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Full Name of Author — Nom complet de l'auteur

Milan Joseph Muller

Date of Birth — Date de naissance	Country of Birth — Lieu de naissance
July 8 1953	Canada

Permanent Address — Résidence fixe

319 Montmorency Dr.,
Hamilton, Ontario, Canada,
L8K 5H3

Title of Thesis — Titre de la thèse

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1985	Dr. H.P. Baer

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Date	Signature
Nov 10 / 1984	M. J. Muller

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ADENOSINE ACTION AND CYCLIC AMP IN SMOOTH MUSCLE

by

MILAN J. MULLER

A THESIS

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled "Adenosine action and cyclic AMP in smooth muscle", submitted by Milan J. Muller in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Pharmacology.

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Supervisor

....

....

....

....

....

External Examiner

Date December 19 1984

For: Valeria, Milan, Mary and Andy.

ABSTRACT

Although cyclic AMP appears to be the second messenger responsible for many of the biological effects of adenosine, the mechanism of adenosine receptor-mediated relaxation of smooth muscle remains controversial. In light of this controversy and of our current understanding at the molecular level of the adenylate cyclase complex, we have examined the hypothesis that cyclic AMP is involved in the action of adenosine in smooth muscle. Also, the unique properties (discussed below) of the diterpene forskolin allowed us to examine the direct effects of cyclic AMP on the contractility of smooth muscle.

We found that at a high concentration (1 mM) adenosine increased cyclic AMP levels in strips of beef coronary arteries obtained from branches of the left descending coronary artery. At a lower concentration (0.1 mM), adenosine caused a trend towards increasing cyclic AMP levels above basal levels; however, this trend was not significant. This approach proved to be unfruitful and neither supports nor refutes the findings of others.

In broken cell preparations from the guinea pig taenia caeci and the longitudinal muscle of the rabbit small intestine, adenylate cyclase activity was increased by GTP, Gpp(NH)p, NaF and forskolin. Therefore, the guanine nucleotide binding site and the catalytic unit of the adenylate cyclase complex were present and functional in these systems. On the other hand, isoproterenol and adenosine or adenosine analogs did not alter or decrease basal adenylate cyclase activity. Adenosine receptor-mediated stimulation of adenylate cyclase activity was not demonstrated despite a number of measures which were taken to optimize

the conditions. Thus, deoxy-alpha-[^{32}P]-ATP was used as substrate in the assay system. Preparations were treated with adenosine deaminase. GTP or Gpp(NH)p were used in combination with adenosine or analogs of the nucleoside. Finally, the longitudinal muscle from the rabbit small intestine was fractionated. In addition, our results from tissue bath studies with adenosine analogs also support the conclusion that adenosine receptors in smooth muscle differ from those known to be coupled to adenylate cyclase in other cell types.

Forskolin caused a concentration-dependent relaxation of beef and dog coronary arteries, rat aorta, guinea pig taenia caeci and rabbit small intestine. Also, the diterpene caused a concentration-dependent increase in adenylate cyclase activity in broken cell preparations from the intestinal smooth muscles. These data suggest that forskolin-mediated relaxation of smooth muscle occurs via cyclic AMP. Thus cyclic AMP <u>per se</u> can cause smooth muscle relaxation.

We observed inconsistent effects of phosphodiesterase inhibitors on agents which relax smooth muscle. Thus forskolin action was significantly potentiated by Ro 20-1724 in the guinea pig taenia caeci and rabbit small intestine but by MIX in the latter tissue only. Neither phosphodiesterase inhibitor altered the relaxation by forskolin of beef coronary arteries. These data are difficult to interpret and exemplify the problem of using phosphodiesterase inhibitors to investigate the role of cyclic AMP in smooth muscle.

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1 INTRODUCTION

1.1 OVERVIEW

In this dissertation the smooth muscle relaxant action of adenosine is explored, with particular reference to the suggestion of Sutherland and Rall (1960) that receptor-mediated relaxation of smooth muscle occurs via a mechanism involving cyclic AMP (adenosine 3':5'-cyclic monophosphate). Experimental proof of this has to be concerned with two aspects. Firstly, the interaction of hormones or mediators with receptors must elevate cyclic AMP levels. Secondly, cyclic AMP must cause relaxation.

In the first part of the introduction the current understanding of receptor-adenylate cyclase interactions and also the regulation of smooth muscle contractility by cyclic AMP are reviewed. Two things become apparent. First, we are beginning to understand the intricacies of the hormone-receptor/adenylate cyclase complex, albeit in systems other than smooth muscle. Second, the molecular events comprising cyclic AMP-mediated smooth muscle relaxation remain speculative. The remainder of the introduction deals with the evidence that two hormones in particular, adrenaline and adenosine, relax smooth muscle via a cyclic AMP mechanism. In addition, the unique properties of forskolin which make it a useful tool to study the role of cyclic AMP in biological systems are discussed.

1.2 MOLECULAR ASPECTS OF HORMONE-MEDIATED ALTERATION OF CYCLIC AMP AND SUBSEQUENT SMOOTH MUSCLE RELAXATION

The molecular components and kinetics of adenylate cyclase which catalyse the conversion of ATP (adenosine 5'-triphosphate) to cyclic AMP and PPi, have been the subject of intensive research in recent years. It should be noted that these studies have involved systems other than smooth muscle. A schematic representation, of the adenylate cyclase complex and the possible mechanism(s) by which cyclic AMP could cause smooth muscle relaxation, is shown in figure 1.

1.2.1. COMPONENTS OF THE ADENYLATE CYCLASE COMPLEX

Classically, the adenylate cyclase complex has been envisioned as consisting of two components within the cell membrane: the hormone receptor and the catalytic site (cf. Perkins, 1973). It is now generally accepted that there are at least three distinct components; the hormone receptor (R), the catalytic site (C) and the guanine nucleotide binding site (N or G/F) (cf. Rodbell, 1980). Since some hormones stimulate while others inhibit adenylate cyclase activity, and in both cases R or N units are required which show distinct properties, Rodbell (1980) has designated the functional stimulatory- and inhibitory- receptor/guanine nucleotide binding site units as RsNs and RiNi, respectively.

Ross and Gilman (1980) have reviewed the biochemical data used to resolve R and C. Support for the concept that R's are proteins distinct from the C comes from kinetic studies (cf. Perkins, 1973),

