EFFECTS OF ISOPROTERENOL AND FORSKOLIN ON TENSION,
CYCLIC AMP LEVELS AND CYCLIC AMP-DEPENDENT
PROTEIN KINASE ACTIVITY IN BOVINE
CORONARY ARTERY

by

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ABSTRACT

The effects of isoproterenol and forskolin on tension, cyclic AMP (cAMP) levels, and cyclic AMP-dependent protein kinase (cA kinase) activity were compared in helical strips of bovine coronary artery.

Isoproterenol and forskolin produced time-dependent and dose-dependent increases in cAMP levels and both compounds relaxed potassium-contracted arteries. Relaxation and cAMP elevation appeared to be well correlated in the isoproterenol experiments. However, in contrast to the results with isoproterenol, cAMP elevation and relaxation were not well correlated at lower concentrations of forskolin. For example, 0.1 μ M forskolin increased cAMP levels in the arteries by approximately 5.5 fold but did not relax the muscles. A smaller elevation of cAMP produced by 1.0 μ M isoproterenol, on the other hand, was accompanied by an almost complete relaxation of arteries.

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). It is possible that low doses of forskolin may elevate cyclic AMP in a compartment which does not have access to these kinases, whereas higher doses may

increase cAMP levels in all compartments. In order to investigate this possibility, optimal conditions were established in our laboratory for the measurement of cA kinase activity in coronary arteries. It has been suggested that addition of 0.5 M NaCl to the buffer used for homogenization of coronary arteries is essential in order to demonstrate hormonal effects on cA kinase in this tissue. However, under our experimental conditions, increasing the ionic strength in the homogenization buffer significantly inhibited total cA kinase activity. Interestingly, in agreement with previous reports in other tissues, an apparent translocation of cA kinase activity from the cytosol to the particulate fraction was observed under conditions associated with high tissue levels of cAMP. For example, relaxation caused by isoproterenol was accompanied by increased cA kinase activity in the particulate fraction and decreased activity in the soluble fraction of the coronary arteries. A similar shift in the distribution of the kinase was caused by various concentrations of forskolin, irrespective of whether the arteries were relaxed or not. Thus, as was observed with cAMP levels, activation of cA kinase did not always correlate well with relaxation in this tissue.

Other workers have suggested that low doses of forskolin can potentiate hormonally-induced effects in several tissues. It was suggested that such a potentiation would constitute evidence in favour of a role for cAMP in the hormonal response. However, in the present experiments in coronary arteries, it was found that low doses of forskolin, which significantly increased cAMP levels, did not potentiate isoproterenol-induced relaxation or elevation of cAMP.

Only higher doses of forskolin ($\geq 1 \mu\text{M}$) potentiated isoproterenol-induced cAMP generation. These results suggest that caution should be exercised in utilizing forskolin as a tool in elucidating a role of cyclic nucleotides in vascular smooth muscle function.

Our results suggest that cAMP may not be the only mechanism responsible for relaxation of coronary arteries by these drugs or alternatively, that some form of functional compartmentalization of cAMP and cA kinase exists in this tissue.

Jack Diamond
Supervisor

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INTRODUCTION

A. CYCLIC AMP : General View

Cyclic AMP was discovered by Sutherland and his colleagues in 1957 as a heat stable mediator of epinephrine's effects on glycogenolysis. The research demonstrating the concept of cyclic AMP (cAMP) as a "second messenger" (Robison, Butcher and Sutherland, 1971) has opened a period of remarkable progress in the understanding of mechanisms whereby many drugs, neurotransmitters and hormones produce some of their well known effects on target tissue.

Figure I illustrates current concepts regarding the role of cAMP as a mediator of hormonal action in physiological responses. The hormone binds to specific receptors on the external surface of the plasma membrane of the cell resulting in activation of the enzyme adenylate cyclase. This activation leads to increased conversion of ATP to cAMP. The increased concentration of cAMP in the cell then leads to activation of the enzyme cyclic AMP-dependent protein kinase. Phosphorylation of specific proteins by the activated kinase is believed to be responsible for the observed physiological response.

Cyclic AMP in the cell is hydrolyzed into 5'-AMP, a reaction catalyzed by one or more cyclic nucleotide phosphodiesterases that exist in all mammalian cells (Robison et al, 1971).

Figure 1: A schematic diagram of the mechanism by which hormones and drugs are thought to produce physiological responses in different tissues by elevating cyclic AMP and activating cyclic AMP-dependent protein kinase. R, regulatory subunit of cyclic AMP-dependent protein kinase; C, catalytic subunit of cyclic AMP-dependent protein kinase.

For the past twenty years, regulation of cellular responses by cyclic nucleotides has been a subject of intense investigation. This resulted in an exponential increase in the number of publications in the cyclic nucleotide research area. 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 (eds.), 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.

B. Role of cAMP in the Control of Smooth Muscle Contraction

The precise role of cyclic nucleotides 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 to review all the information is beyond the scope of this thesis. However, this topic has been excellently reviewed by several authors presenting evidence for and against a role for cyclic nucleotides in smooth muscle function (Namm and Leader, 1976; Diamond, 1978; Namm, 1980; Hardman, 1981; and Kukovetz et al, 1981). Sutherland and Rall (1960) first suggested that epinephrine-induced

relaxation of smooth muscle might be related to an elevation of cAMP levels in that tissue. From that time onwards, many researchers have attempted to test the general hypothesis of cAMP mediated relaxation of different smooth muscles. 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 stimulants and other substances and their relaxant effects on smooth muscle. However there is also evidence that is inconsistent with this concept.

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. Extensive evidence for a mediator role of cAMP in smooth muscle relaxation produced by β -adrenergic drugs and other stimulants of adenylate cyclase activity 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 its relaxant effects. Kukovetz et al have shown that the extent of changes in cAMP is consistent with a mediator role in the relaxant response. Many researchers have studied the ability of various agents to elevate cAMP levels and to alter tension in several smooth muscles, as shown in Table 1. The majority of the studies support a role of cAMP in the relaxation of smooth muscle. It should be noted that many of these studies lack time-dependent and dose-dependent correlations in demonstrating the cause-effect relationships (Kramer and Hardman, 1980).

Table I. Effects of various agents on cyclic AMP levels and tension in blood vessels.

