclic AMP**

Cyclic AMP levels in the frozen tissues were determined by using radioimmunoassay kits supplied by Becton Dickinson Canada, Inc. Briefly, the frozen samples were homogenized in 6% trichloroacetic acid; the trichloroacetic acid was removed by ether extraction and radioimmunoassays were performed on the aqueous extracts. Results are expressed as picomoles of cAMP per gram wet weight of tissue.

4. **Measurement of Cyclic GMP**

Cyclic GMP (cGMP) levels in the frozen muscles were determined using radioimmunoassay as described by Janis and Diamond (1979). Briefly,
aqueous extracts of the samples were prepared similar to cAMP measurements as described above. Before the cGMP assay, aliquots of the aqueous extracts were acetylated as suggested by Harper and Brooker (1975) in order to increase the sensitivity of the cGMP assay. Results are expressed as picomoles of cGMP per gram wet weight of tissue.

5. Preparation of Extracts and Assay of Cyclic AMP-Dependent Protein Kinase

Approximately 30-40 mg of frozen tissue was suspended at 4°C in 8 volumes of buffer (pH 6.8) containing 10 mM potassium phosphate, 10 mM EDTA, 0.5 mM MIX and 0.5 mM 1,4-dithiothreitol as suggested by Silver _et al._ (1982). The tissue was homogenized with a Polytron homogenizer (setting the speed at 8 for 30 seconds) and the homogenate was immediately centrifuged at 30,000 x g for 15 min (Sorvall, RC-2B centrifuge) at 4°C to form soluble (supernatant) and particulate (pellet) fractions. The supernatant fraction was immediately assayed for cA kinase activity.

The pellet containing the particulate fraction was gently washed twice with 8 volumes of homogenizing buffer to remove loosely adhering material. The washed pellet was homogenized with a hand homogenizer in 4 volumes of homogenizing buffer containing 0.2% Triton X-100. After homogenization, the sample was kept on ice for 10 min and centrifuged at 30,000 x g for 15 min. The supernatant fraction from the Triton-treated pellet was used to estimate particulate protein kinase activity.

The cA kinase activity was determined by measuring the transfer of $^{32}$P from [$y^{32}$P] ATP to histone in the presence and absence of 2 μM
cAMP as described by Corbin and Reiman (1975). The assay reaction was started by adding 20 μl of the supernatant to 50 μl of the reaction mixture containing 20 mM potassium phosphate (pH 6.8), 100 μM [γ-32P] ATP (50-100 cpm/pmol), 10 mM magnesium chloride, 100 μg histone II-A, and 10 mM sodium fluoride, in the presence and absence of 2 μM cAMP. The incubation was carried out at 30°C for 10 min. The reaction was terminated by pipetting 50 μl aliquots of the reaction mixture on to filter paper discs (Whatman 3 mm, 2.3 cm diameter) which were immediately dropped into 10% ice cold trichloroacetic acid with 2.5% pyrophosphate. The filter papers were washed in 5% trichloroacetic acid with 2.5% pyrophosphate by a modified procedure suggested by Fiscus et al. (1984). This procedure included two hot washes at 90°C and two washes at room temperature for 15 min each. The final washes were in 95% ethanol and ether for 5 min each. Use of the hot washes resulted in very low blanks (usually 5 pmoles or less).

The filter papers were then dried and transferred into scintillation vials. These vials were filled with ACS scintillation fluid (5 ml) and the radioactivity counted in a MARK III scintillation counter. The cA kinase activity was expressed as pmoles phosphate transferred per mg protein per min. The extent of cA kinase activation was assessed by calculating the activity ratio, which is the ratio of kinase activity in the absence of added cAMP to that in the presence of enough cAMP to fully activate the enzyme.
6. **Protein Determination**

The concentration of protein in the supernatant and the pellets of the protein kinase experiments was determined by a modified micro Lowry assay method as reported by Peterson (1977), using bovine serum albumin as a standard. Briefly, the assay consists of two steps.

(a) **Precipitation Step**: Samples containing between 0.5-15.0 µg of protein were brought to a total volume of 1.5 ml with distilled water. To this, 12.5 µl of 2% (w/v) DOC was added, mixed and allowed to stand at room temperature for 10 min. Afterwards, 0.5 ml of 24% trichloroacetic acid was added, mixed and centrifuged at 2,500 rpm for 60 min. The supernatant was discarded and the pellet was subjected to micro-Lowry assay.

(b) **Micro Lowry Assay**: The procedure is the same as the Standard Lowry assay (Lowry et al., 1951) except that all quantities are reduced to give 0.5 - 15.0 µg of protein in a total volume of 660 µl.

To the pellet obtained in the precipitation step, 600 µl of freshly prepared copper reagent (500 µl each of 2% sodium, potassium tartrate and 1% copper sulphate added to 50 ml of 2% sodium carbonate in 0.1 N sodium hydroxide) was added, vortexed and allowed to stand at room temperature for 10 min. Then, 60 µl of diluted folin phenol reagent (1:1 dilution of 2N phenol reagent) was added and vortexed immediately. After 60 min, samples were read at 660 nm in microcuvettes using a Gilford spectrophotometer. A standard curve was run with each assay. A representative standard curve for the microassay using bovine serum albumin is shown in Fig. 3. The protein concentrations of the samples were read from the standard curve.
FIGURE 3: Standard curve for the micro Lowry protein assay using bovine serum albumin as a standard. Samples are processed through the DOC-TCA precipitation step prior to colour reaction as described in Methods.
7. Statistical Analyses

Values in the treated groups were compared to controls using an unpaired Students t-test. When comparisons were made between different treatment groups the data were further analyzed using ANOVA followed by Neuman-Keul's range test. A probability (P) of less than 0.05 was accepted as the level of statistical significance. At least one control tissue, in addition to several test tissues, were obtained from each animal, so that control and drug-treated tissues were obtained from the same population of animals. In the results, N represents the number of strips or rings used in these experiments. All the results were expressed as mean ± standard error of the mean (SEM). Approximately, 8 rings were obtained from each rabbit and 16 strips from each bovine heart.
RESULTS

A. Effects of Prostacyclin on Bovine Coronary Arteries

Time courses for elevation of cAMP and relaxation of potassium-contracted bovine coronary arteries by 30 μM PGI$_2$ are shown in Fig. 4. This concentration of PGI$_2$ was a maximally effective one with respect to relaxation in this tissue. A significant increase in cAMP levels was observed 30 seconds after addition of PGI$_2$ to the organ bath, at which time relaxation had not yet occurred. At 1 min after PGI$_2$, cAMP levels were further increased, and the muscles had now begun to relax. Maximum relaxation was usually obtained within 10 min, and cAMP levels had almost plateaued within that time.

The effects of various concentrations of PGI$_2$ on cAMP levels and tension in coronary arteries are shown in Table 2. PGI$_2$ (0.3-30.0 μM) increased cAMP levels and relaxed potassium-contracted arteries in a concentration-dependent manner.

B. Effects of Prostacyclin on Rabbit Aortic Rings

The effects of PGI$_2$ on rabbit aortic rings were studied in experiments analogous to those described above for bovine coronary arteries. Time courses for alteration of cAMP levels and tension of rabbit aortic rings by 30 μM PGI$_2$ are shown in Fig. 5. As was the case in the coronary arteries, PGI$_2$ produced a significant increase in cAMP
FIGURE 4: Effects of 30 µM PGI$_2$ on tension and cyclic AMP levels in potassium-contracted strips of bovine coronary artery. Tension (o) and cyclic AMP (●) are plotted as percentages of the values in potassium-contracted control muscles. Values represent means ± SEM of 5-8 experiments. Asterisks indicate values significantly different from control (p < 0.05).
## TABLE 2
EFFECTS OF PROSTACYCLIN (PGI₂) ON CYCLIC AMP LEVELS
AND TENSION IN POTASSIUM-CONTRACTED BOVINE CORONARY ARTERIES

<table>
<thead>
<tr>
<th>Treatment</th>
<th>N</th>
<th>Cyclic AMP (pmoles/g tissue)</th>
<th>% Relaxation of KCl Contraction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>15</td>
<td>163 ± 31</td>
<td>-</td>
</tr>
<tr>
<td>PGI₂ (0.3 μM, 10 min)</td>
<td>9</td>
<td>270 ± 46</td>
<td>12 ± 2*</td>
</tr>
<tr>
<td>PGI₂ (3 μM, 10 min)</td>
<td>11</td>
<td>352 ± 53*</td>
<td>42 ± 6*</td>
</tr>
<tr>
<td>PGI₂ (30 μM, 10 min)</td>
<td>12</td>
<td>356 ± 50*</td>
<td>48 ± 7*</td>
</tr>
</tbody>
</table>

All muscles were precontracted with 30 mM KCl as described in Methods. Control values represent cAMP levels in muscles frozen 20 min after addition of KCl. PGI₂-treated preparations were exposed to the indicated concentrations of PGI₂ for the last 10 min of the KCl treatment. Results represent means ± SEM of the number of experiments shown (N).