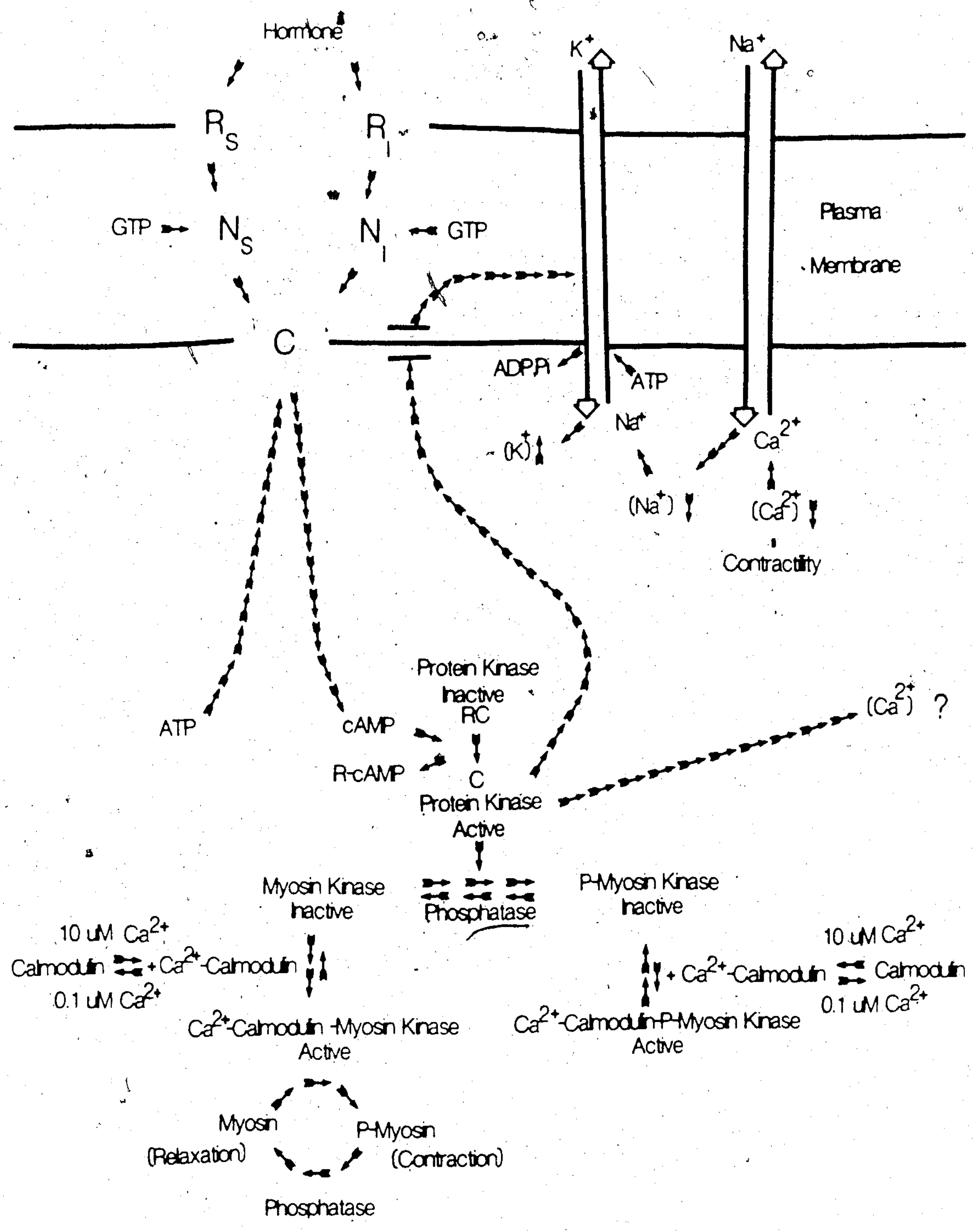


FIG. 1 Schematic representation of hormone effects on the adenylate cyclase complex and subsequent events leading to smooth muscle relaxation. This is a composite diagram of models proposed by Rodbell (1980), Adelstein _et al_. (1980) and Scheid _et al_. (1979).

observations of their independent ontogenetic regulation (Charness et al., 1976; Bilezekian et al., 1977; Harden et al., 1977; Harden et al., 1979), and studies utilizing chemical (Schramm and Naim, 1970; Schramm, 1976) or genetic (Insel et al., 1976) manipulations. The cell fusion experiments of Orly, Schramm and coworkers (Orly and Schramm, 1976; Schramm et al., 1977; Schulster et al., 1978; Schramm, 1979) provided more direct evidence. Sendai virus was used to fuse Friend erythroleukemia cells to turkey erythrocytes. The former have C but lack R, while the latter were treated with N-ethylmalemide to inactivate C and thus only contained R. Fusion of the Friend cells with turkey erythrocytes resulted in cells which displayed catecholamine-stimulatable adenylate cyclase activity. Finally, with the physical separation and molecular characterization of adenylate cyclase and beta-adrenoceptor, utilizing detergent solubilization and separation by gel exclusion chromatography and sucrose density gradient centrifugation (Haga et al., 1977; Limbird and Lefkowitz, 1977) or a beta-adrenoceptor affinity matrix (Vauquelin et al., 1977), there is little doubt that these components of the adenylate cyclase complex are separate proteins.

Two approaches have been used to demonstrate that N is a protein distinct from either R or C. Pfeuffer (Pfeuffer and Helmreich, 1975; Pfeuffer, 1977; Cassel and Pfeuffer, 1978; Pfeuffer, 1979) solubilized adenylate cyclase from pigeon erythrocyte membranes with Lubrol PX and separated the components by passing the extract over a column of GTP-substituted agarose (guanosine 5'-triphosphate substituted agarose). Using the photoaffinity ligand GTP-gamma-azidoanilide, Pfeuffer showed that N was a heat-stable 42,000 molecular weight protein. Ross and Gilman (1977 and 1978) showed that C and N cyclase units were separate

proteins by utilizing their differential heat-stabilities and somatic cell variants that were genetically deficient in one or the other protein (Ross and Gilman, 1977; Ross et al., 1978). It is now thought that N in its native state is oligomeric with a molecular weight of 90,000-130,000 (Ross and Gilman, 1980). The subunits have molecular weights of 42,000 and 55,000 with the latter acting as a substrate for ADP-ribosylation (adenosine 5'- diphosphate). A 35,000 dalton component, that appears to be essential for transduction but is not ADP-ribosylated by C, has also been isolated (Sternweis et al., 1981).

1.2.1.1 INTERACTION OF THE COMPONENTS OF THE ADENYLATE CYCLASE COMPLEX

Several models have been proposed to explain the interaction of the components of the adenylate cyclase complex. The "mobile receptor hypothesis" states that receptors, which can diffuse independently in the plane of the membrane, reversibly associate with effectors to regulate their activity. Furthermore, the affinity for the effector is greater when the receptor is occupied by hormone (Jacobs and Cuatrecasas, 1976.) Bergman and Hechter (1978) proposed a "random-hit matrix model" to explain the dynamic and steady state relationships between occupation of bovine renal medullary receptors by (Lys-8) vasopressin and neuro-hypophyseal hormones and activation of adenylate cyclase. Although these models merit attention and explain many of the experimental observations concerning receptor-mediated adenylate cyclase activation, neither takes into account the N component of the cyclase complex.

Two models have emerged which account for the interaction of R, N and C: the "disaggregation-coupling model" of Rodbell (1980) and the "exchange collision-coupling model" of Levitzki (1982).

Rodbell (1980) reviewed evidence, obtained by the technique of target size analysis in liver membranes (Schlegel et al., 1979; Rodbell, 1980), to suggest an association between R and N under steady state conditions. The R/N units are thought to exist as oligomers, constituting a domain distinct from C. The "turn on" process involves hormone- and GTP-binding to R and N, respectively. The primary event is the disaggregation of R/N oligomers to R/N monomers which can then interact with C. The R/N/C complex is the active form which converts ATP to cyclic AMP and PPi. In this model, the "turn off" reaction was suggested to be the reaggregation of the R/N units into oligomers as oppossed to GTP-ase activity (cf. Rodbell, 1980).

In turkey erythrocyte membranes, data obtained by target size analysis suggests that R/N units do not exist as oligomers (Rodbell, 1980). In this system the beta-adrenoceptor/adenylate cyclase complex has been studied in detail by Levitzki and coworkers (Cassel et al. 1977; Tolkovsky and Levitzki, 1978; Arad and Levitzki, 1979). They made the following observations: the rate constants "k-on" and "k-off" follow first order kinetics, neither GTP nor GDP (guanosine 5'-diphosphate) are rate limiting, the binding of regulatory ligands (hormones and GTP) is random and independent, and "k-on" appears to be linearly dependent on the receptor concentration. According to Levitzki (1982), the "exchange collision-coupling model" is the only model which could account for these data. The model states that, when hormone and GTP are added to the system, there is a transient encounter between an activated form of R/N and an inactivated form of N/C. The N components are exchanged to yield an activated form of N/C which then catalyses the conversion of ATP to cyclic AMP and PPi. The exchange of N units between R and C

therefore constitutes the primary event in the "turn-on" reaction. Although hydrolysis of GTP to GDP by GTP-ase depicts the "turn-off" reaction in this model, evidence for this is lacking (cf. Rodbell, 1980; Levitzki, 1982).