Animal and Vessel	Agent	Mechanical Effect	Cyclic AMP
Rat			
Aorta	Angiotensin	Contraction	No change
	Angiotensin + theophylline	Not reported	Decreased
	Cyclopropane	Contraction	Decreased
	Isoflurane	Relaxation	Increased
	Halothane	Relaxation	Increased
Tail artery	Angiotensin	Contraction	Decreased
Portal vein	Angiotensin + theophylline	Contraction	Decreased
	Mepivacaine	Relaxation	Increased
Rabbit			
Pulmonary artery	Serotonin	Contraction	No change
	Nitroglycerin	Relaxation	Increased
	D-600	Relaxation	No change
	Dipyridamole	Relaxation	Increased
Mesenteric artery	Histamine	Contraction	Increased
	Histamine + metiamide	Increased contraction	No change
	Histamine + mepyramine	Decreased contraction	Increased
Mesenteric vein	Diazoxide	Relaxation	No change
	MnCl ₂	Relaxation	No change
	EGTA	Relaxation	No change
Dog			
Femoral artery	Carbachol	No change	No change
	Nitroglycerin	Relaxation	No change
Mesenteric artery	PGE ₁	Relaxation	Increased
Lobar artery	PGF _{2α}	No change	No change
	PGE ₁	Relaxation	Increased
Lobar vein	PGF _{2α}	Contraction	No change
	PGE ₁	Relaxation	Increased
Saphenous vein	PGF _{2α}	Contraction	No change
	PGE ₂	Relaxation	Increased
	Acetylcholine	Contraction	No change
Cow			
Mesenteric artery	Diazoxide	Relaxation	Increased
	Hydralazine	Relaxation	Increased
	Histamine	Contraction	Decreased at 15 s, increased at 5 min
Facial artery	KCl	Relaxation	Increased
Common dorsal digital vein	KCl + β-methylidigoxin	Contraction	Increased
	PGF _{2α}	Contraction	No change
	PGE ₂	Relaxation	Increased
Pig			
Coronary artery	Histamine	Contraction	Increased
Human			
Umbilical artery	Acetylcholine	Contraction	No change
	Histamine	Contraction	No change
	Bradykinin	Contraction	No change
	Serotonin	Contraction	No change
	KCl	Contraction	No change
	PGE ₁	Relaxation	Increased
	PGA ₂	Contraction	No change
	PGF _{2α}	Contraction	No change
	Ionophore A-23187	Contraction	No change
	Oxygen	Contraction	No change

EGTA, ethylene glycol bis(β-aminoethyl ether) tetraacetic acid; PG, prostaglandin.

Kramer and Hardman (1980)

In direct contrast to the above findings, it has been demonstrated in some smooth muscles 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. Supporting 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 questioned the exclusive role of cAMP in the relaxation of smooth muscle.

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 G. 1982). 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. This situation might result

in erroneous conclusions because of the apparent dissociation between estimated total tissue cAMP levels and contractile responses observed in such studies.

Some authors tried to explain their controversial results by suggesting compartmentalization of cAMP in the smooth muscle. (For example, see Vesin and Harbon, 1974). Elucidation of a role of cyclic AMP-dependent protein kinase in several cellular processes led to the suggestion that not only cAMP but also cyclic AMP-dependent protein kinase might be compartmentalized by selective activation of hormones in some tissues (Corbin et al, 1977; Brunton et al, 1980). For example, in their classical experiments in the heart, 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 particulate protein kinase suggesting specific pools of cAMP and its protein kinase might be important in the elucidation of hormonal action. Based on these results and also on extensive evidence existing in the literature, Hayes and Brunton hypothesized the compartmentalization of cyclic nucleotide action. In their recent excellent review article, (Hayes and Brunton, 1982) it was stated that "Hormonally specific compartmentation of cAMP occurs in cardiac myocytes and probably in many other cell types. The accumulation of cAMP at precise and limited intracellular loci leads to localized activation of cAMP-PK and the phosphorylation of some, but not

necessarily all, substrates of cAMP-PK. As a result of this compartmentation, we observe experimentally that not all cellular cAMP interacts with all endogenous cAMP-PK, and that not all catalytic subunits of cAMP-PK interact with all protein substrates. We offer this as a corollary to the protein kinase hypothesis of cAMP action." Even though verification of this theory is difficult at this stage, 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.

We should not exclude the possibility that to some extent, the technical limitations in measurement of total cAMP levels might contribute to the controversy. For example, Sala et al, (1979) observed a dissociation between cAMP levels and steroid production in response to ACTH on steroid producing tissues. However, this discrepancy was resolved when they demonstrated a rise in protein kinase activity even in the apparent absence of a change in cAMP levels in these tissues. They further suggested that instead of cAMP, measurement of cyclic AMP-dependent protein kinase (cA Kinase) might be the most meaningful parameter in demonstrating a role of cyclic nucleotides in physiological responses.

It is unfortunate to note that very little is known about the ability of pharmacological agents to control the activity of cA kinase in intact smooth muscle in general and vascular smooth muscle in particular.

C. 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 cyclic nucleotide. Kuo and Greengard (1969) extended this concept by identifying this enzyme in a wide range of mammalian tissues. This 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 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. These two isozymes appear to share a common catalytic subunit but have different regulatory subunits which might be responsible for the difference in their properties (Corbin and Keely, 1977). It was implied that specific activation of either of these isozymes might be responsible for the cAMP-mediated cellular processes in different tissues (Schwoch, 1978; Corbin et al, 1977; Medieks 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 (also see Fig. 1).

In the basal state of the cell, i.e., in the absence of hormonal stimulation, cA kinase exists predominantly in a holo-enzyme form (R_2C_2) in which a regulatory subunit dimer (R_2) is bound to two catalytic (C) subunits. In this form the enzyme is inactive since the regulatory dimer exerts an inhibitory effect on the activity of the catalytic subunits. When the cAMP concentration of the cytosol increases, the nucleotide interacts with cAMP binding sites on the regulatory dimer and promotes the dissociation of R_2 from the catalytic subunit to produce the active form of the enzyme (C). This is reflected in vitro as an increase in the -cAMP/+cAMP activity ratio of the enzyme. The catalytic subunit of cA kinase catalyzes the phosphorylation of intracellular enzymes and other proteins using ATP as the phosphoryl donor. This phosphorylation alters the functional activity of many of the enzymes i.e., causes either activation or inactivation. These activity changes, in turn, might be responsible for many of the physiological actions of the drugs.

In smooth muscle, microsomal proteins (Krall et al, 1978) and myosin light chain kinase (Adelstein et al, 1981) have been suggested to be substrates for cA-kinase. Phosphorylation of these proteins

has been implicated to be associated with smooth muscle relaxation. Although the mechanism by which cAMP can regulate the intracellular free Ca^{++} levels is not known, the essential role of Ca^{++} and its direct modulation of actin-myosin interactions in the regulation of smooth muscle contraction also must be considered. (Magaribuchi and Kuriyama, 1972; Webb and Bhalla, 1976; Marshall, 1977; Webb and Bohr, 1981; Stull, 1980).

D. Forskolin and its Role in Cyclic Nucleotide Research

In recent years, a novel drug called forskolin has produced a major impact on cyclic nucleotide research. Forskolin is a cardio-active diterpene derivative isolated from the Indian plant coleus forskohlii. It has been shown in membranes and intact cells that forskolin is a potent stimulant of adenylate cyclase and the activation appears to be rapid and reversible (Seamon et al, 1981). However, the mechanism by which forskolin activates adenylate cyclase is not yet clear. Several biochemical studies revealed that forskolin has no direct effect on sodium-potassium ATPase, cyclic nucleotide phosphodiesterase (Lindner et al, 1978), guanylate cyclase, cyclic AMP-dependent protein kinase and calcium-magnesium ATPase of sarcoplasmic reticulum (Metzer and Lindner, 1981). The effects produced by forskolin were also not blocked by a variety of receptor blockers ruling out a role of receptor-mediation in its activation. Seamon and Daly (1981a) reported that forskolin does not require a functional guanine nucleotide regulatory subunit to activate the catalytic subunit of the enzyme and it was suggested that forskolin might possibly be

acting directly on the catalytic subunit.