*Significantly different from KCl controls (p < 0.05).
FIGURE 5: Effects of 30 μM PGI₂ on tension and cyclic AMP levels in rabbit aortic rings. Tension (o) and cyclic AMP (●) are plotted as percentages of the values in control muscles. Values represent means ± SEM of 5-16 experiments. Asterisks indicate values significantly different from control (p < 0.05).
levels within 1 min after addition to the organ bath. Levels of the cyclic nucleotide were further increased at 5 and 10 min. In contrast to the results in bovine coronary arteries however, PGI$_2$ produced contractions of rabbit aortic rings under these conditions.

The effects of various concentrations of PGI$_2$ on cAMP levels and tension in rabbit aortic rings are shown in Table 3. Similar to the results in coronary artery, PGI$_2$ produced dose-dependent increases in cAMP levels in the rabbit aortic rings. However, these concentrations of the drug produced contractions of the aortic rings rather than relaxation as had been observed with the coronary arteries. Similarly, these concentrations of PGI$_2$ produced further contractions of rabbit aortic rings which were already submaximally contracted by 0.5 μM phenylephrine (PE) (data not shown).

Finally, the effects of isoproterenol on PGI$_2$-induced contractions and on cAMP levels in rabbit aortic rings are illustrated in Fig. 6. Ethanol, at the same concentration used to prepare the stock solutions of PGI$_2$, had no effect on tension or cAMP levels in these preparations. As previously shown in Table 3, 30 μM PGI$_2$ significantly elevated cAMP levels and contracted the aortic rings. In separate experiments, isoproterenol alone caused a time-dependent elevation of cAMP levels accompanied by relaxation of rabbit aortic rings (Fig. 7). For example, 1 μM isoproterenol significantly elevated cAMP levels (from 194 ± 21 to 274 ± 28 pmoles/g tissue, N = 7) and relaxed PE-contracted aortic rings by 46 ± 5% within 2 min after addition to the organ bath (also see Fig. 7). As illustrated in Fig. 6, that concentration of isoproterenol also
### TABLE 3
EFFECTS OF PROSTACYCLIN (PGI₂) ON CYCLIC AMP LEVELS AND TENSION IN RABBIT AORTIC RINGS

<table>
<thead>
<tr>
<th>Treatment</th>
<th>N</th>
<th>Cyclic AMP (pmoles/g tissue)</th>
<th>Change in Tension (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>8</td>
<td>204 ± 19</td>
<td></td>
</tr>
<tr>
<td>PGI₂ (0.3 μM, 10 min)</td>
<td>4</td>
<td>270 ± 28*</td>
<td>+ 0.6 ± 0.2*</td>
</tr>
<tr>
<td>PGI₂ (3 μM, 10 min)</td>
<td>7</td>
<td>579 ± 97*</td>
<td>+ 1.9 ± 0.4*</td>
</tr>
<tr>
<td>PGI₂ (30 μM, 10 min)</td>
<td>16</td>
<td>768 ± 131*</td>
<td>+ 3.8 ± 0.7*</td>
</tr>
</tbody>
</table>

*Significantly different from control (p < 0.05).
FIGURE 6: Effects of prostacyclin (PGI$_2$) and isoproterenol (ISO) on tension and cyclic AMP levels in rabbit aortic rings. Representative tracings for each experimental protocol are shown on the left. The double lines indicate points at which the tissues were frozen for cyclic AMP estimation. Cyclic AMP values represent means ± SEM for the number of experiments indicated in parentheses. Control values are the same as those used in the dose-response data shown in Table 3. Preparations were exposed to PGI$_2$ for 10 min and to ISO for 1 or 2 min. Asterisks indicate significant elevation of cyclic AMP from ethanol-treated controls and significant relaxation of PGI$_2$-contracted muscles (p < 0.05).
<table>
<thead>
<tr>
<th>ETHANOL (0.01%)</th>
<th>Cyclic AMP (pmol/g tissue)</th>
<th>% Relaxation Caused by ISO</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>204 ± 19 (8)</td>
<td>--</td>
</tr>
<tr>
<td>PGI2 (30μM)</td>
<td>768 ± 131 (16)*</td>
<td>--</td>
</tr>
<tr>
<td>PGI2 (30μM)</td>
<td>780 ± 125 (13)*</td>
<td>33 ± 5*</td>
</tr>
<tr>
<td>ISO (1μM)</td>
<td>780 ± 162 (13)*</td>
<td>44 ± 7*</td>
</tr>
</tbody>
</table>
relaxed PGI₂-contracted aortic rings to a similar extent. However, when cAMP levels were estimated 1 and 2 min after addition of isoproterenol (i.e., during isoproterenol-induced relaxation), no further elevation of total cAMP levels (p > 0.05, ANOVA followed by Neuman-Keul's test) was observed beyond that produced by 30 μM PGI₂ alone (Fig. 6). Similar results were obtained when the effects of 1 μM isoproterenol were studied against contractions induced by a lower concentration (3 μM) of PGI₂ (data not shown).

C. Effects of Isoproterenol on Cyclic AMP Levels and Tension in Rabbit Aortic Rings

Time courses for elevation of cAMP and relaxation of rabbit aortic rings by 1 μM isoproterenol are shown in Fig. 7. Aortic rings were pre-contracted with 0.5 μM PE before addition of the isoproterenol. The 1 μM concentration of isoproterenol was a maximally effective one with respect to relaxation in these preparations. At 15 sec after addition of isoproterenol to the muscle bath, a small elevation of cyclic AMP levels were observed, but this was not statistically significant. The muscles had not yet begun to relax at this time point. However, at 1 and 2 min after addition of isoproterenol, cAMP levels were significantly increased and this was accompanied by relaxation of the aortic rings. Maximal relaxation and cAMP elevation had occurred within 2 min.
FIGURE 7: Time course for cyclic AMP elevation and relaxation of PE-contracted rabbit aortic rings by 1 μM isoproterenol. Cyclic AMP levels and tension were measured in the same aortic rings as described in Methods. Values represent means ± SEM for eight experiments. Stars indicate values significantly different from the corresponding zero time controls (p < 0.05).
D. Effects of PGE₁ on Cyclic AMP Levels and Tension in Rabbit Aortic Rings

As shown in Table 4, PGE₁ produced an increase in cAMP levels in rabbit aortic rings. However, in contrast to the results with isoproterenol, PGE₁ caused contraction of the aortic rings rather than relaxation. The higher concentration of PGE₁ (10 µM) significantly increased cAMP levels within 1 min after addition to the muscle bath, which was the earliest time point at which muscle contraction could be detected. Cyclic AMP levels had further increased at the 10 min time point, and the maximum contraction had occurred within that time.

E. Effect of Isoproterenol on Contractions of Rabbit Aortic Rings Induced by Various Agonists

The relaxant effect of isoproterenol was first studied against PE-induced contractions of rabbit aortic rings. A cumulative dose-response relationship for PE on this tissue is shown in Fig. 8. When a maximally relaxant concentration of isoproterenol (1 µM) was added 1 min prior to PE, it produced a shift of the dose response curve to the right.