In summary, although the components of the adenylate cyclase complex (R, N and C) may be identical in all cell types, the structural arrangement and the interaction of these components may differ considerably among cell types. In the author's opinion, both of the above models of the interaction of the components of the adenylate cyclase complex may be correct depending on the cell type concerned; neither model is necessarily applicable to smooth muscle cyclase.

1.2.2 POSSIBLE MECHANISM(S) OF CYCLIC AMP-MEDIATED SMOOTH MUSCLE RELAXATION.

Activation of cyclic AMP-dependent protein kinases by cyclic AMP is the common primary event in any mechanism involving the second messenger. Phosphorylation of specific proteins by cyclic AMP-dependent protein kinase constitutes the common secondary event. Since there is little doubt that the intracellular calcium concentration and smooth muscle contractility are associated (for review see: Somlyo and Somlyo, 1968), the possible mechanisms by which cyclic AMP causes smooth muscle relaxation must either describe regulation of calcium levels or sensitivity of the contractile apparatus to calcium by some phosphorylation process.

Scheid _et al_. (1979) studied the mechanism of beta-adrenergic relaxation of isolated smooth muscle cells from the stomach of _Bufa_

marinus. Their model (see figure 1) is based on activation of sodium-potassium ATPase by phosphorylation of an associated protein, resulting in increased intracellular concentration of potassium and decreased intracellular concentration of sodium. The increased sodium gradient would accelerate the rate of sodium/calcium exchange at the plasma membrane, sarcoplasmic reticulum and/or mitochondrial membranes, resulting in increased efflux of calcium from the cytoplasm. Ultimately, the change in free intracellular calcium would decrease contraction.

Models of cyclic AMP-mediated smooth muscle relaxation which do not depend on the control of the intracellular calcium concentration but rather on the phosphorylation of the contractile proteins, myosin and actin are as follows. The biochemical events, by which phosphorylation of myosin kinase by cyclic AMP-dependent protein kinase can lead to smooth muscle relaxation, are shown in figure 1 (Adelstein *et al*., 1980). Inherent in this model, the reversible phosphorylation of myosin is thought to be the dominant regulatory system controlling actin-myosin interactions in smooth muscle, and the calcium-binding protein plays a major role in this regard (for review see: Adelstein and Eizenberg, 1980). Finally, it has been suggested by Wallach *et al*. (1977) that filamin, an actin-binding protein which is phosphorylated by protein kinase and is found in a variety of smooth muscles, may be involved in cyclic AMP-mediated smooth muscle relaxation. However, the biochemical events leading to smooth muscle relaxation via filamin have not been studied.

Thus, evidence and theories exist to suggest that cyclic AMP-mediated relaxation of smooth muscle occurs via mechanisms involving

calcium, actin and myosin. There is insufficient evidence to accept one mechanism and reject the others. The possibility, that all of these mechanisms co-exist to varying degrees in different smooth muscles, cannot be ruled out; the relative importance of these mechanisms in a particular smooth muscle remains speculative.

1.3 EVIDENCE FOR THE INVOLVEMENT OF CYCLIC AMP IN HORMONE-MEDIATED SMOOTH MUSCLE RELAXATION

Sutherland *et al*. (1968) suggested four criteria which must be satisfied to establish cyclic AMP involvement in hormone-mediated smooth muscle relaxation. Thus, in a cell free system the hormone should stimulate the adenylate cyclase enzyme. Cell free systems and broken cell preparations are terms used to designate that the tissues or cells have been homogenized. An exogenous cyclic nucleotide or phosphodiesterase inhibitor should cause relaxation of the smooth muscle. Phosphodiesterase inhibitors should potentiate the relaxant effect of the hormone, and finally, hormones should increase cyclic AMP levels in intact cells in a dose- and time-dependent manner consistent with the dose- and time-dependence of relaxation.

1.3.1 MECHANISM OF ACTION OF BETA-AGONISTS IN SMOOTH MUSCLE

To catalogue the observations that support or refute cyclic nucleotide involvement in beta-agonist-induced smooth muscle relaxation is beyond the scope of this introduction. Fortunately, this area has been reviewed extensively (for review see: Baer, 1974; Andersson *et al*., 1975; Namm and Leader, 1976; Schultz and Hardman, 1976; Andersson and

Nilsson, 1977; Hardman et al., 1977; Diamond, 1978; Kramer and Hardman, 1980; Hardman, 1981). At best, reviewers have concluded that evidence does exist to at least "superficially" satisfy the four criteria for implicating a role for cyclic AMP in smooth muscle relaxation caused by beta-agonists (cf. Hardman, 1981). A more definite conclusion is not possible since many conflicting reports have been published.

It has become obvious that technical problems exist both at the level of the broken cell preparation and the intact cell when attempting to satisfy the criteria in the case of smooth muscle. The lack of hormone-sensitive adenylate cyclase in broken cell preparations is often thought to result from an uncoupling of the cyclase complex. In this regard, addition of GTP to some cell free systems has been shown to restore cyclase activity which can be stimulated by beta-agonists (cf. Hardman, 1981). The pharmacological tools used to examine cyclic AMP-mediated relaxation of smooth muscle, phosphodiesterase inhibitors and cyclic nucleotides, lack specificity and thus interpretation of the data is complicated (Hardman, 1981). The question of whether cyclic AMP can cause smooth muscle relaxation is dealt with separately below. Questions have arisen concerning the validity of using cyclic AMP levels in intact cells as a measure of adenylate cyclase activity. Some investigators claim that measurement of cyclic AMP-dependent protein kinase activity is more meaningful (cf. Kramer and Hardman, 1980; Hardman, 1981). Cell homogeneity and the postulate of specific pools of cyclic AMP-dependent protein kinase further complicate interpretation of data obtained in intact cells (cf. Hardman, 1981).

1.3.2 MECHANISM OF ACTION OF ADENOSINE IN SMOOTH MUSCLE

Adenosine has been shown to alter cyclic AMP levels in a variety of cell types including guinea pig cerebral cortex slices (Sattin and Rall, 1970; Shimizu and Daly, 1970), neuroblastoma cells (Schultz and Hamprecht, 1973; Blume et al., 1973), astrocytoma cells (Clark et al., 1974), bone cells (Peck et al., 1974), thrombocytes (Mills and Smith, 1971; Haslam and Rossom, 1975) and rat lung (Palmet, 1971). These observations coupled with the initial suggestions by Sutherland and Rall (1960), that hormone-induced relaxation of smooth muscle may result from increasing intracellular levels of cyclic AMP, have provided the impetus for a relatively small number of investigators to address themselves to the hypothesis that cyclic AMP is involved in adenosine receptor-mediated relaxation of smooth muscle. Others have examined the effects of adenosine on the intracellular concentration of calcium. Since these studies on the role of cyclic AMP and/or calcium in adenosine-mediated smooth muscle relaxation are of primary importance to this thesis, they are examined in some detail below.

1.3.2.1 CYCLIC AMP INVOLVEMENT IN ADENOSINE-MEDIATED SMOOTH MUSCLE RELAXATION

In 1979 Kukovetz and coworkers compiled and reviewed their studies (Wurm et al., 1976; Kukovetz et al., 1977; Kukovetz et al., 1978; Rinner and Wurm, 1978) which provide evidence to support the hypothesis that cyclic AMP mediates adenosine receptor-induced relaxation of smooth muscle. Increases in cyclic AMP levels and the relaxant effects mediated by adenosine, in beef and pig coronary arteries contracted by potassium,

were dose- and time-dependent with the former preceeding the latter. Theophylline antagonized both adenosine-mediated relaxations and increases in cyclic AMP levels, suggesting that both of these adenosine effects were mediated via the adenosine receptor. Adenosine had a biphasic effect on adenylate cyclase from broken cell preparations of beef coronary arteries, ie. stimulatory at low concentrations (1-100 nM) and inhibitory at higher concentrations. Theophylline antagonized only the stimulatory action. This is consistent with the suggestion that the former is a receptor-mediated event while the latter is a "P site" interaction of adenosine. Kukovetz and coworkers went one step further and showed that solubilization of adenylate cyclase with digitonin or polyoxyethylene abolished only the stimulatory effects of adenosine, and this could be partially restored by addition of phospholipids with phosphatidylserine appearing to be the most potent in this regard (1978).