Forskolin has been shown to increase cAMP levels in a variety of tissues (Seamon et al, 1981). Several forskolin-induced cellular responses have been correlated to increased cyclic AMP 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 also been reported to relax a variety of smooth muscles including vascular smooth muscle (Dubey et al, 1981; Muller and Baer, 1983; Burka, 1983; Vegesna and Diamond, 1983). As mentioned earlier, relaxation of vascular smooth muscle by β -adrenergic amines is generally assumed to be mediated by an increase in intracellular accumulation of cyclic AMP (Kukovetz et al, 1981). If elevation of cAMP is responsible for relaxation of vascular smooth muscle, forskolin-induced elevation of cAMP should be correlated with relaxation. Evidence consistent with this hypothesis has been presented by Muller and Baer (1983). Forskolin was capable of relaxing isolated preparations of rat aorta, bovine coronary artery, canine coronary artery, guinea pig taenia coli and rabbit small intestine. The relaxant effects of forskolin on guinea pig taenia coli and rabbit small intestine were potentiated by phosphodiesterase inhibitors and forskolin was found to directly stimulate adenylate cyclase from these intestinal smooth muscles. Although cAMP levels were not actually measured, it was concluded from these results that relaxation of smooth muscle by forskolin was mediated by cAMP. However, to this date, no data correlating forskolin-induced smooth muscle relaxation with

increases in cAMP levels have been published.

Another striking aspect of forskolin effects observed was its potentiation of hormonally-induced effects on cyclic AMP generation. It has been suggested 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, PGE_2 and VIP in a number of hormonally responsive cells including rat adipocytes, rat and guinea pig brain slices, rat glioma cells, rat adrenal cells, guinea pig thyroid glands and human platelets (see for review, Seamon and Daly, 1981b). The intricate mechanism by which forskolin elicits the tremendous potentiation of hormonal responses is not yet clear. It is still unknown whether or not forskolin can potentiate the hormonal response in smooth muscle.

From the aforementioned information, it is clear that, although elevation of cAMP does not appear to be the primary mechanism of action of most 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 were done relating changes in cAMP levels to relaxation of vascular smooth muscle. (Kramer and Hardman, 1980, for review). Estimation of cyclic-AMP dependent protein kinase might further promote our understanding of a role of cAMP in the relaxation of vascular smooth muscle. Forskolin may be a possible tool to explore the cAMP-mediated effects in smooth muscle. It would also be interesting to see whether forskolin can potentiate hormonally-induced responses in smooth muscle.

The present study has been undertaken to answer some of these questions. Attempts have been made to correlate changes in cAMP levels and protein-kinase activity with relaxation of bovine coronary arteries by forskolin. The effects of forskolin were compared to those of isoproterenol, a β -adrenergic agonist generally believed to relax smooth muscles by virtue of its ability to increase cyclic AMP levels.

SPECIFIC GOALS OF THE PRESENT INVESTIGATION

1. To study time-dependent and dose-dependent effects of forskolin on cyclic AMP levels and tension in isolated bovine coronary arteries and to determine whether a correlation exists between elevation of cAMP and relaxation of the vascular smooth muscle. These effects would be compared with those of isoproterenol, a β -adrenergic agonist.
2. To see whether low doses of forskolin can potentiate isoproterenol-induced effects in vascular smooth muscle, as is the case in other tissues.
3. To establish optimal conditions for the estimation of the enzyme cyclic AMP-dependent protein kinase in bovine coronary arteries.
4. To study the effects of forskolin on cyclic AMP-dependent protein kinase activity and tension in coronary arteries and compare these effects with those of isoproterenol.

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, dithiothreitol, Histone II-A, sodium fluoride, cyclic AMP, bovine serum albumin and (-)-isoproterenol.

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 [r - 32 p] ATP (20 ci/mMole) were purchased from Amersham.

Forskolin was obtained from Calbiochem. A 1mM stock solution of forskolin was prepared in 95% ethanol. This solution was diluted with water and added to the muscle baths to give the desired final concentration. Control strips were treated with the appropriate concentration of ethanol.

Cyclic-AMP radio-immunoassay kits were purchased from Becton Dickinson Canada Ltd.

Fresh bovine hearts were obtained from a local slaughter house (Intercontinental Packers) for this entire study.

B. METHODS

I. Preparation of muscle samples

Bovine hearts were obtained immediately after slaughter, immersed in ice cold regular Krebs solution and transported to the laboratory. Branches of the left anterior descending and circumflex coronary arteries were dissected free and helical strips were prepared. The strips were suspended at 37°C under 2 g tension in Krebs bicarbonate solution with the following composition (mM): NaCl, 118; KCl, 5.7; MgSO₄, 2.33; CaCl₂, 1.26; NaHCO₃, 2.5; NaH₂PO₄, 1.17 and glucose, 11. Solutions were aerated with 5% CO₂ and 95% O₂, which maintained the pH at approximately 7.4. Alterations in tone were monitored isometrically using force transducers. After a 2 hour equilibrium 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)(see Figs. 9 and 10). 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 cyclic AMP and cyclic AMP-dependent protein kinase assays. Tension and cyclic AMP or cyclic AMP-dependent protein kinase were thus determined in the same muscle strips.

II. Measurement of cyclic AMP

Cyclic AMP levels in the frozen tissues were determined using

radio-immunoassay kits supplied by Beckton Dickinson Canada, Inc. Briefly, the frozen samples were homogenized in 6% trichloroacetic acid, the trichloroacetic acid removed by ether extraction and radio-immunoassay performed on the aqueous extracts. Results are expressed as pmoles of cyclic AMP per g wet weight of tissue.

III. Preparation of extracts and assay of cyclic AMP-dependent protein kinase

Approximately 100-200 mg of frozen tissue was suspended at 4°C in 10 volumes of buffer (pH 6.8) containing 10mM potassium phosphate, 10 mM EDTA, 0.5 mM methyl isobutylxanthine and 0.5 mM 1,4-dithiothreitol, with and without 0.5 M NaCl 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 protein kinase activity.

When assayed, the pellet containing the particulate fraction was gently washed twice with 10 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 0.1% 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 protein kinase activity was determined by measuring the transfer of 32 P from [γ - 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 - ^{32}P] ATP (50-100 cpm/pmol), 10 mM magnesium chloride, 100 μg histone II-A (Sigma), and 10 mM sodium fluoride, in the presence and absence of 2 μM cyclic AMP. The incubation was carried out at 30°C for 10 min. The reaction was terminated by pipetting 60 μl aliquots of the reaction mixture on to filter paper discs (Whatmann 3 mm, 2.3 cm diameter) which were immediately dropped into 10% ice cold trichloroacetic acid. The filter papers were washed 4 times (15 min each) in trichloroacetic acid followed by one 5 min wash in 95% ethanol and one in ether. 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 protein kinase activity was expressed as pmoles phosphate transferred per mg protein. The extent of cyclic AMP-dependent protein kinase activation in the supernatant was assessed by calculating the activity ratio, which is the ratio of kinase activity in the absence of added cyclic AMP to that in the presence of enough cyclic AMP to fully activate the enzyme.

IV. Protein determination

The concentration of protein in the supernatant and the pellet was determined according to the Lowry procedure (Lowry et al., 1951) using bovine serum albumin as a standard.

V. Statistical Analyses:

Data were evaluated statistically by means of a student's t test. A probability of less than 0.05 was accepted as the level of statistical significance.

RESULTS

A. CONDITIONS FOR THE ASSAY OF CYCLIC AMP-DEPENDENT PROTEIN KINASE (CA KINASE) IN INTACT TISSUE

An attempt was made to standardize the protein kinase assay conditions in our laboratory. The following experiments were undertaken to determine optimum conditions for the assay.