Representative tracings of the effects of isoproterenol on PE-, PGE₁-, and KCl-induced contractions are shown in Fig. 9. All three agents produced sustained contractions. In the case of PGE₁ and KCl studies, tissues were pretreated with 10 µM phentolamine. Maximum relaxation of PE-, PGE₁-, and KCl-contracted tissues was observed within
TABLE 4
EFFECTS OF PROSTAGLANDIN E₁ (PGE₁) ON CYCLIC AMP LEVELS
AND TENSION IN RABBIT AORTIC RINGS

<table>
<thead>
<tr>
<th>Treatment</th>
<th>N</th>
<th>Cyclic AMP (pmoles/g tissue)</th>
<th>Change in Tension (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>8</td>
<td>204 ± 19</td>
<td></td>
</tr>
<tr>
<td>PGE₁ (1 μM, 10 min)</td>
<td>5</td>
<td>540 ± 66*</td>
<td>+ 0.24 ± 0.03</td>
</tr>
<tr>
<td>PGE₁ (10 μM, 1 min)</td>
<td>5</td>
<td>333 ± 46*</td>
<td>+ 0.30 ± 0.10</td>
</tr>
<tr>
<td>PGE₁ (10 μM, 10 min)</td>
<td>10</td>
<td>833 ± 158*</td>
<td>+ 2.00 ± 0.30</td>
</tr>
</tbody>
</table>

*Significantly different from control (p < 0.05).
FIGURE 8: Effect of isoproterenol on the cumulative dose response relationship of PE in rabbit aortic rings. Preparations were exposed to cumulative doses of PE, washed and then recontracted in the presence of isoproterenol. Responses in the absence (▲—▲) and in the presence of (■...■) of 1 μM isoproterenol are shown. Each point represents the mean of two experiments.
FIGURE 9: Representative tracings of the effect of isoproterenol on PE-, PGE₁- and KCl-induced contractions of rabbit aortic rings. Tissues were pretreated with 10 μM phentolamine 10 min prior to contraction in the case of PGE₁- and KCl-induced contractions.
PE (0.5μM) 20 min in ISO (1μM)
PGE1 (10μM) 20 min
KCL (145mM) 20 min
2 min after addition of 1 μM isoproterenol. The relaxation pattern appears to be transient and similar with all three contractile agonists. Tension returned to control level within 10 min after addition of isoproterenol.

Finally, the effect of different concentrations of isoproterenol was studied on PE- and PGE1-induced contraction of rabbit aortic rings. As shown in Fig. 10, isoproterenol in a dose-dependent manner relaxed the aortic rings. Also the percentage of relaxation appears to be the same whether the tissue is contracted by PE or PGE1. Prior treatment of the tissues with 1 μM propranolol abolished the isoproterenol-induced relaxation in both cases.

F. Effect of Dibutyryl Cyclic AMP on PE- and PGE1-induced Contraction of Rabbit Aortic Rings

Dibutyryl cAMP (db-cAMP), a lipid soluble analogue of cAMP has been suggested to produce relaxation in vascular smooth muscle by a cAMP-dependent mechanism. The effect of this agent on PE- and PGE1-induced contraction is shown in Fig. 11. Dibutyryl cAMP produced a dose-dependent relaxation of PE- and PGE1-induced contraction. Maximum relaxation was observed 20 min after addition of high concentrations of db-cAMP. The relaxation appears to be slow compared to that caused by isoproterenol where maximum relaxation was observed within 2 min (see Fig. 9). This could be due to the time required for db-cAMP to enter the cell. At higher concentrations of db-cAMP (> 500 μM), the percentage relaxation is the same against both PE- and PGE1-induced
FIGURE 10: Effects of isoproterenol on PE- and PGE₁-induced contractions of rabbit aortic rings. Aortic rings were incubated as described in the Methods, contracted with 0.5 μM PE or 10 μM PGE₁, and isoproterenol was added for 2 min. Values represent means ± SEM of 10-16 experiments.
FIGURE 11: Effect of db-cAMP on PE- and PGE₁-induced contractions in rabbit aortic rings. Dibutyryl cAMP was added for 20 min to rings pre-contracted with 0.5 μM PE or 10 μM PGE₁. After completion of one concentration of db-cAMP, rings were washed, and experiments repeated in the same manner for the other concentrations. Values represent mean ± S.E. of 6-9 experiments. Asterisks indicate significant difference from PE controls (p < 0.05).
- 64 -

% RELAXATION

DIBUTYRYL cAMP (mM)

PE (0.5μM)
PGE1 (10μM)
contractions (comparable to isoproterenol effect in Fig. 10). However at lower concentrations, the percentage relaxation of db-cAMP on PGE$_1$-induced contraction is significantly greater than on PE-induced contractions. This might actually reflect the slower rate of relaxation of PE contractions (compared to PGE$_1$ contractions) at lower concentrations of db-cAMP. The relaxing effect could be due to the toxic effect of butyrate ion rather than to cAMP itself (Buibring and Hardman 1976). To check this possibility the effect of sodium butyrate on the rabbit aortic rings was studied. No change in tension was observed when 1 mM sodium butyrate was added to PE- and PGE$_1$-contracted rings (data not shown).

G. Effects of Isoproterenol on PE- and PGE$_1$-induced Contractions and on Cyclic AMP Levels in Rabbit Aortic Rings

The effects of 1 µM isoproterenol on PE- and PGE$_1$-induced contractions and on cAMP levels in aortic rings are shown in Fig. 12. No change in the basal level of cAMP was observed in PE-contracted arteries when compared to the relaxed controls (see Table 4). At 2 min after addition of isoproterenol, PE-contracted aortic rings were relaxed by about 46%. When added to relaxed muscles, PGE$_1$ alone significantly elevated cAMP levels (4.5 fold) and contracted the muscles. Finally, the effect of isoproterenol on PGE$_1$-induced contraction and on cAMP levels was studied. Isoproterenol relaxed the PGE$_1$-contracted aortic rings with no further change in total cAMP levels, (p > 0.05, ANOVA followed by Neuman-Keul's test) x, which were already markedly elevated
FIGURE 12: Effects of isoproterenol (ISO) on PE- and PGE₁-induced contractions and on cyclic AMP levels in rabbit aortic rings. Cyclic AMP levels and tension were measured in the same aortic rings as described in Methods. Representative tracings for each experimental protocol are shown on the left hand side of the figure. Aortic rings were frozen at the points indicated by the double lines. Cyclic AMP levels in these preparations are given in the middle column (values represent means ± SEM of the number of experiments indicated in parentheses). Preparations were exposed to PE for 15 min, PGE₁ for 10 min and ISO for 2 min. Stars indicate significant elevation of cyclic AMP from PE controls and significant relaxation of PE- and PGE₁-contracted muscles (p < 0.05).
REPRESENTATIVE TRACING

<table>
<thead>
<tr>
<th>CYCLIC AMP (pmol/g tissue)</th>
<th>% RELAXATION CAUSED BY ISO</th>
</tr>
</thead>
<tbody>
<tr>
<td>194 ± 21 (5)</td>
<td>—</td>
</tr>
<tr>
<td>274 ± 28 (7)*</td>
<td>46 ± 5*</td>
</tr>
<tr>
<td>926 ± 91 (25)*</td>
<td>—</td>
</tr>
<tr>
<td>1044 ± 71 (18)*</td>
<td>40 ± 6*</td>
</tr>
</tbody>
</table>
by the PGE₁. It should be noted that isoproterenol relaxed the PGE₁-contracted tissues to approximately the same extent as it did the PE-contracted aortic rings (also see Fig. 10).

In another set of experiments, cGMP levels were estimated in rabbit aortic rings under conditions analogous to those described above. As shown in Table 5, no significant changes in cGMP levels were observed under any of these conditions.