Herlihy et al. (1976) have also examined the role of cyclic AMP in the adenosine-induced relaxation of vascular smooth muscle. This group reported that adenosine caused relaxation of hog carotid median strips contracted with norepinephrine and potassium. Adenosine up to 100 μM did not alter the cyclic AMP content of these strips or of dog coronary artery. Higher concentrations of adenosine (1 mM) did increase cyclic AMP levels significantly. Herlihy et al. (1976) concluded that cyclic AMP was not involved in adenosine-mediated relaxation of vascular smooth muscle. This group's findings and those of Verhaeghe (1977) on calcium involvement in adenosine relaxations in smooth muscle are discussed below.

Verhaeghe (1977) also found that adenosine did not alter intracellular cyclic AMP levels in vascular smooth muscle, although the nucleoside did relax in a dose-dependent manner the dog saphenous vein strips which were contracted with acetylcholine. He concluded that the depression of smooth muscle reactivity by adenosine was not mediated by the 3'5'-cyclic-nucleotide system.

Interestingly, Schror and Rosen (1979) found that adenosine-induced relaxation in isolated bovine coronary artery strips was accompanied by a decrease in cyclic AMP. A low dose of adenosine (0.2 μM) which produced minimal relaxation of the coronary arteries was found to reduce cyclic AMP levels by 68 percent of the control value. By way of a control experiment, Schror and Rosen (1979) showed that norepinephrine relaxed the coronary artery strips and increased the cyclic AMP content of these strips.

There has been only one report (McKenzie et al., 1977), concerning the involvement of cyclic AMP in adenosine action in smooth muscle other than vascular smooth muscle. In the longitudinal muscle of the rabbit small intestine, McKenzie et al. (1977) found that adenosine and various adenosine analogs caused inhibition of smooth muscle spontaneous activity. However, adenosine did not alter cyclic AMP levels in intact tissues. Furthermore, adenylate cyclase which was prepared by gentle homogenization of a smooth muscle fraction isolated by collagenase treatment was inhibited by adenosine and various adenosine analogs. Since the relaxant effects of adenosine and analogs was not related to their inhibition of adenylate cyclase, these authors concluded that cyclic AMP was not related to the mechanism of adenosine-

mediated smooth muscle relaxation.

Huang and Drummond (1979) have isolated microvessels which presumably consist of capillaries and arterioles from guinea pig cerebral cortex and found that Gpp(NH)p (guanosine 5'-imido diphosphate) activated adenylate cyclase in broken cell preparations. Further, the rate of Gpp(NH)p activation was enhanced by adenosine, various adenosine analogs and a variety of other hormones. Activation by adenosine or analogs occurred only when adenosine deaminase was added to destroy endogenously generated adenosine. Huang and Drummond (1979) concluded that at present it is not known whether the stimulation of adenylate cyclase by any of the agents tested relates to their action on vascular tone.

Recently, Anand-Srivastava et al. (1983) have been able to demonstrate stimulation of adenylate cyclase activity in cultured vascular smooth muscle from rat aorta by adenosine and a number of analogs. This stimulation was dependent on guanine nucleotides and magnesium concentration and could be blocked by MIX (3-isobutyl-1-methylxanthine). Adenosine and 2-chloroadenosine stimulated adenylate cyclase at low concentrations and inhibited at higher concentrations, while 2'-deoxyadenosine only inhibited the cyclase. These investigators concluded that vascular smooth muscle contains both Ra and "P site" with the former being responsible for stimulation of adenylate cyclase and hence probably was responsible for smooth muscle relaxation.

Thus, experimental evidence which supports the hypothesis that cyclic AMP mediates adenosine-induced smooth muscle relaxation has been found in bovine and porcine coronary arteries (Kukovetz et al., 1979). Furthermore, stimulation of adenylate cyclase activity by

adenosine has been demonstrated in microvessels from guinea pig cerebral cortex (Huang and Drummond, 1979) and cultured smooth muscle from rat aorta (Anand-Srivastava et al., 1983). However, the hypothesis remains controversial due to the contradictory findings of a number of investigators (Herlihy et al., 1976; Verhaeghe, 1977; Schror and Rosen, 1979). The sole study dealing with this hypothesis in smooth muscle other than vascular smooth muscle (McKenzie et al., 1977) found no support for a role for cyclic AMP in the relaxant effects of adenosine.

1.3.2.2 CALCIUM INVOLVEMENT IN ADENOSINE-MEDIATED SMOOTH MUSCLE RELAXATION

Evidence to suggest that adenosine mediates smooth muscle relaxation via alteration of calcium fluxes has come from a number of different types of studies including mechanical studies with coronary arteries (Herlihy et al., 1976; Schnar and Sparks, 1972) and dog saphenous vein (Verhaeghe, 1977). Schnar and Sparks (1972) compared the mechanical responses of large and small canine coronary arteries to adenosine and various other smooth muscle relaxant agents, and found that adenosine induced a greater relaxation of small than large coronary arteries while the reverse was the case with nitroglycerin. From experiments with high potassium solutions and variation of calcium concentrations, Schnar and Sparks, (1972) concluded that relaxation to adenosine occurred without changes in the electrical state of the membrane and that the nucleoside acted via alteration of calcium fluxes. Since norepinephrine is thought to primarily release intracellular-bound calcium and potassium primarily effects extracellular-bound calcium (Van Breeman et al., 1973) and

Herlihy et al. (1976) found that adenosine relaxed arteries contracted to norepinephrine better than those contracted to potassium, these authors suggested that adenosine to some extent inhibited calcium influx. However, their data with arteries contracted to potassium with various calcium concentrations suggested that adenosine also had an effect on intracellular calcium stores. Verhaeghe (1977) also found evidence to suggest that adenosine affects both intracellular and extracellular calcium pools in dog saphenous veins. This author suggested that the main effect of adenosine was on intracellular calcium pools on the basis of the following evidence: 1) Verapamil blocked spontaneous contractions of the preparation while adenosine did not. 2) Adenosine relaxed norepinephrine-induced contractions in both the presence and absence of calcium and after inhibition of calcium flux by verapamil. 3) Adenosine antagonized calcium-induced contraction in depolarized strips after alpha-noradrenergic blockade in a non-competitive manner. 4) Finally, adenosine relaxed barium-induced contractions. Askar and Mustafa (1982) have also found that adenosine-induced relaxation of beef coronary arteries contracted to KCL was not altered by calcium antagonists D-600, nifedipine, verapamil or diltiazem. In contrast, Merrill et al. (1982) studied the interaction of adenosine and nifedipine, a calcium antagonist known to block calcium uptake in vascular smooth muscle (Nayler and Pool-Wilson, 1981), in intact dog heart. They found that nifedipine attenuated excess coronary flow production by intra-coronary infusion of adenosine and markedly decreased reactive hyperemia following the release of a 30 s interval of left anterior coronary artery occlussion.