- 1) Linearity with time: The effect of incubation time on the protein kinase activity in coronary arteries was examined. A typical experiment is shown in Fig. 2. The experiment method was the same as described earlier except the incubation time was varied. The reaction rate was linear at 30°C up to 40 min in the presence of 2 μ M cAMP and up to 20 min in the absence of cAMP. The protein kinase activity ratio remained constant up to 20 min (data not shown).
- 2) Linearity with protein concentrations: The effect of enzyme concentration on the protein kinase activity is shown in Fig. 3. Dilution of the supernatant fraction was made with the homogenization buffer. The protein kinase activity in this fraction was determined as described under experimental procedures. Histone phosphorylation by the coronary artery protein kinase was

linear with respect to the amount of enzyme added up to 30 μg of protein per assay. Protein kinase ratio remained constant up to this concentration.

3) Effect of magnesium chloride on cA kinase activity: The assay was run under routine conditions stated in methods with the exception that Mg^{++} concentration was varied. Fig. 4 shows that maximum protein kinase activity was observed at 15-20 mM MgCl_2 and further increasing the concentration inhibited the enzyme activity.

4) Effect of NaF on protein kinase activity: Crude enzyme preparations may contain ATPases and addition of NaF minimizes the effects of these ATPases. Assays were performed in the presence of 2 μM cAMP. Experimental procedure was same as described earlier except NaF concentration was varied. As shown in Fig. 5, the total kinase activity was not changed by lower concentrations of NaF (10-20 mM) but higher concentrations inhibited the kinase activity.

5) Effect of Triton X-100 concentration on particulate protein kinase activity: Frozen arterial strips were homogenized in the normal buffer, the homogenate divided into several aliquots and particulate fractions (pellets) of these aliquots were prepared as described in the methods. Then the pellets were suspended in the buffer containing different concentrations of Triton X-100 and centrifuged. The supernatant fractions from these Triton-treated pellets were used to estimate particulate protein kinase activity. The assays were run under routine conditions, in the

presence and absence of 2 μ M cAMP in the final incubation mixture. As shown in Fig. 6, the activity of the pellet was low in the absence of Triton X-100, and increased to a maximum at 0.2%, indicating solubilization of the enzyme by Triton. At higher concentrations, protein kinase activity was inhibited by the detergent.

In summary, the following standard conditions were used: The assay was routinely run for 10 min at 30°C, with 10 mM MgCl₂, 10 mM NaF and 20 μ l of the supernatant containing 10-25 μ g protein in the final incubation mixture. In the pellet experiments, 0.1% Triton X-100 was included in the homogenization buffer used for suspending the pellet.

B. TIME COURSE STUDIES OF ISOPROTERENOL AND FORSKOLIN ON CYCLIC AMP LEVELS AND TENSION IN BOVINE CORONARY ARTERIES

Time courses for elevation of cyclic AMP and relaxation of bovine coronary arteries by 1 μ M isoproterenol are shown in Fig. 7. This concentration of isoproterenol was a maximally-effective one with respect to relaxation. Cyclic AMP levels were significantly increased within 1 minute after addition of isoproterenol to the muscle bath, which was the earlier time point at which muscle relaxation could be detected. Cyclic AMP levels were further increased at 4 and 10 minute points. Maximal relaxation had occurred within 10 minutes.

A similar experiment with a maximally-relaxant concentration of forskolin is shown in Fig. 8. At 1 minute after addition of forskolin to the bath, no relaxation and no cyclic AMP elevation were seen. At 2 minutes, cyclic AMP levels had increased significantly, and the muscles had begun to relax. Within 8 minutes, cyclic AMP levels had increased approximately 30 fold, and the muscles had completely relaxed.

C. EFFECTS OF VARIOUS CONCENTRATIONS OF ISOPROTERENOL AND FORSKOLIN ON cAMP LEVELS AND TENSION IN CORONARY ARTERIES

The effects of various concentrations of isoproterenol and forskolin on cyclic AMP levels and tension in bovine coronary arteries are shown in Table II. Both drugs caused concentration-dependent increases in cyclic AMP levels. However, at lower concentrations of forskolin (0.01-0.1 μ M), marked increases in cyclic AMP levels were observed with no muscle relaxation.

Addition of 1 μ M isoproterenol in combination with 10 μ M forskolin increased cyclic AMP levels by approximately 360 fold (Table II). This is much greater than the sum of the increases produced by isoproterenol or forskolin alone (3.7 and 30 fold, respectively). The combination relaxed the muscles by 111% (i.e., the KCl-induced tone was completely eliminated, and a partial relaxation of the 2 g resting tone also occurred). (See Fig. 10, for experimental protocol)

D. EFFECTS OF FORSKOLIN AND ISOPROTERENOL, ALONE AND IN COMBINATION,
ON CYCLIC AMP LEVELS AND TENSION IN BOVINE CORONARY ARTERIES

The effects of 0.1 μ M and 10 μ M forskolin on the cyclic AMP elevation and the relaxation caused by two concentrations of isoproterenol are shown in Figure 11. Isoproterenol alone increased cyclic AMP levels and relaxed the coronary arteries in a dose-dependent manner. The lower concentration of forskolin (0.1 μ M) did not potentiate the effects of either dose of isoproterenol on cyclic AMP levels or tension. In fact, the combination of 0.1 μ M forskolin plus 0.1 μ M isoproterenol increased cyclic AMP levels less than did 0.1 μ M forskolin alone (Figure 11). However, the higher dose of forskolin (10 μ M) markedly potentiated the effects of both concentrations of isoproterenol on cyclic AMP levels (Figure 11). The magnitude of the cyclic AMP increase caused by a combination of isoproterenol and 10 μ M forskolin was much greater than the sum of the increases caused by either agent alone. The 10 μ M concentration of forskolin alone caused almost complete relaxation of the arteries. Therefore, under these conditions, we were unable to demonstrate a potentiation of the relaxant effects of isoproterenol. (See Figs. 9 and 10, for experimental protocol).

The above results indicate that potentiation of the effects of isoproterenol on cyclic AMP levels in these preparations occurred with 10 μ M forskolin but not with 0.1 μ M. A closer approximation of the concentration of forskolin needed to potentiate the effects of isoproterenol is provided in the results of the experiment shown in Figure 12. The effects of 0.1 μ M isoproterenol together with 0.01, 0.1, 1 and 10 μ M forskolin on cyclic AMP levels and tension in coronary

arteries are shown in the figure. The effects on cyclic AMP levels were less than additive at 0.01 μ M forskolin, approximately additive at 1 μ M forskolin. Therefore, potentiation occurred only with concentrations above 1 μ M. It should be noted that, as previously shown in Table II, lower doses of forskolin alone (0.01-0.1 μ M) significantly increased cyclic AMP levels (2-5 fold) but did not relax the arteries.