H. Effects of Forskolin and PGE₁ on Cyclic AMP Levels and Tension in Phenylephrine-contracted Rabbit Aortic Rings

The effects of forskolin, and its interactions with PGE₁ and isoproterenol, on cAMP levels and tension in PE-contracted aortic rings are shown in Fig. 13. As reported by others (Pfaffman and Chu-Sun, 1979; Wheeler and Weiss, 1980), addition of PGE₁ to the PE-contracted muscles elicited a further increase in tension. This was accompanied by significant elevation of cAMP levels. The effects of forskolin on PE-induced contractions and on cyclic AMP levels are also shown in Fig. 12. Significant muscle relaxation could be detected with concentrations of forskolin as low as 0.1 μM. As shown in Fig. 12, 0.1 μM forskolin produced only a small elevation of cAMP which was not statistically significant. It has been previously demonstrated that low doses of forskolin, which produce small elevations of cAMP, can potentiate hormone-induced elevation of cAMP as well as cAMP-mediated physiological responses in several tissues (Seamon and Daly, 1981b). The effects of PGE₁ on cAMP levels and tension were therefore examined in the presence
TABLE 5

EFFECTS OF PHENYLEPHRINE (PE), PROSTAGLANDINE E\textsubscript{1} (PGE\textsubscript{1})
AND ISOPROTERENOL (ISO) ON CYCLIC GMP LEVELS
IN RABBIT AORTIC RINGS

<table>
<thead>
<tr>
<th>Treatment</th>
<th>N</th>
<th>Cyclic GMP (pmoles/g tissue)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>5</td>
<td>4.8 ± 0.9</td>
</tr>
<tr>
<td>PE Control (0.5 μM, 15 min)</td>
<td>12</td>
<td>3.5 ± 0.6</td>
</tr>
<tr>
<td>PE + ISO (1 μM, 2 min)</td>
<td>4</td>
<td>4.0 ± 0.5</td>
</tr>
<tr>
<td>PGE\textsubscript{1} (10 μM, 10 min)</td>
<td>4</td>
<td>5.7 ± 1.6</td>
</tr>
<tr>
<td>PGE\textsubscript{1} (10 μM, 10 min) + ISO (1 μM, last 2 min)</td>
<td>4</td>
<td>4.9 ± 0.3</td>
</tr>
</tbody>
</table>
FIGURE 13: Effects of prostaglandin E₁ (PGE₁), forskolin (FORSK) and isoproterenol (ISO) on phenylephrine (PE)-induced contractions and on cyclic AMP levels in rabbit aortic rings. Representative tracings for this series of experiments are shown on the left. The double lines indicate points at which the tissues were frozen for cyclic AMP estimation. Cyclic AMP values represent means ± SEM for the number of experiments indicated in parentheses. Values for tension indicate percent change from PE control at the time the muscles were frozen. Preparations were exposed to FORSK for 15 min, PGE₁ for 10 min and ISO for 2 min. Stars indicate values significantly different from the PE controls (p < 0.05).
REPRESENTATIVE TRACING

<table>
<thead>
<tr>
<th>CYCLIC AMP (pmol/g tissue)</th>
<th>TENSION (% change from PE control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>206 ± 14 (9)</td>
<td>-</td>
</tr>
<tr>
<td>549 ± 130 (5)*</td>
<td>+43 ± 7*</td>
</tr>
<tr>
<td>237 ± 16 (5)</td>
<td>-32 ± 1*</td>
</tr>
<tr>
<td>3364 ± 659 (6)*</td>
<td>+20 ± 1*</td>
</tr>
<tr>
<td>422 ± 62 (5)*</td>
<td>-94 ± 3*</td>
</tr>
<tr>
<td>6666 ± 1164 (5)*</td>
<td>-30 ± 9*</td>
</tr>
<tr>
<td>5360 ± 742 (5)*</td>
<td>-72 ± 3*</td>
</tr>
</tbody>
</table>

Legend:
- PE (0.5μM)
- PGE1 (10μM)
- FORSK (0.1μM)
- FORSK (1μM)
- ISO (1μM)
of 0.1 μM forskolin. Consistent with the earlier reports, 0.1 μM forskolin markedly potentiated the PGE₁-induced elevation of cAMP in rabbit aortic rings. The magnitude of the cAMP increase caused by a combination of 10 μM PGE₁ and 0.1 μM forskolin was much greater (15 fold) than the sum of the increases caused by either agent alone. However, even under these conditions, PGE₁ produced a contraction of the aortic rings. A higher concentration (1 μM) of forskolin alone significantly elevated cAMP levels and this was accompanied by almost complete relaxation of the aortic rings. In the presence of this higher concentration of forskolin, PGE₁ produced an even more marked elevation of cAMP levels (30 fold), but this was still accompanied by contraction (i.e., PGE₁ partly reversed the forskolin-induced relaxation). Finally, the effect of isoproterenol on forskolin-treated, PGE₁-contracted muscles was studied. Isoproterenol relaxed these muscles with no further change in total cAMP levels compared to the corresponding forskolin-PGE₁ combination alone (p > 0.05, ANOVA followed by Neuman-Keul's test).

In some experiments, the effects of forskolin and isoproterenol were studied on cAMP levels and tension of tissues contracted with PGE₁ alone. As shown in Table 6, forskolin and forskolin plus isoproterenol were able to partially relax the PGE₁-contracted aortic rings. However, very high tissue levels of cAMP were observed under these conditions.

Thus there does not appear to be a good correlation between changes in cAMP levels and changes in tension in rabbit aorta under various conditions.
TABLE 6
EFFECTS OF FORSKOLIN ON PGE₁-INDUCED CONTRACTION AND ON CYCLIC AMP LEVELS IN RABBIT AORTIC RINGS

<table>
<thead>
<tr>
<th>Treatment</th>
<th>N</th>
<th>Cyclic AMP (pmoles/g tissue)</th>
<th>% Relaxation of PGE₁ Contraction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>8</td>
<td>204 ± 19</td>
<td></td>
</tr>
<tr>
<td>PGE₁ (10 μM) 25'</td>
<td>3</td>
<td>411 ± 30*</td>
<td></td>
</tr>
<tr>
<td>PGE₁ (10 μM) 25' + Forsk (1 μM) last 15'</td>
<td>7</td>
<td>5,509 ± 987*</td>
<td>68 ± 10</td>
</tr>
<tr>
<td>PGE₁ (10 μM) 25' + Forsk (1 μM) 15' + ISO (1 μM) last 2'</td>
<td>3</td>
<td>6,288 ± 196*</td>
<td>75 ± 6</td>
</tr>
</tbody>
</table>

*Significantly different from control (p < 0.05).
I. Effects of Isoproterenol, Prostaglandin E₁ and Forskolin on Cyclic AMP-dependent Protein Kinase (cA kinase) Activity in the Soluble and Particulate Fractions of Rabbit Aortic Rings

In these experiments, tissue samples were prepared analogous to the conditions shown in Fig. 13, and protein kinase activity in the supernatant and particulate fraction were estimated as discussed in the Methods. The results obtained in these experiments are shown in Table 7 and expressed in terms of specific activity and activity ratio of the cA kinase.

Isoproterenol relaxed the PE-contracted aortic rings and also produced a small but significant change in cAMP levels (see Fig 7). When the cA kinase activity was estimated in the supernatant fraction, there was no significant difference in the basal activity (activity estimated in the absence of added cAMP in the assay medium), total activity (activity estimated in the presence of 2 µM cAMP in the assay medium) or activity ratio of the tissues treated with isoproterenol compared to the control. PGE₁ and forskolin activated the protein kinase (a significant increase was seen in the basal activity as well as activity ratio). However, PGE₁ contracted the aortic rings whereas forskolin relaxed the PE-contracted aortic rings. Also isoproterenol relaxed the PGE₁-contracted rings without further significant change in cA kinase activity, which was already activated by PGE₁ alone. Changes in the activity ratios observed above are due to changes in catalytic activity (-cAMP) rather than increases in total holoenzyme activity (+ 2 µM cAMP). This change in catalytic activity reflects the activation of
TABLE 7