Early studies on electrical activity in guinea pig taenia caeci

using sucrose gap demonstrated that inhibition of spontaneous spike discharge and hyperpolarization by adenosine amongst other compounds were associated with their relaxant effects. These effects have been attributed to a decrease in sodium permeability (Imai and Takeda, 1967) or a decrease in calcium influx (Axelsson and Holmberg, 1969). Harder et al. (1979) used intracellular microelectrodes to study the electrical properties of isolated large and small coronary arteries of the dog. Action potentials were induced by electrical stimulation of the arteries in the presence of tetraethylammonium (TEA) and they found that calcium carries most of the inward current since the amplitude of the action potential increased as a function of log extracellular calcium concentration; the slope of the curve being 30 mV/decade. These action potentials have also been shown to depend mainly on the inward calcium current in guinea pig mesenteric artery (Harder and Sperelakis, 1978) and rabbit ear artery (Droogmans et al., 1977). Adenosine blocked the calcium inward current preferentially in small coronary arteries and nitroglycerin blocked the calcium inward current preferentially in large coronary arteries (Harder et al., 1979). Verapamil blocked the calcium inward current in both large and small coronary arteries (Harder et al., 1979).

Fenton et al. (1982) have studied the effects of adenosine on uptake of 45-calcium in primary vascular smooth muscle monolayers from rat aorta and porcine carotid artery strips. In both preparations, adenosine reduced the calcium uptake in cells stimulated with 25 mM potassium and had no apparent effect on the efflux of intracellular 45-calcium. This is the only direct evidence in smooth muscle for inhibition of calcium influx by adenosine. These authors also found evidence that adenosine

has the potential to reduce intracellular free calcium levels by enhancing the binding of calcium intracellularly, since adenosine induced an increase in cellular exchangeable calcium levels under conditions that would not promote vascular smooth muscle contraction.

In summary, alteration of calcium fluxes by adenosine have been reported in smooth muscle. Evidence obtained from contractility studies, electrophysiological studies and tracer studies with 45-calcium suggest that extracellular and intracellular pools of calcium are affected by the nucleoside. These investigations do not exclude the possibility of cyclic AMP involvement in adenosine action. Furthermore, if cyclic AMP is involved in adenosine action, the above studies would suggest that the intracellular mediator acts via an effect on calcium levels as opposed to phosphorylation of proteins which constitute the contractile apparatus of smooth muscle.

1.3.3 ADENOSINE ACTION IN BIOLOGICAL SYSTEMS

In 1929 Drury and Szent-Gyorgi originally described the hypotensive, sedative, antispasmodic and vasodilatory actions of adenosine. Berne's (1963) suggestion, that adenosine may serve as the physiological mediator of autoregulation of coronary flow, renewed interest in the nucleoside. In 1970 Burnstock et al. proposed the "purinergic" nerve hypothesis which postulated an inhibitory neurotransmitter role for adenosine and/or ATP in non-cholinergic, non-adrenergic inhibitory nerves (for review see: Burnstock, 1972). Since then, adenosine has been shown to affect cell functions in many cell types other than smooth muscle. These have recently been tabulated in a review by Daly (1982) and include: centrally mediated relaxation of striated muscle (Buckle

and Spence, 1981), and sedation (Snyder et al., 1981), negative chronotropic and inotropic effects in heart (Prasad et al., 1980), inhibition of lipolysis in adipocytes (Trost and Stock, 1977; Londos et al., 1978), inhibition of platelet aggregation (Born et al., 1975; Agarwal and Parks, 1980), stimulation of steroidogenesis in adrenals (Londos et al., 1980), inhibition of lymphocyte function (Wolberg et al., 1978), potentiation of glucagon release in pancreas (Petrack et al., 1981), potentiation of histamine release in mast cells (Fredholm, 1980), inhibition of central neurons (Phillis et al., 1979) and finally inhibition of neurotransmitter release (Hedquist et al., 1978; Kuroda, 1978; Paton, 1981; Stone, 1981).

In general, the biological effects of adenosine can be antagonized by theophylline (see refs cited above). In addition, numerous studies with adenosine and xanthine analogs and derivatives support the existence of specific adenosine receptors which are responsible for mediating various biological effects elicited by adenosine. Two lines of evidence support the view that the ligand binding site for adenosine on this receptor faces the external surface of the cell membrane. First adenosine uptake inhibitors such as dipyridamole and nitrobenzylthioguanosine usually potentiated the effects of adenosine (Ebert and Schwabe, 1972; Haslam and Rossen, 1975; Wolff and Cook, 1977; Marquardt et al., 1978; Okwuasaba et al., 1978; Muller and Paton, 1979). Secondly, adenosine has been linked to stacchyose (Olsson et al., 1976; Fain and Shepherd, 1979), polylysine (Olsson et al., 1977) or carbonic anhydrase (Schrader et al., 1977) to yield high molecular weight adenosine analogs which do not readily cross the cell membrane. The effects of these compounds are qualitatively and quantitatively similar to that of adenosine in blood vessels, cardiac cells and fat cells.

Adenosine has been shown to alter cyclic AMP levels in many cell types (see section 1.3.2) and in some cases this appears to be related to the biological effect of the nucleoside (see reviews: Daly, 1982; Londos et al., 1983). It can have different effects on adenylate cyclase, such as stimulation and inhibition via extracellular receptors and inhibition via the so called "P site".

1.3.4 CLASSIFICATION OF ADENOSINE RECEPTORS COUPLED TO ADENYLATE CYCLASE

Adenosine receptors have been classified, in systems where they are coupled to adenylate cyclase, as A1 and A2 receptors by van Calker et al. (1979) or as Ri and Ra receptors by Londos et al. (1980). A1/Ri receptors inhibit adenylate cyclase activity while A2/Ra receptors stimulate adenylate cyclase activity. The Ri/Ra nomenclature of Londos and [illegible] will be adopted for the purposes of this thesis. In addition [illegible] Ra receptors, adenosine can act at a so called "P site" which inhibits adenylate cyclase and is not considered to be a receptor site.

1.3.4.1 DISTINGUISHING Ra OR Ri RECEPTORS FROM THE "P" SITE"

Haslam and Lynham (1972) were the first to demonstrate adenosine receptor-mediated stimulation of adenylate cyclase in a broken cell preparation. Adenylate cyclase from platelets was stimulated by low concentrations of adenosine and inhibited by raising the concentration of the nucleoside. Only the stimulatory effect was blocked by caffeine. Consequently, similar observations have been reported by a number of groups with a variety of adenylate cyclase preparations (Birnbaumer et al., 1974; Clark and Seney, 1976; Cooper and Londos, 1979; Jakobs

et al., 1979; Londos and Wolff, 1977; Peck et al., 1976; Penit et al., 1976). Thus, adenosine appeared to have two sites of action. More recently, the stimulatory R site and the inhibitory "P site" have been discriminated by using adenosine analogs. With the exception of NECA (5'-N-ethylcarboxamide adenosine) the receptor accepts a wide variety of N-6- and 2-substituted adenosine analogs but few compounds modified in the ribose moiety. To the contrary, the "P site" accepts a wide variety of modifications in the ribose moiety but few substitutions in the purine ring (cf. Londos and Wolff, 1977; Londos et al., 1978; Londos et al., 1979).

The task of distinguishing between "P site"- and R site- mediated inhibition of adenylate cyclase activity was met by the group of Londos and coworkers (1978 and 1979). They found that, if background adenosine was reduced by either adenosine deaminase or with the use of deoxy-ATP as substrate and GTP was added to purified rat fat cell membranes, adenosine analogs which stimulate Ra receptors would inhibit adenylate cyclase activity. This effect was blocked by methylxanthines, while the inhibition of cyclase produced by 2',5'-dideoxyadenosine which is "P site" specific was not affected. Adenosine and 2-chloroadenosine which activate both R and "P site" produced biphasic inhibitory curves (Cooper and Londos, 1979; Londos et al., 1978). Only the initial inhibitory phase which occurred at agonist concentrations in the nanomolar range was blocked by methylxanthines. Finally, N-6-substituted adenosine analogs such as PIA (phenylisopropyladenosine) which have been shown by Trost and Stock (1977) to inhibit lipolysis and decrease cyclic AMP levels in intact fat cells were potent inhibitors of adenylate cyclase

in the membrane preparation (Londos et al., 1978; Londos et al., 1979). To date the isolated fat cell membrane is the only system where the existence of Ri receptors for adenosine has been firmly established.