E. ACTIVATION OF CYCLIC AMP-DEPENDENT PROTEIN KINASE IN BOVINE CORONARY ARTERIES BY ISOPROTERENOL AND FORSKOLIN

Silver et al. (1982) have recently reported that relaxation of bovine coronary arteries by isoproterenol is associated with activation of soluble cyclic AMP-dependent protein kinase. They also reported that it was necessary to add 0.5 M NaCl to the homogenization medium in order to demonstrate isoproterenol-induced activation of the kinase in these preparations. Similar conditions were used, in the present study, to compare the effects of isoproterenol and forskolin on soluble cyclic AMP-dependent protein kinase activity in bovine coronary arteries. The effects of several concentrations of forskolin and isoproterenol on the kinase activity measured with and without 0.5 M NaCl in the homogenization buffer are presented in Table III. When 0.5 M NaCl was not included in the homogenization buffer, no significant increase in kinase activity ratios were observed in coronary arteries treated with 1 μ M isoproterenol or with 0.1 μ M forskolin. This is in spite of the fact that cyclic AMP levels were significantly increased by these concentrations of the drugs (see Figure 11). However, when 0.5 M NaCl was added to the homogenization buffer as suggested by Silver et al. (1982), kinase activity ratios

were found to be significantly increased (Table III). Activity ratios in control preparations were also increased under these conditions. These results therefore agree with those of Silver et al. (1982) for isoproterenol. Higher doses of forskolin, which produced very marked increases in cyclic AMP levels, significantly increased protein kinase activity ratios even in the absence of 0.5 M NaCl in the homogenization medium (Table 3). A combination of 10 μ M forskolin and 1 μ M isoproterenol resulted in extremely high tissue levels of cyclic AMP (Figure 11) and caused almost maximal activation of cyclic AMP-dependent protein kinase with and without 0.5 M NaCl in the homogenization medium.

F. EFFECT OF NaCl ON SOLUBLE PROTEIN KINASE ACTIVITY

It can be seen from Table 3 that total kinase activities (measured in the presence of 2 μ M cyclic AMP) were inhibited by as much as 60% in the high NaCl experiments. Control as well as drug-treated activities were affected. This is in contrast to the results of Silver et al. (1982), who noted that the addition of 0.5 M NaCl to the homogenization medium did not cause any significant changes in total kinase activity. We therefore decided to further investigate the effect of NaCl on the soluble kinase activity by measuring control and hormonally-stimulated cyclic AMP-dependent protein kinase activity in the presence of increasing concentrations of NaCl. As shown in Figure 13, increasing the concentration of NaCl in the homogenization medium caused a progressive decrease in total kinase activity in control preparations. No consistent changes in basal activity were seen. Similar results were

also obtained in muscles treated with 1 μ M isoproterenol. (See Figure 14). When NaCl was removed from the solutions by dialysing for 2 hr against regular homogenization buffer, enzyme activity was returned toward control levels (Figure 15). Thus, addition of NaCl to the homogenization medium appeared to inhibit total kinase activity and this probably accounts for the increased activity ratios observed in our experiments under these conditions.

G. EFFECT OF ADDITION OF CYCLIC AMP TO THE HOMOGENATE ON SUPERNATANT AND PARTICULATE PROTEIN KINASE ACTIVITY

It can be seen from the left hand panel of Table 3 that drugs which caused large increases in tissue cyclic AMP levels also caused decreases in total cyclic AMP-dependent protein kinase activity estimated in the absence of added NaCl. This decreased activity in the supernatant may be due to translocation of the enzyme from the soluble to the particulate fraction. Similar observations have previously been noted by Krall et al. (1978) and Harbon et al. (1978) in rat myometrium treated with catecholamines, suggesting that translocation of the enzyme to a membrane fraction might have occurred in that tissue as well.

In order to further investigate this possibility, frozen arterial strips were homogenized, the homogenate divided into two aliquots, and 2 μ M cyclic AMP added to one of them. As shown in Figure 15, addition of cyclic AMP to the homogenization buffer decreased the total kinase activity in the supernatant fraction. This is similar to the decreases observed in arteries in which high endogenous cyclic AMP levels had

been induced by drug treatment (see Table III). When the kinase activity was determined in the particulate fraction of these muscles as described in Methods, it was found that the decrease in total kinase activity in the supernatant was accompanied by a corresponding increase in activity in the pellet (Figure 16). Basal cyclic AMP activity (measured in the absence of cyclic AMP in the final incubation mixture) is also increased in the pellet obtained from preparations homogenized in the presence of 2 μ M cyclic AMP. This altered distribution or translocation of kinase activity was also observed even if the coronary arteries were homogenized in buffer containing 0.5 M NaCl (data not shown).

H. EFFECTS OF ISOPROTERENOL AND FORSKOLIN ON PROTEIN KINASE ACTIVITY IN SUPERNATANT AND PARTICULATE FRACTIONS. RELATIONSHIP TO RELAXATION OF THE CORONARY ARTERIES

As shown earlier (see Figure 7), relaxation of coronary arteries by isoproterenol was correlated in a time-dependent manner with elevation of cyclic AMP in the tissues. Similarly, in the present experiment, cyclic AMP levels were significantly elevated after a 1 min exposure to 1 μ M isoproterenol, at which time the muscles had just begun to relax (Table IV). After a 10 min exposure to the isoproterenol, cyclic AMP levels were further elevated and the muscles were maximally relaxed. Even though cyclic AMP levels were elevated in these preparations, no activation of the protein kinase in the supernatant fractions was observed if NaCl was omitted from the homogenization (Table IV, see also Table III). However, when the protein kinase activity was estimated in

the particulate fractions from these same arteries, a significant increase in activity was observed even at the 1 min time (Table IV). As was noted earlier, 0.1 μ M forskolin significantly increased cyclic AMP levels without producing any relaxation of the arteries. In the present experiments, this dose of forskolin also significantly increased the particulate protein kinase activity without causing relaxation (Table IV). Higher doses of forskolin (1-10 μ M) produced larger increases in cyclic AMP levels, relaxed the muscles, and also stimulated soluble and particulate protein kinase activity.

Figure 2:

The effect of incubation time on cyclic AMP-dependent protein kinase activity in coronary arteries. Protein kinase activity was measured at different time points, both in the presence and absence of 2 μ M cAMP in the final incubation mixture. Values represent mean of 2 experiments.

Figure 3:

The effect of enzyme concentration on cyclic-AMP-dependent protein kinase activity in coronary arteries. Supernatant fraction was diluted with homogenization buffer and protein kinase activity estimated in the presence and absence of 2 μ M cAMP in the final incubation mixture. Values represent mean of 2 similar experiments.

Figure 4:

The effects of magnesium chloride on cyclic AMP-dependent protein kinase activity in coronary arteries. Protein kinase activity estimated in the presence and absence of 2 μ M cAMP in the final incubation mixture. Values represent mean of 2 similar experiments.

Figure 5:

Effect of sodium fluoride on cyclic AMP-dependent protein kinase activity in coronary arteries. Total protein kinase activity was estimated in the presence of 2 μ M cAMP in the final incubation mixture. Values represent mean of 2 similar experiments.

(x) (+) CAMP (N=2)

Figure 6:

Effect of Triton X-100 concentration on particulate cyclic AMP-dependent protein kinase activity in coronary arteries. Protein kinase activity of the pellet was estimated, both in the presence and absence of 2 μ M cAMP in the final incubation mixture, as described in Methods. Values represent mean of 2 similar experiments.

Figure 7:

Time course for cyclic AMP elevation and relaxation of KCl-contracted bovine coronary arteries by 1 μ M isoproterenol. Cyclic AMP levels and tension were measured in the same arterial strips as described in Methods. Values represent means $\pm$ SEM for 5 experiments. Asterisks indicate values significantly different from the corresponding zero times controls ($p < 0.05$).

Figure 8:

Time course for cyclic AMP elevation and relaxation of KCl-contracted bovine coronary arteries by 10 μ M forskolin. Cyclic AMP levels and tension were measured in the same arterial strips as described in Methods. Values represent means $\pm$ SEM for 5 experiments. Asterisks indicate values significantly different from the corresponding zero time controls ($p < 0.05$).

TABLE II. Effects of isoproterenol (ISO) and forskolin (FORSK) on cyclic AMP levels and tension in bovine coronary artery.