EFFECTS OF ISOPROTERENOL (ISO), PROSTAGLANDIN E₁ (PGE₁) AND FORSKOLIN (FORSK) ON SOLUBLE AND PARTICULATE CYCLIC AMP-DEPENDENT PROTEIN KINASE ACTIVITY IN RABBIT AORTIC RINGS.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>N</th>
<th>Protein Kinase Activity (p moles/mg protein/min)</th>
<th>SUPERNATANT</th>
<th>PELLET</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>-cAMP</td>
<td>+2 µM cAMP</td>
</tr>
<tr>
<td>Control</td>
<td>12</td>
<td>133 ± 11</td>
<td>727 ± 63</td>
<td>0.18 ± 0.01</td>
</tr>
<tr>
<td>PE Control (0.5 µM)</td>
<td>12</td>
<td>152 ± 10</td>
<td>781 ± 60</td>
<td>0.20 ± 0.01</td>
</tr>
<tr>
<td>PE + ISO (1 µM) 2'</td>
<td>5</td>
<td>183 ± 9</td>
<td>912 ± 64</td>
<td>0.20 ± 0.01</td>
</tr>
<tr>
<td>PGE₁ (10 µM) 10'</td>
<td>6</td>
<td>240 ± 20*</td>
<td>902 ± 67</td>
<td>0.26 ± 0.02*</td>
</tr>
<tr>
<td>PGE₁ (10 µM) + ISO (1 µM) 2'</td>
<td>6</td>
<td>184 ± 17*</td>
<td>813 ± 82</td>
<td>0.23 ± 0.01*</td>
</tr>
<tr>
<td>PE + Forsk (1 µM) 15'</td>
<td>5</td>
<td>202 ± 22*</td>
<td>759 ± 39</td>
<td>0.25 ± 0.01*</td>
</tr>
<tr>
<td>PE + Forsk (1 µM) + PGE₁ (10 µM) 10'</td>
<td>6</td>
<td>338 ± 17*</td>
<td>557 ± 17*</td>
<td>0.66 ± 0.02*</td>
</tr>
<tr>
<td>PE + Forsk (1 µM) + PGE₁ (10 µM) + ISO (1 µM) 2'</td>
<td>5</td>
<td>346 ± 35*</td>
<td>489 ± 51*</td>
<td>0.71 ± 0.01*</td>
</tr>
</tbody>
</table>

NOTE: Results are means ±SEM of the number of experiments indicated (N). Details of the experiments are similar to figure legend 9. Asterisks indicate significant differences from corresponding controls (p < 0.05).
cA kinase in the tissue. In the presence of forskolin, PGE₁ produced very large increases in protein kinase activity. But PGE₁ contracted the preparations under these conditions (see Fig. 13). Isoproterenol was able to relax these forskolin-treated, PGE₁-contracted muscles with no further increase in cA kinase activity (p > 0.05, ANOVA followed by Neuman–Keuls test). Thus the protein kinase activity estimated in the supernatant fraction appears to be very consistent with the cAMP data obtained under similar conditions (see Fig. 13).

It can be seen from the left hand panel of Table 7 that drugs which caused large increases in tissue cyclic AMP levels also caused decreases in total holoenzyme activity. This decreased activity in the supernatant may be due to a shift or translocation of the enzyme from the soluble to the particulate fraction. Such a phenomenon was observed in bovine coronary arteries treated with forskolin and isoproterenol (Vegesna and Diamond, 1984), suggesting that translocation of the enzyme to a membrane fraction might have occurred in this tissue as well. It was hoped that such a trend might possibly explain the differences in pharmacological effects observed in the rabbit aortic rings.

To further investigate this possibility, cA kinase activity was estimated in the particulate fractions obtained in the above studies as described in Methods. The results are shown in the right-hand panel of Table 7. In the case of the isoproterenol and PGE₁ studies, there appeared to be an increase in basal kinase activity (not significant) and total kinase activity (significant) in the particulate fractions. Significant increases in particulate kinase activity were observed with
forskolin and also with the combination of forskolin, PGE<sub>1</sub> and isoproterenol. These results indicate that activation of the cA kinase occurred in the particulate fractions under some conditions whether the tissues were relaxed or contracted. In other words, the changes in the particulate kinase activity in the rabbit aortic rings do not explain the differences in pharmacological response to these agents.

F. Characterization of Contractile Responses of PE and PGE<sub>1</sub> and the Effect of Isoproterenol in Rabbit Aortic Rings

The rabbit aortic rings were contracted with PE and PGE<sub>1</sub>. The contribution of intracellular and extracellular Ca<sup>2+</sup> to the PE and PGE<sub>1</sub> contraction was examined using zero Ca<sup>2+</sup> buffer containing EGTA and a Ca<sup>2+</sup> antagonist (D-600).

As shown in Fig. 14, when Ca<sup>2+</sup>-free Krebs bicarbonate buffer containing 5 mM EGTA was substituted for the normal physiological buffer (with 1.26 mM Ca<sup>2+</sup>), PE produced a phasic contraction of the aortic rings. Calcium antagonists such as D-600, are known to block entry of extracellular Ca<sup>2+</sup>. In preliminary experiments, 1 μM D-600 inhibited 90% of the 145 mM KCl-induced contraction of the aortic rings (data not shown). In order to further rule out the possibility that extracellular Ca<sup>2+</sup> entry is contributing to the PE-induced phasic contraction, 1 μM D-600 was added in addition to zero Ca<sup>2+</sup> and EGTA. As shown in Fig. 14, even under these conditions, PE produced a phasic contraction. When 1 μM isoproterenol was added prior to the addition of PE, complete inhibition of the phasic contraction was observed. These results
FIGURE 14: Effects of reduced calcium concentration and isoproterenol on the contractile response in rabbit aortic rings to 0.5 μM PE. All rings were initially contracted after an equilibration period of at least 2 hr in the Krebs bicarbonate buffer. After the maximum contractile tension was developed, the rings were washed with fresh buffer until basal tension was restored. At this point various manipulations were performed. A, effect of normal Krebs bicarbonate buffer on contraction to 0.5 μM PE; B, effect of Ca\(^{++}\)-free Krebs plus 5 mM EGTA on contraction to 0.5 μM PE; C, effect of Ca\(^{++}\)-free, 5 mM EGTA Krebs plus 1 μM D-600 on contraction to 0.5 μM PE; D, effect of prior treatment with 1 μM isoproterenol on 0.5 μM PE-induced contraction in Ca\(^{++}\)-free Krebs plus 5 mM EGTA. In these experiments, tissues were incubated in Ca\(^{++}\)-free, EGTA buffer and D-600 for 10 min prior to the treatment with PE.
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- INCUBATION MEDIUM -

1.26mM Ca

0 Ca
EGTA (5mM)

0 Ca
EGTA (5mM)
D-600 (1μM)

0 Ca
EGTA (5mM)

PE (0.5μM)

PE (0.5μM)

PE (0.5μM)

ISO PE (0.5μM)
(1μM)

5min
suggest that isoproterenol may be exerting its effects in rabbit aortic rings by altering intracellular Ca\(^{++}\) mobilization.

Similarly, the effect of PGE\(_1\) was studied on rabbit aortic rings incubated in Ca\(^{++}\) free, EGTA buffer containing 1 \(\mu\)M D-600. As shown in Fig. 15, PGE\(_1\) still produced a sustained contraction of rabbit aortic rings. Again isoproterenol was able to relax these tissues contracted with PGE\(_1\) in the absence of extracellular calcium.
FIGURE 15: Effects of reduced calcium concentration and isoproterenol on the contractile response in rabbit aortic rings to 10 μM PGE₁. All rings were initially contracted after an equilibration period of at least 2 hr in the Krebs bicarbonate buffer. After the maximum contractile tension was developed, the rings were washed with fresh buffer until basal tension was restored. At this point various manipulations were performed. A, effect of normal Krebs bicarbonate buffer on contraction to 10 μM PGE₁; B, effect of Ca⁺⁺-free 5 mM EGTA Krebs plus 1 μM D-600 on contraction to 10 μM PGE₁; C, effect of 1 μM isoproterenol on 10 μM PGE₁ induced contraction in Ca⁺⁺-free, 5 mM EGTA containing buffer with 1 μM D-600. In these experiments, tissues were incubated in Ca⁺⁺-free, EGTA buffer and D-600 for 10 min prior to the treatment with PGE₁.
INCUBATION MEDIUM

1.26 mM Ca

0 Ca
EGTA (5 mM)
D-600 (1 μM)

0 Ca
EGTA (5 mM)
D-600 (1 μM)

PGE1 (10 μM)

ISO (1 μM)
DISCUSSION

In the present study, PGI$_2$ elevated cAMP levels and produced relaxation of potassium-contracted helical strips of bovine coronary artery. This agrees with previous reports in the literature (Kukovetz et al., 1979, Holzman et al., 1980). As was the case in the earlier reports, cAMP levels were elevated by PGI$_2$ even in the absence of phosphodiesterase inhibitors, and cAMP elevation appeared to be correlated with relaxation in both a time-dependent and dose-dependent manner. Our results in bovine coronary arteries therefore confirm the previous results of Kukovetz and coworkers (1979) and tend to support their conclusion that PGI$_2$-induced relaxation of bovine coronary arteries is mediated by elevation of cAMP.