1.3.4.2 SIMILARITIES AND DIFFERENCES BETWEEN Ra AND Ri RECEPTORS FOR ADENOSINE

Ra and Ri receptors have a number of common properties. First, they appear to be located on the outer cell surface of the plasma membrane. Evidence for the location of Ra and Ri receptors comes from studies in intact cells with blockers of adenosine uptake (Clark et al., 1974; Fredholm et al., 1978; Green and Stanberry, 1977; Haslam and Rosson, 1975; Huang and Daly, 1974; Huang and Drummond 1976 and 1978; Marone et al., 1978) and a study with a stacchyose-adenosine compound (Fain and Shepherd, 1979). Second, methylxanthines which have long been known to block Ra receptors (Haslam and Lynham, 1972; Sattin and Rall, 1970) also block Ri receptors in fat cells (Londos et al., 1978; Cooper and Londos, 1979). Third, Ra and Ri receptors both appear to require GTP for expression of adenylate cyclase activity (Londos et al., 1978; Cooper and Londos, 1979).

There are a number of differences in the properties of Ra and Ri receptors for adenosine which necessitate such a classification. The most obvious difference is that stimulation of Ra receptors mediates stimulation of adenylate cyclase activity (Haslam and Lynham, 1972) and stimulation of Ri receptors mediates inhibition of adenylate cyclase activity (Londos et al., 1978). Two adenosine analogs in particular, NECA and PIA, have been found to be useful for discrimination between

Ra and Ri receptors. NECA has been shown to be more potent than PIA with respect to stimulation of adenylate cyclase activity from rat liver, Leydig tumor cells, turkey erythrocytes and adrenal Y-1 tumor cells as well as stimulation of steroidogenesis in Leydig and adrenal tumor cells (Londos et al., 1980). On the other hand, PIA has been shown to be more potent than NECA with respect to inhibition of adenylate cyclase from fat cells as well as inhibition of isoproterenol-stimulated lipolysis (Cooper et al., 1980; Londos et al., 1980) and reduction of cyclic AMP levels in intact adipocytes (Fain and Malbon, 1979; Trost and Stock, 1977). Therefore, NECA and PIA appear to show selectivity for Ra and Ri receptors, respectively. These two adenosine analogs are thought not to interact with the "P site". There is an apparent difference in the affinity of Ra and Ri receptors for adenosine. Ri receptors appear to have a high affinity for adenosine in the 15 to 100 nanomolar range (Cooper and Londos, 1979; Marquardt et al., 1978; van Calker et al., 1979). On the other hand, Ra receptors appear to have an affinity towards adenosine in the 0.5 to 20 micromolar range (Braun and Levitzki, 1979; Clark and Seney, 1976; Cooper and Londos, 1979; Haslam and Rosson, 1975; Peck et al., 1976). Finally, Londos et al. (1981) have found that sodium greatly enhances the magnitude of adenosine receptor-mediated inhibition of adenylate cyclase activity in rat fat cells. They suggested that this "sodium effect" may be common to all hormone-mediated inhibition of adenylate cyclase. In this regard, hormone-mediated inhibition of adenylate cyclase activity has been demonstrated with alpha-adrenergic agents in platelets (Jakobs et al., 1978) and neuroblastoma x glioma hybrid cells (Sabol and Nirenberg, 1978), as well as with opiates in

the former system (Blume et al., 1979). Furthermore, Blume et al. (1979) found that sodium enhanced the inhibition of adenylate cyclase activity by opiates.

1.3.4.3 "P SITE"

The "P site" has been shown to be distinct from adenosine receptors in a number of ways. It is thought to be located on the cytoplasmic surface of the cell membrane, unlike adenosine receptors which appear to face the extracellular space. Evidence for the location of the "P-site" comes from studies by the group of Haslam and colleagues (Haslam and Rossen, 1975; Haslam et al., 1978; Haslam et al., 1979). They found that adenosine decreased cyclic AMP levels in intact platelets and that inhibitors of the transport of adenosine into cells blocked this effect of adenosine. Furthermore, 2'-deoxyadenosine 3'-monophosphate, which is assumed not to cross the membrane, did not alter cyclic AMP levels in the platelets. Also adenosine or analogs have been shown to compete with the substrate for adenylate cyclase in a non-competitive manner (cf Londos et al., 1981). Therefore, Londos et al. (1981) conclude that the "P site" is not the catalytic site on the catalytic component of the adenylate cyclase complex, and the exact location of the "P site" remains unknown.

Stimulation of the "P site" invariably results in inhibition of adenylate cyclase activity, and unlike Ra/Ri receptors the "P site" appears to be present in all adenylate cyclase systems studied to date.

The agonist specificity of the "P site" has been mentioned above. Generally, many ribose-modified analogs of adenosine are agonists while purine-modified analogs particularly at the N-6

position are poor effectors at the "P site". Examples of analogs of adenosine which do not react with adenosine receptors but inhibit adenylate cyclase activity via "P site" interaction include 2',5'-dideoxyadenosine (Fain and Weiser, 1975; Fain et al., 1972, Londos and Wolff, 1977; Haslam et al., 1978), 9-beta-D-arabinofuranosyladenine (Londos and Wolff, 1977), 9-beta-D-xylofuranosyladenine (Londos and Wolff, 1977), 9-(tetrahydrofuranosyl)-adenine (Weinryb and Michel, 1974), and 2'-deoxyadenosine, 3'-monophosphate (Sahyoun et al., 1976a and 1976b).

Methylxanthines have been shown to be competitive antagonists at Ra/Ri receptors, but these compounds do not antagonize the inhibition of adenylate cyclase by adenosine or analogs at the "P site" (Londos and Wolff, 1977). To date no compound has been found which specifically blocks the "P site" (cf. Londos et al., 1981).

Another unique property of the "P site" is that divalent cations such as magnesium and manganese increase expression of this site but reduce expression at adenosine receptors. This has been demonstrated with adenylate cyclase from lung (Weinryb and Michel, 1974), liver (Londos and Preston, 1977), thyroid (Wolff et al.,1978) and Leydig tumor cells (Londos et al., 1979b).

Finally, the "P site" has a much lower affinity for adenosine than do Ra/Ri receptors; in the 25 to 1 millimolar range. Phosphorylation, deamination (for review see: Arch and Newsholme, 1978; Fox and Kelly, 1978), and entry into the S-adenosyl homocysteine pathway (cf. Usdin et al., 1978) maintain a low intracellular concentration of adenosine. Thus, Londos et al. (1981) concluded that a physiological

role for the "P site" as a regulator of adenylate cyclase activity remains tenuous.

1.3.4.4 BINDING STUDIES

Early binding studies utilizing radioactive adenosine failed to characterize binding sites which could be classified as Ri or Ra receptors (Malbon et al., 1978; Schwabe et al., 1979; Dutta and Mustafa, 1980; Newman et al., 1981). Binding studies to brain membranes have been attempted with adenosine analogs such as 2-chloroadenosine, L-N-6-phenylisopropyladenosine and N-6-cyclohexyladenosine which were rendered radioactive with tritium (Bruns et al., 1980; Schwabe and Trost, 1980; Williams and Risley, 1980; Wu and Phillis, 1980; Wu et al., 1980). The data obtained with the various ligands in these studies generally agree with each other and appear to characterize Ri receptors (cf. Daly, 1982).