Treatment	N	Cyclic AMP (pmoles/g tissue)	% relaxation of KCl contraction
Control	14	96 ± 15	--
ISO (0.1 µM)	5	196 ± 22*	54 ± 4*
ISO (1 µM)	5	369 ± 56*	83 ± 4*
FORSK (0.01 µM)	6	239 ± 48*	0
FORSK (0.1 µM)	6	531 ± 160*	0
FORSK (0.5 µM)	6	662 ± 120*	29 ± 5*
FORSK (1 µM)	8	914 ± 230*	46 ± 12*
FORSK (10 µM)	6	3020 ± 830*	100 ± 2*
ISO (1 µM) + FORSK (10 µM)	4	35941 ± 4358*	111 ± 3*

NOTE: Results are means ± SEM of the number of experiments indicated (N). Preparations were exposed to ISO for 10 min and to FORSK for 8 min. In the ISO + FORSK experiment, preparations were exposed to the combination of drugs for 8 min.

*Significantly different from control ($p < 0.005$).

Figure 9:

Experimental protocol showing actions and interactions of low doses of isoproterenol (150) and forskolin (FORSK) on coronary arteries. Tissues were incubated for 2 hrs prior to the addition of 124 mM KCl.

Figure 10:

Experimental protocol showing actions in interactions of high doses of isoproterenol (ISO) and forskolin (FORSK) on coronary arteries. Tissues were incubated for 2 hrs prior to the addition of 124 mM KCl.

Figure 11:

The effects of forskolin (0.1 μ M and 10 μ M) on isoproterenol-induced relaxation (●---●) and cyclic AMP elevation (○—○) in bovine coronary arteries. Preparations were exposed to isoproterenol for 10 min and to forskolin for 8 min. In the combination experiments, preparations were exposed to the combination of drugs for 8 min. Cyclic AMP levels and tension were measured in the same arterial strips. Values represent averages of 5-7 experiments. Standard errors were within the limits of 10% in all the values and were not included in the figure for the sake of clarity.

Figure 12:

The effect of isoproterenol ($0.1 \mu\text{M}$) on forskolin-induced relaxation ($\bullet---\bullet$) and cyclic AMP elevation ($\circ---\circ$) in bovine coronary arteries. Preparations were exposed to isoproterenol for 10 min and to forskolin for 8 min. In the combination experiments, preparations were exposed to the combination of drugs for 8 min. Cyclic AMP levels and tension were measured in the same arterial strips. Values represent averages of 5-7 experiments. Standard errors were within the limits of 10% in all the values and were not included for the sake of clarity.

Table III. Effects of isoproterenol (ISO) and forskolin (FORSK) on soluble cyclic AMP-dependent protein kinase activity measured in the presence and absence of 0.5 M NaCl. Coronary arteries were exposed to isoproterenol for 10 min and to forskolin for 8 min. In the combination experiments, preparations were exposed to the combination of drugs for 8 min. Values are expressed as means $\pm$ SEM of the number of experiments indicated (N). Asterisks indicate significant differences from corresponding controls ($p < 0.05$).

TREATMENT	N	PROTEIN KINASE ACTIVITY (pmoles P/ mg protein)					
		-NaCl			+NaCl		
		-cAMP	+cAMP	-cAMP/+cAMP	-cAMP	+cAMP	-cAMP/+cAMP
CONTROL	8	579 $\pm$ 70	3023 $\pm$ 281	0.19 $\pm$.02	576 $\pm$ 83	1214 $\pm$ 157	0.46 $\pm$.02
ISO (1 μ M)	8	588 $\pm$ 40	2565 $\pm$ 171	0.22 $\pm$.01	694 $\pm$ 41	1053 $\pm$ 72	0.65 $\pm$.01*
FORSK (0.1 μ M)	7	518 $\pm$ 43	3083 $\pm$ 172	0.17 $\pm$.06	720 $\pm$ 66	1244 $\pm$ 95	0.58 $\pm$.03*
FORSK (1 μ M)	6	625 $\pm$ 71	2394 $\pm$ 247	0.28 $\pm$.04*	648 $\pm$ 60	981 $\pm$ 79	0.66 $\pm$.03*
FORSK (10 μ M)	6	803 $\pm$ 90	2141 $\pm$ 315	0.38 $\pm$.02*	740 $\pm$ 74	1150 $\pm$ 160	0.66 $\pm$.03*
ISO (1 μ M) +FORSK (10 μ M)	4	1263 $\pm$ 290*	1523 $\pm$ 386*	0.84 $\pm$.04*	899 $\pm$ 218	1063 $\pm$ 222	0.84 $\pm$.03*

Figure 13:

Effect of NaCl concentration in the homogenizing buffer on basal (without added cyclic AMP in the final incubating mixture, ●---●) and total (with 2 μ M cyclic AMP in the final incubation mixture, ●—●) protein kinase activity in the supernatant fractions prepared from control coronary arteries. A whole homogenate (10 ml/g) of coronary artery was prepared in NaCl-free buffer, aliquots were taken, and varying amounts of NaCl were added. Aliquots were centrifuged at 30,000 x g for 15 min to obtain supernatant fractions. Assays were carried out under the routine conditions described in Methods. Values represent means $\pm$ SEM for 4 experiments.

Figure 14:

Effect of NaCl concentration in the homogenizing buffer on basal (without added cyclic AMP in the final incubation mixture, ●---●) and total (with 2 μ M cyclic AMP in the final incubation mixture, ●—●) protein kinase activity in the supernatant fractions prepared from coronary arteries treated with 1 μ M isoproterenol. A whole homogenate (10 ml/g) of coronary artery was prepared in NaCl-free buffer, aliquots were taken, and varying amounts of NaCl were added. Aliquots were centrifuged at 30,000 x g for 15 min to obtain supernatant fractions. Assays were carried out under the routine conditions described in Methods. Values represent means $\pm$ SEM for 4 experiments.

Figure 15:

Inhibition of soluble protein kinase activity by addition of NaCl to the homogenizing medium and reversal of the NaCl-induced enzyme inhibition by dialysis. A whole homogenate of coronary artery (10 ml/g) was prepared in NaCl-free buffer and divided into 3 aliquots. No NaCl was added to the first aliquot (control), whereas 0.5M NaCl was added to the remaining two aliquots. Aliquots were centrifuged at 30,000 x g for 15 min. The supernatant fractions from the first two aliquots were kept on ice, and the third aliquot was subjected to dialysis for 2 hrs using dialysis tubing which can remove ions less than 12,000 mol. wt from the supernatant. After 2 hrs, protein kinase activity in these three aliquots was estimated as described in Methods. Values represent means $\pm$ for 6 experiments.

Figure 16:

Effect of addition of cyclic AMP to the whole homogenate on protein kinase activity in supernatant and particulate fractions. Basal and total protein kinase activities were estimated in the absence and presence of 2 μ M cyclic AMP in the final incubation mixture as described in Methods (mean data from 2 experiments).

Table IV. Effects of isoproterenol (ISO) and forskolin (FORSK) on cyclic AMP levels, soluble and particulate cyclic AMP-dependent protein kinase activity and tension in bovine coronary artery. Results are expressed as means $\pm$ SEM of the number of experiments indicated (N). Asterisks indicate significant differences from corresponding controls ($p < 0.05^*$; $p < 0.01^{**}$).