As noted in the introduction, one of the earlier studies in bovine coronary artery with PGI$_2$ reported a decrease in cAMP levels (Schrör and Rösen, 1979). Other workers reported an increase in cAMP levels but only in the presence of phosphodiesterase inhibitors (Dembinska-Kiec et al., 1979, Miller et al., 1979). These results differed from those reported by Kukovetz et al. (1979) and from the results found in the present study. The reason for these differences is not entirely clear. However, there appear to be major differences in the experimental procedures used by the various groups. For example, Schrör and Rösen (1979) did not freeze clamp the bovine coronary artery rings used for
cAMP determination and it is possible that slow freezing could alter intracellular cAMP levels. There are also differences in the incubation conditions in some of these experiments. Many of these studies lack data on the time courses of the effects of PGI$_2$ on cAMP levels. Even in those experiments in which complete time- and dose-response studies were reported, mechanical response and cAMP were estimated in separate tissues (Kukovetz et al., 1979). In the present experiments, the tissues were clamp frozen while tension was being monitored and cAMP levels were determined in the same preparations used for tension measurements. In our opinion this approach is more likely to give accurate results than the techniques used in the previous studies.

Similar to the results in bovine coronary artery, PGI$_2$ also elevated cAMP levels in rabbit aortic rings in a time- and dose-dependent manner. However, in direct contrast to the results in the coronary arteries, PGI$_2$ produced contractions of the aortic rings rather than relaxation. In the present experiments, the correlation between cAMP elevation and contraction in rabbit aortic rings appeared to be as good as that between cAMP elevation and relaxation in the bovine coronary arteries. Taken together, these results demonstrate a dissociation between elevation of cAMP and relaxation in at least one type of vascular smooth muscle.

In the next series of experiments, the effects of another prostaglandin, PGE$_1$ and of isoproterenol and forskolin on cAMP levels and tension were studied in rabbit aortic rings to further determine whether a correlation exists between elevation of cAMP and tension in this tissue.
In agreement with previous reports (Meisneri and Van Breemen, 1982), isoproterenol elevated cAMP levels and caused relaxation of rabbit aortic rings. The cAMP elevation appeared to be correlated with relaxation in a time-dependent manner (Fig. 7). Furthermore, as predicted from the ability of prostaglandins to activate adenylate cyclase obtained from rabbit aortic smooth muscle cells (Oliva et al., 1984), PGE₁ markedly increased cAMP levels in intact rings of rabbit aorta. However, in contrast to the results obtained with isoproterenol, PGE₁ caused contraction of the aortic rings rather than relaxation. For example, a 1.5 fold elevation of cAMP caused by 1 μM isoproterenol was accompanied by a 46% relaxation of the aortic rings, whereas a 4.5 fold elevation of cAMP caused by 10 μM PGE₁ was accompanied by contraction of the muscles (see Fig. 12). These results are similar to the effect of PGI₂ in rabbit aortic rings discussed previously.

A similar dissociation between elevation of cAMP and tension has previously been reported with PGE₁ (Harbon et al., 1978) and PGI₂ (Vesin et al., 1979) in another tissue, the rat myometrium. In that tissue, 1 μM epinephrine increased cAMP levels and relaxed the uterus, whereas 10 μM PGE₁ elevated cAMP levels to a similar extent but contracted the myometrium. Thus the present results in vascular smooth muscle are analogous to the earlier observations in a nonvascular smooth muscle.

The contractile effect of PGI₂ and PGE₁ in rabbit aortic rings does not appear to involve release of other endogenous prostaglandins, since the presence of cyclooxygenase inhibitors such as indomethacin, suprofen and meclofenamic acid did not change the tension response to PGI₂ and
PGE₁ in this preparation (Hadhazy et al., 1984; Forstermann et al., 1984). Endogenous release of norepinephrine can be excluded as the mechanism of PGE₁ action, since phentolamine does not block the direct contractile effect of PGE₁ in rabbit aortic preparations (Wheeler and Weiss, 1980; see also Fig. 9). Relaxing factors derived from endothelium (Furchgott and Zawadzki, 1980) did not contribute to the effects of PGI₂ in either of the blood vessels, or those of PGE₁ in the rabbit aortic rings used in our studies because the endothelium was intentionally removed from both preparations (see Methods). Thus the contraction observed in the rabbit aortic rings appears to be a direct action of PGE₁ and PGI₂ on the smooth muscle cells themselves. Furthermore, cGMP does not seem to be playing a role in these responses, since no changes in the level of that cyclic nucleotide were observed under any of these conditions (see Table 5).

If cAMP elevation normally mediates vascular smooth muscle relaxation, which would be consistent with our observations in bovine coronary arteries, then some explanation must be provided for our observation that PGE₁- and PGI₂-induced elevation of cAMP in rabbit aortic rings is accompanied by contraction rather than relaxation. It can be argued that prostaglandins may have several independent actions in rabbit aortic rings. In addition to activating adenylate cyclase and increasing cAMP levels, prostaglandins may induce contraction of the muscles via other mechanisms such as enhancing calcium influx or promoting release of intracellular calcium (Wheeler and Weiss, 1980). Assuming that cAMP elevation normally leads to relaxation of smooth
muscles, the cAMP elevation caused by PGE$_1$ and PGI$_2$ in rabbit aorta must not be sufficient to overcome the contractile effects of the drugs in this tissue. This would suggest that prostaglandin-induced contractions are more resistant to cAMP-dependent relaxation than phenylephrine (PE)-induced contractions, since isoproterenol was able to relax PE-contracted muscles with only small elevations of cAMP. In order to test this possibility, we studied the effect of isoproterenol and db-cAMP on the mechanical response of PE- and PGE$_1$-induced contractions in rabbit aortic rings. As shown in Fig. 9, the relaxation pattern of isoproterenol appears to be the same whether the tissues were contracted with PE, PGE$_1$ or even with high KCl. Different concentrations of isoproterenol also relaxed PE- and PGE$_1$-induced contractions equally (Fig. 10). Finally, db-cAMP, a lipid soluble analogue of cAMP, is as effective against PGE$_1$-induced contractions as it was against PE-induced contraction (Fig. 11). The ability of db-cAMP to relax these tissues suggests that cAMP-dependent relaxation mechanisms may exist in the rabbit aorta and that they are capable of relaxing prostaglandin-induced contractions. The question remains, however, as to why the marked elevations of cAMP caused by the prostaglandins themselves are incapable of relaxing the muscles.

As indicated earlier, if we assume that isoproterenol generally exerts its relaxant effects in vascular smooth muscles by virtue of its ability to increase tissue levels of cAMP, then isoproterenol would be expected to produce an additive effect in the cAMP response when combined with prostaglandins in rabbit aortic rings. However, as
shown in the results (Figs. 6 and 12), isoproterenol relaxed both \( \text{PGI}_2 \) and \( \text{PGE}_1 \) contracted muscles with no further elevation of cAMP beyond that caused by \( \text{PGI}_2 \) and \( \text{PGE}_1 \) alone.

The apparent dissociations observed in the present experiments between cAMP levels and tension in rabbit aortic rings might be explained in several ways. First, isoproterenol may relax rabbit aortic rings by a cAMP-independent mechanism. The ability of isoproterenol to relax smooth muscles via a cAMP-independent mechanism has been suggested by previous results in a nonvascular smooth muscle, the rabbit myometrium (Nesheim et al., 1975; Marshall and Fain, 1985). In both of these reports, isoproterenol was capable of relaxing the myometrial preparations at concentrations which had no effect on cAMP levels in the muscles. Alternatively, prostaglandins and isoproterenol may elevate cAMP levels in different pools or compartments in the rabbit aorta. It would have to be further assumed that only the isoproterenol-sensitive pool can affect the contractility of the muscle and that this pool is small relative to the prostaglandin-sensitive pool. With the total tissue measurements of cAMP used in this study, small changes in a specific pool affected by isoproterenol might not be detected in the presence of large elevations of cAMP in the prostaglandin-sensitive pool.