Recently, two groups simultaneously describe the successful radioiodination of the adenosine analog hydroxy-PIA and characterized binding sites in rat brain (Munshi and Baer, 1982; Schwabe et al., 1982). It remains to be seen whether a radioactive adenosine analog of high specific activity will be useful in characterization of binding sites in cells with low densities of adenosine receptors.

Binding studies with an adenosine receptor antagonist 1,3-diethyl-8-[^{3}H]-phenylxanthine and a "P site" specific adenosine analog 2'5'-dideoxy[^{3}H]-adenosine have been attempted (Bruns et al., 1980; cf. Daly, 1982). The significance of the sites characterized with these ligands remains obscure.

1.4 EVIDENCE THAT CYCLIC AMP CAN CAUSE SMOOTH MUSCLE RELAXATION

The last part of the introduction deals with the question of whether cyclic AMP can cause smooth muscle relaxation. Usually this question is not asked and, despite the lack of convincing experimental evidence, it is widely assumed that cyclic AMP can cause relaxation.

Phosphodiesterase inhibitors and nucleoside derivatives such as dibutyryl- cyclic AMP have been used as tools to investigate this hypothesis. Multiple forms of phosphodiesterase are known to catalyze the hydrolysis of cyclic AMP to 5'-AMP (for review see: Wells and Hardman, 1977). A wide variety of chemical compounds inhibit phosphodiesterase (reviews see: Amer and Kreighbaum, 1975; Chasin and Harris, 1976). Furthermore, the inhibitors are not specific at the concentrations used (cf. Kramer and Hardman, 1980; Hardman, 1981). Dibutyryl-cyclic AMP, which has been used extensively to study the direct effects of cyclic AMP on smooth muscle contractility, breaks down to butyrate and adenosine and both of these have direct effects on smooth muscle (cf. Baer, 1974; Kramer and Hardman, 1980; Hardman, 1981). Few properly controlled studies using dibutyryl-cyclic AMP have been published (cf. Baer, 1974; Hardman, 1981). Thus, the interpretation of the data obtained with the available pharmacological tools to study the role of cyclic AMP on smooth muscle contractility have not been conclusive.

In this regard, much interest has been generated by the discovery of forskolin. This diterpene appears to possess several unique properties which suggest that it will be useful as a specific pharmacological tool in the study of cyclic AMP involvement in smooth muscle

contractility.

1.4.1 FORSKOLIN

The diterpene forskolin was isolated in 1977 by Bhat *et al.* from the roots of *Coleus forskohlii*, a plant which was used in ancient Hindu and Aurvedic medicine and is still used in India in folk medicines as remedies for such ailments as heart diseases, abdominal colic, respiratory disorders, painful micturition, insomnia and convulsions (cf. Seamon and Daly, 1981). Shortly following its isolation, forskolin was shown to possess positive inotropic and chronotropic actions in isolated guinea pig heart and positive inotropic actions in guinea pig atria and papillary muscle (Lindner *et al.*, 1978). Besides the cardiac effects, forskolin also caused hypotension in various species including dogs, cats, renal-hypertensive rats and spontaneous-hypertensive rats (Lindner *et al.*, 1978). The cardiac and hypotensive effects of forskolin could not be attributed to stimulation of beta-adrenoceptor or effects on sodium-potassium ATPase or cyclic nucleotide phosphodiesterase (Lindner *et al.*, 1978). Furthermore, unlike the depolarizing agent, veratridine, the effect of forskolin was not altered by the local anesthetic tetracaine. In addition, calcium-magnesium ATPase of sarcoplasmic reticulum, actomyosin ATPase and soluble or membrane bound guanylate cyclase were not affected by forskolin (cf. Seamon and Daly, 1981).

The first report suggesting that forskolin acted via stimulation of adenylate cyclase was by Metzger and Lindner (1981). In rabbit heart slices forskolin activated endogenous cyclic AMP-dependent protein kinase not directly but via stimulation of adenylate cyclase

and the latter was correlated with forskolin-mediated augmentation of contractile force in guinea pig atria.

Simultaneously and independently Seamon et al. (1981) found that forskolin stimulated adenylate cyclase from many different mammalian tissues. The forskolin-mediated stimulation of adenylate cyclase was rapid and reversible and desensitization did not occur upon repeated administration of the diterpene. Seamon et al. (1981) showed that forskolin-mediated increases in cyclic AMP levels in rat brain slices were not inhibited by phentolamine, propranolol, 8-phenyltheophylline, cimetidine or diphenhydramine; thus suggesting that forskolin did not interact with these extracellular hormone receptors. In this same study, Seamon et al. (1981) suggested that forskolin acts via a site distinct from the site of action of GTP or NaF. GDP, GDP-betaS and manganese inhibited Gpp(NH)p-induced stimulation of adenylate cyclase but these compounds did not alter the action of forskolin. Therefore, forskolin appeared to act on a site different from that of GTP. Since forskolin and NaF had different temperature dependencies and GDP-betaS inhibited the activity of NaF, forskolin did not act on the same site as NaF.

A later study by Seamon and Daly (1981) provided evidence to suggest that forskolin-mediated activation of adenylate cyclase resulted from interaction of forskolin with the cyclase unit directly. Forskolin stimulated adenylate cyclase in the mutant cells of murine S49 lymphoma cell line, cyc- which did not contain a functional guanine nucleotide binding subunit and are not stimulated by isoproterenol, NaF, GTP or cholera toxin. In addition, water soluble adenylate

cyclase from rat testes, lacking the guanine nucleotide binding subunit, was stimulated by forskolin up to 3.5 fold but not by Gpp(NH)p or NaF.

Insel et al. (1982) have confirmed the stimulation of adenylate cyclase in the cyc- S49 lymphoma cell line by forskolin. In ram sperm membranes which also lack the guanine nucleotide binding site but contain a functional cyclase (Cooper and Ahern, 1979) forskolin did not stimulate the cyclase. The following evidence obtained by Insel et al. (1982) suggest that forskolin may have an effect on the guanine nucleotide binding subunit in human platelet membranes; 1) The forskolin response was enhanced by GTP to a greater extent than could be explained by GTP alone. 2) The dose-response patterns for NaF and Gpp(NH)p were changed by forskolin. 3) They observed a blunting in inhibition by epinephrine of adenylate cyclase activated by forskolin and prostaglandin E-1. Therefore, there is evidence to suggest that forskolin activates adenylate cyclase directly or at least via a protein which is closely associated with cyclase and is different from the sites of action of hormones, NaF, GTP or cholera toxin. However, there is also evidence that forskolin can enhance the ability of the cyclase subunit to interact with the guanine nucleotide binding subunit.

Low concentrations of forskolin can potentiate increases in cyclic AMP elicited by certain hormones. In brain slices, forskolin increased the magnitude of increases in cyclic AMP levels elicited by histamine and noradrenaline, while the potency of prostaglandin E-2 and vasoactive intestinal peptide were increased by forskolin (Seaman et al., 1981). Similar results have been reported in human platelets and rat adipocytes where the increases in cyclic AMP levels mediated by

forskolin have been correlated with the physiological effects, ie., inhibition of platelet aggregation and lipolysis, respectively (cf. Seamon and Daly, 1981). There is one demonstration that forskolin can augment hormone-induced stimulation of adenylate cyclase in broken cells; in rat striatal membranes forskolin potentiated dopamine-mediated stimulation of adenylate cyclase (Seamon et al., 1981).