TREATMENT	N	PROTEIN KINASE ACTIVITY (pmoles P/mg protein)				% INCREASE IN cAMP	% RELAXATION
		SUPERNATANT		PELLET			
		-cAMP	+cAMP	-cAMP	+cAMP		
CONTROL	16	487 ± 47	2548 ± 201	382 ± 34	1672 ± 106	—	—
ISO (1 μM, 1 min)	5	516 ± 48	2310 ± 142	625 ± 94*	2329 ± 238*	43 ± 13*	21 ± 2**
ISO (1 μM, 10 min)	8	552 ± 43	2430 ± 148	733 ± 115**	2346 ± 238**	284 ± 58**	82 ± 3**
FORSK (0.1 μM, 8 min)	5	412 ± 48	2617 ± 255	614 ± 60**	2395 ± 228**	452 ± 167**	0
FORSK (1 μM, 8 min)	5	522 ± 50	2059 ± 142	639 ± 53**	2526 ± 98**	851 ± 241**	46 ± 2**
FORSK (10 μM, 8 min)	3	849 ± 116*	1784 ± 19**	1340 ± 171**	2521 ± 106**	3045 ± 873**	94 ± 5**

DISCUSSION

In agreement with previous reports in the literature (Kukovetz et al., 1979), isoproterenol elevated cyclic AMP levels and caused relaxation of potassium-evoked contractions in helical strips of bovine coronary artery. The cyclic AMP elevation appeared to be correlated with relaxation in both a time- and dose-dependent manner. In addition, our results show that the diterpene, forskolin, can also produce time- and dose-dependent increases in cyclic AMP levels in these preparations. Higher concentrations of forskolin (0.5-10 μM) markedly elevated cyclic AMP levels and relaxed the muscles. The cyclic AMP elevation and the relaxation caused by a maximally-effective dose of forskolin (10 μM) were well correlated on a temporal basis. However, cyclic AMP elevation and relaxation were not well correlated at lower concentrations of the drug. For example, 0.1 μM forskolin increased cyclic AMP levels approximately 5.5 fold, but did not relax the muscles. This is in marked contrast to the results with isoproterenol, where a much smaller elevation of cyclic AMP by 1 μM isoproterenol was accompanied by an almost complete relaxation of the arteries (see Table 2).

If cyclic AMP elevation is responsible for the relaxation caused by isoproterenol, as suggested by Kukovetz et al., (1979) the question arises as to why a large elevation of cyclic AMP produced by 0.1 μM

forskolin is not accompanied by relaxation. Hormonal effects such as vascular relaxation may be a consequence of selective activation of specific cyclic AMP-dependent protein kinases (cA kinases). It is conceivable that low doses of forskolin may elevate cyclic AMP in a compartment which does not have access to these kinases, whereas higher doses may increase cyclic AMP levels in all compartments. Experiments were undertaken to determine whether differences in activation of cA kinases by forskolin and isoproterenol can explain our results.

It has recently been reported that bovine coronary arteries contain cA kinase and that stimulation of this kinase is well correlated to relaxation of the arteries by isoproterenol (Silver et al., 1982). These authors further noted that activation of the enzyme by isoproterenol could be demonstrated only if the tissues were homogenized in the presence of 0.5 M NaCl. Similar results were obtained in the present study. For example, dose-dependent increases in cA kinase activity ratios by isoproterenol were observed in our experiments in the presence, but not in the absence, of 0.5 M NaCl. The results of our experiments with isoproterenol are therefore consistent with the conclusion of Silver et. al., that activation of kinase may play a role in the relaxation of coronary arteries by isoproterenol. However, using the same techniques, we were able to demonstrate a similar activation of the protein kinase by a concentration of forskolin (0.1 μ M) which did not relax the arteries. Thus, as was the case with cyclic AMP levels, activation of cA kinase did not always correlate well with relaxation in this tissue.

One striking difference between our results and those of Silver et al., (1982) is the effect of NaCl on total cA kinase activity (measured in the presence of 2 μ M cyclic AMP). Silver et al. noted that high NaCl did not affect the total kinase activity, and they suggested that the reason the isoproterenol-induced activation of the enzyme could be observed only in the presence of high NaCl was that the high ionic strength prevented the reassociation of the catalytic and the regulatory subunits during the assay procedure. However, in our experiments, total cA kinase activity was markedly inhibited by high NaCl (see Results). This decrease in total kinase activity is largely responsible for the increase in kinase activity ratios observed in our experiments in the presence of high NaCl, in both control and drug-treated arteries. The reason for this difference between our results and those of Silver et al., is not clear. Our results do agree with those of Palmer et al., (1980) and Singh (1981) who reported that high ionic strength inhibited the total kinase activity in liver and bovine carotid artery, respectively. Furthermore, Jimenez et al., (1982) suggested that high concentrations of salt could induce conformational changes in sulfhydryl groups in the catalytic subunit of protein kinase isolated from rabbit skeletal muscle, with the resultant inactivation of the enzyme. The results of the present study in bovine coronary artery are consistent with these earlier reports in other tissues.

In the presence of high tissue levels of cyclic AMP, an apparent translocation of total protein kinase activity from the cytosol to a particulate fraction has been observed in several tissues (Palmer et al., 1974; Korenman et al., 1974). It was suggested that this

translocated protein kinase might phosphorylate certain specific membrane-bound substrates to produce the physiological responses attributed to cyclic AMP elevation in these tissues. However, the physiological relevance of this translocation phenomenon was questioned by Keely et al., (1975) and Corbin et. al., (1977). These authors suggested, based on studies in cardiac muscle, that the observed translocation of the kinase to a particulate fraction might be due to nonspecific binding of the catalytic subunit to membrane fragments after homogenation, as a result of the low ionic strength used in the homogenization buffer. They suggested that the addition of NaCl to the homogenization medium would prevent this nonspecific binding and would yield results more representative of conditions in the intact cells. In a later paper, Krall et al., (1978) demonstrated a translocation of the enzyme into a particulate fraction in isoproterenol-treated rat myometrium even in the presence of high ionic strength in the homogenization medium. It was suggested that translocation might be a unique phenomenon associated with hormonal regulation of smooth muscle function. Similar results have been obtained by Harbon et al., (1978), also in rat myometrium, and by Mednieks and Hand (1982) in rat parotid gland.

In the present experiments in bovine coronary arteries, a significant loss of total cA kinase activity from the supernatant fraction was observed under conditions associated with high tissue levels of cyclic AMP (see Table III). The possibility that the kinase might be translocating to the particulate fraction, as described above, was therefore considered. Some evidence in favour

of this possibility was obtained in the experiments showing that addition of 2 μM cyclic AMP to the homogenate produced a decrease in total kinase activity in the supernatant, and a corresponding increase in the particulate fraction (Figure 16). The relative distribution of protein kinase activity in the soluble and particulate fractions in bovine coronary artery was therefore studied in preparations treated with isoproterenol or forskolin.

Significant increases in kinase activity in the particulate fraction were observed in all cases. Silver et al., (1982) were unable to demonstrate an increase in the soluble protein kinase activity ratio in coronary arteries exposed to 1 μM isoproterenol for 1 min. However, a significant increase in protein kinase activity in the particulate fraction was observed in the present study, even at this time point, although cyclic AMP levels in the tissue were only slightly elevated and the muscles had just begun to relax (Table 2). Thus, in our experiments, determination of the kinase activity in the pellet appeared to be a good indicator of expected enzyme activation in the cells and appeared to correlate well with relaxation by isoproterenol. It was hoped that when the effects of forskolin were examined using this technique, differences in activation of the kinase might be apparent which would help to explain the lack of a relaxant effect of low doses of forskolin. However, a significant increase in particulate protein kinase activity was observed with 0.1 μM forskolin, which had no relaxant effect on these preparations. Thus, even when measured in this way, activation of cA kinase was not always accompanied by relaxation in this tissue.