The experiments with forskolin further indicate that the inability of the prostaglandin-elevated cAMP to relax prostaglandin contractions is not simply due to the fact that the cAMP levels are not high enough.
Forskolin has been known to potentiate prostaglandin-induced elevation of cAMP levels in several tissues (Seamon and Daly, 1981b). We decided to use forskolin as a tool to test the possibility that PGE₁-induced elevation of total tissue levels of cAMP might be below the threshold for regulation of tension in this tissue.

Consistent with reports in other vascular smooth muscle preparations (Lincoln and Simpson, 1983; Vegesna and Diamond, 1983; Jones et al., 1984), forskolin caused relaxation and cAMP elevation in rabbit aortic rings. However, cAMP elevation and relaxation were not always well correlated at lower concentrations of the drug. In previous studies on bovine coronary arteries, cAMP levels were markedly elevated (5.5 fold) by concentrations of forskolin which did not relax the arteries (Vegesna and Diamond, 1983) and in the present experiments, 0.1 µM forskolin relaxed the rabbit aortic rings with no significant elevation of cAMP (Fig. 13). In agreement with previous reports that forskolin potentiates hormone-induced elevation of cAMP levels, in the present experiments 0.1 or 1 µM forskolin markedly potentiated the cAMP elevating effect of PGE₁ in rabbit aortic rings (Fig. 13). In the presence of 1 µM forskolin, 10 µM PGE₁ produced a 30-fold elevation of cAMP. However, even under these conditions, PGE₁ caused a contractile response in the aortic rings. Once again, isoproterenol was able to relax these preparations with no further elevation of cAMP. These results indicate that the magnitude of the increase in tissue levels of cAMP produced by PGE₁ is not responsible for its inability to relax rabbit aorta.
Our results with the forskolin-PGE\textsubscript{1} combination study further demonstrate a clear dissociation between total tissue level of cAMP and tension in rabbit aortic rings under various conditions. One possible explanation for these observations would be to assume that cAMP does not play an important role in the control of vascular smooth muscle tone. However, in view of the substantial amount of evidence in favour of a role for cAMP as a mediator of smooth muscle relaxation (see Introduction for references), alternate explanations, such as compartmentation of cAMP (as mentioned earlier), should be sought. This might be partly explained on the basis of the heterogeneity of the rabbit aortic rings used in this study; i.e., it is possible that PGE\textsubscript{1} might produce contractions of the aorta by a direct action on the smooth muscle cells while increasing cAMP levels in the nonmuscle cells. However, the observation that PGE\textsubscript{1} as well as PGI\textsubscript{2} can directly activate adenylate cyclase from isolated rabbit aortic smooth muscle cells (Nicosia et al., 1984) suggests that at least part of the cAMP elevation caused by PGE\textsubscript{1} in aortic rings occurs in the muscle cells themselves.

Hormonal effects such as vascular smooth muscle relaxation, may be a consequence of selective activation of specific cyclic AMP-dependent protein kinases (cA kinases). Although several possibilities for compartmentation of cyclic nucleotides in various tissues were addressed recently (Harper et al., 1985), at least two mechanisms may be proposed to explain how compartmentation (or selective activation) of cAMP and/or cA kinase can occur in vascular smooth muscle. First, selective activation of cA kinase can occur in different pools such as soluble vs
particulate fractions of the cell. Alternatively, it may be possible to selectively activate the two isozymes of cA kinase which are known to exist in many tissues.

The first possibility has been suggested in cardiac tissue by Brunton and coworkers (Hayes and Brunton, 1982; Buxton and Brunton, 1983) who provided evidence that isoproterenol and PGE₁ affect different pools of the protein kinase in that tissue. Isoproterenol activated both membrane-associated and cytosolic cA kinase, while PGE₁ stimulated only cytosolic cA kinase in rat heart. Although isoproterenol and PGE₁ elevated cAMP and activated the kinase, only isoproterenol produced a positive inotropic effect on the heart. These authors excluded the second possibility of selective activation of isozymes when they obtained similar results in both guinea pig heart (which comprises > 90% of isozyme II) and rat heart (which comprises > 80% of isozyme I) (Hayes et al., 1980). It is possible that similar mechanisms may be operating in rabbit aorta as well. To date, very few studies have been done attempting to elucidate the role of cA kinases in the regulation of vascular tone (Silver et al., 1982, 1984, 1985; Lincoln and Simpson, 1983; Vegesna and Diamond, 1984). Experiments were undertaken to determine whether PGE₁, isoproterenol and forskolin may differ in their ability to activate these kinases in different pools (soluble and particulate) in rabbit aortic rings, which might explain the differences in pharmacological actions observed in the present study.

The effects of isoproterenol, forskolin and PGE₁ on soluble and particulate protein kinase activity in rabbit aortic rings are shown in
Table 7. In the supernatant fraction, we were unable to see a significant change in protein kinase activity with 1 μM isoproterenol, although this concentration of the drug produced a small but significant elevation in cAMP levels (Fig. 7). This could be partly due to the technical limitations of the assay. It is possible that isoproterenol might be producing small but functionally relevant changes in the kinase activity and our assay may not be sensitive enough to detect these small changes. When the protein kinase activity was estimated with forskolin and PGE₁, both of them significantly increased the kinase activity, although PGE₁ contracted the muscle. Isoproterenol relaxed these PGE₁-contracted muscles without any further change in protein kinase activation. Forskolin also potentiated the effects of PGE₁ on protein kinase activity. The magnitude of cA kinase activation caused by a combination of forskolin and PGE₁ is much greater than the sum of the activation caused by either agent alone. Still, PGE₁ contracted the forskolin-relaxed aortic rings. Once again, isoproterenol was able to relax these preparations without further activating the kinase. Thus, activation of the kinase in the supernatant fraction occurred whether the muscles were relaxed or contracted.

In view of the earlier reports in cardiac tissue (Buxton and Brunton, 1983), we hypothesized that a difference in activation in the particulate kinase activity might possibly explain our results. However, as shown in Table 7, the increase in cA kinase activity in the pellet appears to be the same with isoproterenol and PGE₁ alone or in combination. Significant increases in cA kinase activity were observed
with forskolin and also with the combination of forskolin, PGE₁ and isoproterenol. In bovine coronary arteries, we observed a decrease in the total kinase activity in the supernatant fraction which was accompanied by a corresponding increase (both basal and total activity) in the particulate fraction with different drug treatments (Vegesna and Diamond, 1984). In the present experiments in rabbit aortic rings, there appears to be a similar shift in the basal and total kinase activity under some conditions. However, some of these changes are not statistically significant compared to their respective controls. This could be partly due to the complexity involved in handling small tissue samples and sensitivity of the assay.

Although we were unable to see a difference in activation of the kinase in soluble and particulate fractions, our results do clearly demonstrate an increase in the protein kinase activity under various conditions whether the tissues are relaxed or contracted. Thus, activation of cA kinase does not appear to be a crucial step in the regulation of vascular smooth muscle tension.

Similar findings were reported in other vascular smooth muscle tissues. For example, Silver et al. (1985) compared the effect of forskolin on cA kinase activity in the normotensive and spontaneously hypertensive rats to determine if alteration of the kinase might be responsible for the decreased relaxation responsiveness of arterial strips in spontaneously hypertensive rats. They observed that, although the extent of relaxation of arterial strips with forskolin was markedly less in spontaneously hypertensive rats, the extent of kinase activation
was the same in both groups. They concluded that components of the cAMP system proximal to and including cA kinase are probably not responsible for the altered responsiveness in the arterial strips. It was further suggested that phosphorylation of specific proteins by cA kinase under these conditions might possibly explain the results. Similar to this report, Tsujimoto and Hoffman (1984) studied the relationship between desensitization of β-adrenergic receptor-mediated smooth muscle relaxation of rat mesenteric arteries and cAMP levels. The extent of relaxation of mesenteric arteries by isoproterenol was decreased (86% vs 43%) in the desensitized rats compared to the control. However, the ability of isoproterenol to stimulate cAMP production was the same in both desensitized and control groups. Similar conclusions as above were reached, even though these authors did not estimate cA kinase activity in their experiments.