It has been suggested that in many cases, potentiation of a hormonal response by low doses of forskolin may be a good indicator of the involvement of cyclic AMP in the response (Seamon and Daly, 1983). In the present experiments, when high doses of forskolin ($> 1 \mu\text{M}$) and isoproterenol were added in combination, the cyclic AMP increase observed in the coronary arteries was substantially greater than the sum of the increase produced by each agent alone. These results are consistent with the potentiation of hormonal effects on cyclic AMP levels observed with forskolin in other tissues (Seamon and Daly, 1981b). However, lower doses of forskolin, which themselves significantly increased cyclic AMP levels, failed to potentiate the effects of isoproterenol on tension or cyclic AMP levels in coronary arteries. In fact, at the lowest concentrations used ($0.01\text{--}0.1 \mu\text{M}$), the combined effects of the agents were less than additive. Thus, the interaction between the two agents appeared to be one of potentiation at high concentrations of forskolin and competition at lower concentrations. At the present time we have no explanation for these results. It is possible that multiple binding sites for forskolin and/or isoproterenol may exist on the adenylate cyclase complex and interactions may depend on the relative affinities of these sites for the agonists. Further studies along these lines may shed some light on the apparently complex interactions between these two agents on adenylate cyclase in this tissue. In any case, it is apparent that potentiation of hormonally-induced activation of adenylate cyclase by low concentrations of forskolin does not occur in all tissues. Thus, although forskolin may still be a useful tool

in elucidating the role of cyclic AMP in various biochemical and physiological processes, caution must be used in interpreting the results of such experiments.

Dissociations between forskolin-induced cyclic AMP elevation, cA kinase activation and physiological responses have also been reported in other tissues. In rat adipocytes, for example, 1 μ M forskolin increased cyclic AMP levels 4 fold without stimulating lipolysis, whereas a similar elevation of cyclic AMP by isoproterenol was accompanied by marked activation of lipolysis. Higher doses of forskolin (10-100 μ M) resulted in very large increases in cyclic AMP followed by increased lipolysis (Litosch et al., 1982). In the thyroid, where many of the effects of TSH are assumed to be mediated through cyclic AMP, forskolin increased cyclic AMP levels and stimulated protein kinase activity without mimicking the effects of TSH on iodide organification or glucose oxidation (Totsuka et al., 1983). These results are analogous to the present observations in bovine coronary arteries. Thus, as Seamon and Daly (1983) have cautioned, a direct correlation between cyclic AMP elevation and a physiological response may not always be observed when comparing the effects of forskolin and other hormones.

If it is assumed that elevation of cyclic AMP and activation of cA kinase are responsible for the vascular relaxation caused by isoproterenol, as suggested by several lines of evidence, then some explanation must be provided for the dissociation between relaxation and protein kinase activation observed in our experiments with lower concentrations of forskolin. One possible explanation is that some

form of functional compartmentalization of the protein kinase exists within the cells as suggested by others (Hayes and Brunton, 1982), and that low doses of forskolin activate the kinase in a compartment which does not affect the contractile apparatus. The arterial preparations used in our experiments contain other cell types in addition to smooth muscle cells, and it is also possible that low doses of forskolin activate protein kinase in nonmuscle cells which again would not affect the contractile activity of the arteries. The apparent similarity between the effects of isoproterenol and forskolin on the distribution of the protein kinase between soluble and particulate fractions in our experiments might tend to argue against the first possibility, but further experiments will be necessary in order to decide whether either explanation can account for our observations. A final possibility which should also be considered is that, in spite of the weight of correlative evidence from various sources, elevation of cyclic AMP and activation of cA kinase may not be directly responsible for the relaxant effects of these drugs in coronary arteries.

A hypothetical model for the possible mechanism of action of isoproterenol and forskolin in vascular smooth muscle is shown in Fig. 17. Isoproterenol, through β -receptor binding, and forskolin, by its direct action, activate the enzyme adenylate cyclase. This activation leads to increased formation of cAMP in the smooth muscle cells. The increased cAMP in the cytosol dissociates the holo-enzyme (RC) into regulatory (R) and catalytic (C) subunits. The dissociated active catalytic subunits translocate into the membrane fraction and phosphorylate certain specific proteins (S).

Figure 17:

Hypothetical model showing the possible mechanism of action of isoproterenol and forskolin on coronary arteries.

We cannot rule out the possibility that the elevated cAMP can directly activate the membrane bound cA kinase. It is known that two isozymes of cA kinase are present in coronary arteries (Silver et. al., 1982). Isozyme I makes up 46% and isozyme II makes up 51% of the total enzyme activity. It has also been noted in several tissues that major portion of isozyme II is bound to the membrane fractions (Corbin and Keely, 1977). It is logical to assume that Isozyme II in coronary arteries may also be bound to the membrane fraction. Silver et. al., (1982) have suggested that isoproterenol might produce relaxation in coronary arteries by specifically activating isozyme II. Therefore, it is possible that elevation of cAMP in this tissue can also stimulate the membrane bound isozyme. This would result in dissociation of catalytic subunits in the membrane. Either this dissociated catalytic subunit in the membrane or the one translocated from the cytosol to the membrane or both, might phosphorylate certain membrane bound specific proteins which are associated with relaxation of vascular smooth muscle.

SUMMARY AND CONCLUSIONS

1. Elevation of cAMP appeared to be correlated with relaxation of bovine coronary arteries by isoproterenol and high doses of forskolin in both a time-dependent and dose-dependent manner.
2. A dissociation between elevation of cAMP and relaxation was obtained with low doses of forskolin. Greater increases in cAMP levels were produced by non-relaxant concentrations of forskolin than by relaxant concentrations of isoproterenol.
3. Estimation of cyclic AMP-dependent protein kinase activity in the coronary arteries demonstrated a possible translocation of protein kinase activity from the soluble to the particulate fractions of the arteries with several treatments.
4. Activation of particulate protein kinase and relaxation of the coronary arteries appeared to be correlated with isoproterenol and high doses of forskolin. However, low doses of forskolin also activated the particulate protein kinase without producing any relaxation.
5. Higher doses of forskolin potentiated isoproterenol-induced elevation of cAMP. However, in contrast to previous results in the literature, low concentrations of forskolin ($\leq 1 \mu\text{M}$), which

themselves markedly elevated cAMP levels in the arteries, did not potentiate the effects of isoproterenol on cAMP levels or tension in these preparations.

In conclusion, a dissociation between elevation of cAMP, activation of cyclic AMP-dependent protein kinase and relaxation of bovine coronary arteries was obtained with low doses of forskolin. This might be partly due to the functional compartmentation of protein kinase or cellular heterogeneity of the tissue. Our results with isoproterenol, appear to be consistent with a causal role of cAMP and cA kinase in β -adrenergic stimulant-induced vascular smooth muscle relaxation. However, results with forskolin do not appear to support a generalization of an exclusive role of cAMP and cA kinase in smooth muscle relaxation. Further studies are necessary to prove or disprove this hypothesis. Our results also suggest that caution should be used in interpreting data using forskolin as an experimental tool to elucidate role of cyclic nucleotides in smooth muscle function.

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