Our results do not exclude the other possible compartmentation by selective activation of isozymes of cA kinase. As discussed in the introduction, two isozymes (Type I and II) of cA kinase exist in various tissues including vascular smooth muscle. The physical and biochemical properties of the catalytic subunits of these isozymes appear to be the same, but the regulatory subunits (R₁ and R₁₁) are different and exhibit different properties. It has been proposed that the Type II holoenzyme exists in vivo mostly in the autophosphorylated form. Novel mechanisms have emerged in recent years implicating changes in the phosphorylation form of regulatory subunits to the regulation of smooth muscle tension. For example, Scott and Mumby (1985) quantitated changes in the extent
of phosphorylation of the R\textsubscript{11} subunit of cA kinase in intact bovine tracheal smooth muscle by using monoclonal antibodies for R\textsubscript{1} and R\textsubscript{11} subunits and immunoblot analysis. They showed that incubation of tracheal smooth muscle with isoproterenol, forskolin and phosphodiesterase inhibitors resulted in dose-dependent decreases in the phosphorylation state of R\textsubscript{11}. This dephosphorylation of R\textsubscript{11} closely paralleled the relaxation in a time- and dose-dependent manner. It was further suggested that dephosphorylation of regulatory subunits might be a sensitive measure to estimate selective activation of the isozymes by different drugs. Recently Harper et al. (1985) discussed the advantage of using immunocytochemical techniques over biochemical means to investigate the discrete changes of cAMP and/or cA kinase in subcellular compartments. Although the extent of the isozymes in rabbit aorta is not well understood, isozyme I makes up 46% and isozyme II makes up 51% of the total enzyme activity in other vascular smooth muscle (Silver et al., 1982). By estimating the activity of the common catalytic subunit of cA kinases with PGE\textsubscript{1}, forskolin and isoproterenol in our experiments, it is possible that we might be missing selective changes in the isozymes (i.e., dephosphorylation of R\textsubscript{1} and R\textsubscript{11}) which might be functionally important for regulation of vascular smooth muscle tension. Experiments such as those described above might be important to further explore these possibilities. A final possibility which should also be considered is that in spite of the weight of correlative evidence from various sources, elevation of cAMP and activation of cA
kinase may not be directly responsible for the regulation of vascular smooth muscle tension.

The importance of Ca\(^{++}\) ions in the excitation-contraction coupling events in vascular smooth muscle suggested that a study of the Ca\(^{++}\)-dependence of the contraction initiated by PE and PGE\(_1\) in rabbit aortic rings could yield valuable information regarding Ca\(^{++}\) utilization in this tissue. Therefore, an attempt was made to study the contribution of Ca\(^{++}\) to the PE- and PGE\(_1\)-induced contractions and the effect of isoproterenol under these conditions.

As shown in Figs. 14 and 15, in the absence of extracellular Ca\(^{++}\) in the incubation medium, PE produced a phasic contraction and PGE\(_1\) produced a sustained contraction. It is known that NE produces part of its contractile response in vascular smooth muscle via the release of intracellular Ca\(^{++}\) (Karaki et al., 1979; also see Introduction). The results of the present study in which PE and PGE\(_1\) were shown to produce a contraction in the presence of zero extracellular Ca\(^{++}\) support the concept that release of intracellular Ca\(^{++}\) is partially responsible for the contractions elicited by PE and PGE\(_1\) in the rabbit aortic rings. To further understand the nature of this Ca\(^{++}\) pool in the relaxation of rabbit aorta, the effect of isoproterenol on PE- and PGE\(_1\)-induced contractions were studied under similar conditions. Pretreatment with isoproterenol resulted in complete inhibition of the PE-induced phasic contraction (Fig. 14). Isoproterenol also relaxed the sustained contraction produced by PGE\(_1\) under similar conditions (Fig. 15).
Meisheri and Van Breemen (1982) studied the effect of isoproterenol on the Ca$^{++}$ fluxes and tension in high-K$^+$-depolarized rabbit aortic rings. In their hands, isoproterenol inhibited the Ca$^{++}$ influx accompanied by relaxation. They concluded that isoproterenol reduces intracellular Ca$^{++}$ and relaxes rabbit aortic rings by inhibiting the Ca$^{++}$ influx at the plasma membrane. However, contrary to their conclusion, isoproterenol inhibited the PE- and PGE$_1$-induced contraction in the absence of extracellular Ca$^{++}$ in the present experiments. Our results suggest that at least part of isoproterenol's relaxant effect is mediated by an action at an intracellular site, as suggested by others in other smooth muscles (Mueller and Van Breemen, 1979; Van Eldere et al., 1982).

From these results it is not clear whether the inhibition of the contraction in Ca$^{++}$ free solution by isoproterenol is caused by a Ca$^{++}$-independent action on the regulatory proteins of the contractile system or by an effect on the cellular Ca$^{++}$. If we assume that cAMP plays a role in the relaxation of vascular smooth muscle, then isoproterenol could theoretically relax rabbit aortic rings in several ways. It could induce relaxation without affecting the intracellular Ca$^{++}$ concentration by decreasing the affinity of MLCK for the Ca$^{++}$/calmodulin complex, or by decreasing cytoplasmic Ca$^{++}$ by stimulating efflux or intracellular sequestration (see also Introduction and Fig. 2).

In view of the inconsistencies observed for a role of cAMP in the regulation of vascular smooth muscle tension in the present study, the
extent of participation of cAMP or the combination of any additional mechanisms by which isoproterenol produces change in Ca^{++} mobilization in vascular smooth muscle remains to be further investigated.
SUMMARY AND CONCLUSIONS

1. In bovine coronary artery, prostacyclin produced time-dependent and dose-dependent elevation of cyclic AMP levels accompanied by relaxation of potassium-contracted coronary arteries.

2. In rabbit aorta, prostacyclin and prostaglandin E₁ time-dependently and dose-dependently elevated cyclic AMP levels. However, in direct contrast to the results in bovine coronary artery, these drugs produced a contraction of the rabbit aortic rings rather than a relaxation.

3. Isoproterenol produced a time-dependent elevation of cyclic AMP levels accompanied by relaxation of rabbit aortic rings.

4. Isoproterenol relaxed the prostaglandin E₁- and prostacyclin-contracted rabbit aortic rings without further change in total cyclic AMP levels which were already elevated by the prostaglandin E₁ and prostacyclin alone.

5. Forskolin alone elevated cyclic AMP levels accompanied by relaxation of the aortic rings. Addition of prostaglandin E₁ to forskolin-treated preparations resulted in a markedly enhanced elevation of cyclic AMP levels, but produced a contraction of the muscles.

6. Forskolin, prostaglandin E₁ and isoproterenol activated the cyclic AMP-dependent protein kinase in supernatant fractions from these preparations whether the tissues were relaxed or contracted. Similar patterns in activation of cyclic AMP-dependent protein
kinase were observed in the particulate fractions under these conditions.

7. Phenylephrine and prostaglandin E<sub>1</sub> produced contractions of rabbit aortic rings in the absence of extracellular calcium in the incubation medium. The ability of isoproterenol to relax these tissues suggests that part of the effect of isoproterenol is through alteration of intracellular Ca<sup>2+</sup> mobilization or by an action on the contractile proteins.

In conclusion, a dissociation between elevation of cyclic AMP, activation of cyclic AMP-dependent protein kinase and tension was observed under various conditions in rabbit aorta. This could be partly due to the functional compartmentation of cyclic AMP-dependent protein kinase in this tissue. Alternatively, a step beyond cyclic AMP-dependent protein kinase activation might be crucial for regulation of vascular smooth muscle tension. Our results with prostacyclin in bovine coronary arteries and with isoproterenol and forskolin under some conditions in rabbit aortic rings appear to be consistent with a causal role of cyclic AMP in the relaxation of vascular smooth muscle. However, results with prostacyclin, prostaglandin E<sub>1</sub> and with forskolin combination studies in rabbit aortic rings do not appear to support a generalization of an exclusive role of cyclic AMP and cyclic AMP-dependent kinase in vascular smooth muscle relaxation. Further studies such as estimation of selective activation of isozymes of cyclic AMP-dependent protein kinase or phosphorylation of specific proteins under various conditions are necessary to prove or disprove the cyclic AMP-relaxation hypothesis.
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