In summary, forskolin is a potent and specific activator of adenylate cyclase in all mammalian cell types tested both in broken cell preparations and in intact cells. This effect of forskolin is rapid, reversible, and appears not to require the hormone receptor or the guanine nucleotide regulatory subunit. Thus, forskolin probably activates adenylate cyclase via an interaction with the cyclase subunit. Low concentrations of forskolin, which have minimal direct effects, potentiate increases in cyclic AMP levels elicited by various hormones in a number of intact cells as well as in rat striatal broken cell preparations. As stated by Seamon and Daly (1981), these unique properties suggest that forskolin is an invaluable tool for the examination of the role of cyclic AMP in physiological responses to hormones.

1.5 RESEARCH OBJECTIVES

In the overview of this thesis it was stated that experimental proof of the suggestion of Sutherland and Rall (1960) concerning the role of cyclic AMP in hormone-mediated relaxation of smooth muscle deals with two questions. 1) Do hormones elevate cyclic AMP levels? 2) Does cyclic AMP *per se* cause smooth muscle relaxation?

The objectives of this thesis are to address these two questions albeit in a limited manner. The limitations involve the receptors and the smooth muscles investigated. Thus, to differing degrees the involvement of cyclic AMP in beta-receptors and adenosine-receptors-mediated relaxation of beef coronary artery, guinea pig taenia caeci and rabbit small intestine were investigated. Specifically, the effects of adenosine on the levels of cyclic AMP in intact cells of beef coronary artery were investigated. This was investigated in light of the controversy existing in the literature concerning the effect that adenosine has on cyclic AMP levels in intact tissues (see Introduction). The effects of isoproterenol, adenosine or analogs of adenosine on adenylate cyclase activity in broken cell preparations of guinea pig taenia caeci and the longitudinal muscle of the rabbit small intestine were investigated. This investigation involved a number of manipulations of the system to optimize the assay conditions and was based on the discoveries of Rodbell (1980) and Londos and coworkers (1982). The question of the usefulness of forskolin as a tool to investigate the effect of cyclic AMP on smooth muscle contractility is also addressed.

Finally phosphodiesterase inhibitors, MIX and Ro 20-1724, were used to investigate the role of cyclic AMP in forskolin-mediated relaxation of smooth muscle.

2 MET[illegible]TERIALS

[illegible]1 CO[illegible]TY STUDIES

2[illegible]1 TI[illegible]E PREPARATIONS

Be[illegible] hearts were obtained from Gainers packing plant immediately aft[illegible]ughter of the animal, placed in ice-cold physiological salt solu[illegible]n and transported to the laboratory. Branches of the descending [illegible]onary artery were carefully removed and cleared of adhering tissue [illegible]nd kept in oxygenated physiological salt solutions (see sectio[illegible].2.4) at 4 degrees C prior to mounting in an organ bath. They were cut into 2-3 mm wide rings according to the method of Furchgott (1981). Two thin wires were inserted through the lumen of the coro[illegible] artery segments. One wire anchored the tissue segment to the bottom of the organ bath while the other wire was connected via thread to a force displacement transducer. For description of recording of responses from all the tissue preparations see below.

Adult mongrel dogs of either sex were killed by intravenous administration of pentobarbitol and the hearts removed and placed in ice-cold physiological salt solution. These hearts were kindly supplied by Cristel Krueger. Descending coronary arteries were excised, cleared of adhering tissue and cut into spiral strips of 1-2 mm width and 15-20 mm length. The tissue strips were kept in oxygenated physiological salt solution at 4 degrees C for less than 15 minutes prior to mounting in organ baths.

Wistar rats of either sex weighing from 200-300 g were killed by a blow to the head and decapitation. The aortae were removed, cleared

of adhering tissue and kept in physiological salt solution at 4 degrees C until mounting in an organ bath. The aortae were cut into spiral strips of 1-2 mm by 15-20 mm.

Guinea pigs of either sex and weighing between 400-600 g were killed by decapitation. Taenia caeci were removed, cut into strips of 10-15 mm and kept in oxygenated physiological salt solution at 4 degrees C until mounting in organ baths.

Adult New Zealand white rabbits of either sex were killed by a blow to the head and exsanguination. The jejunum was removed, cut into 10-15 mm long segments and cleared of any adhering tissue. As with the other preparations, these tissue segments were kept in oxygenated physiological salt solution at 4 degrees C prior to mounting in organ baths.

2.1.2 RECORDING OF RESPONSES TO DRUGS IN TISSUE PREPARATIONS

All the tissue preparations were suspended in 10 ml organ baths containing physiological salt solution (for composition see materials) which was equilibrated with 95% O_2/5% CO_2 and kept at 37 degrees C. Isometric contractions and relaxations were recorded with force displacement transducers (Grass FT03C) and displayed on a polygraph (Grass-Model 7D). Guinea pig taeniae caeci were attached to the transducers by wire springs (0.3 g/cm). All preparations were set to a resting tension of 1 g and allowed to stabilize for a period of at least 60 min prior to addition of drug (Burnstock *et al*., 1970; McKenzie *et al*., 1977; Kukovetz *et al*., 1979; Furchgott, 1981).

2.1.3 EVALUATION OF THE EFFECT OF RELAXANT DRUGS ON SMOOTH MUSCLE CONTRACTILITY

Guinea pig taeniae caeci, beef coronary arteries and rat aortae had very low inherent tone and thus were contracted with 20 mM KCl. With dog coronary arteries 40 mM KCl was used to contract this tissue since 20 mM KCl often did not appreciably contract the preparation. The time required to reach maximal contraction of these tissues with KCl varied between 10 and 30 min, after which the contracted state was maintained for at least 90 minutes. This time was sufficient to construct concentration-response curves to various drugs. The concentrations of KCl which were utilized caused submaximal contractions in the respective tissues. Rabbit small intestine displays inherent tone in the form of spontaneous rhythmic twitch-like contractions. Thus, KCl was not needed for development of tone to study the actions of relaxant drugs.

Cumulative concentration-response curves were obtained to relaxant drugs in all tissues. When maximal relaxation of the tissue was obtained with a certain concentration, usually within 5 min of drug addition, the next higher concentration was added to the organ bath. Upon achieving maximal relaxation with each drug (completion of the concentration-response curve), $NaNO_2$ (10 mM) was added to the bath to achieve 100% relaxation of the respective smooth muscle. Responses obtained to forskolin and other relaxant drugs were expressed as a percentage of this maximum achieved with $NaNO_2$.

2.1.4 EVALUATION OF THE EFFECT OF PHOSPHODIESTERASE INHIBITORS ON RELAXANT DRUG ACTION

Phosphodiesterase inhibitors caused smooth muscle relaxation in all the preparations studied. As described in the results, these compounds were employed in concentrations devoid of "direct" effects. In all cases, experimental smooth muscle preparations were treated with the inhibitors, MIX or Ro 20-1724, for 30 min prior to addition of other drugs. Cumulative concentration-response curves to the relaxant agents were performed in the presence of the phosphodiesterase inhibitors. Each experimental tissue had a paired control from the same animal. Phosphodiesterase inhibitor effects were evaluated by comparing the concentration-effect curves of relaxant drugs from the control and experimental groups on a paired basis.

2.1.5 STATISTICAL ANALYSIS OF DATA

Responses of tissue experiments were expressed as the mean ± standard error of the mean (S.E.M.). The ED_{50} values were extrapolated from each experiment and geometric means with 95% confidence limits were calculated. Significant levels for the difference between groups were estimated using Student's paired "t" test (Hill, 1971) and the difference between groups was considered significant when $P < 0.05$.

2.2 MEASUREMENT OF CYCLIC AMP LEVELS IN BEEF CORONARY ARTERIES

2.2.1 PREPARATION AND TREATMENT OF TISSUES WITH ADENOSINE

As with the measurement of mechanical responses, beef hearts were obtained from Gainers packing plant immediately after slaughter of the animal, placed in ice-cold physiological salt solution and transported to the laboratory. Large and small descending coronary arteries were excised, cleared of adhering tissue and kept in oxygenated physiological salt solution at 4 degrees C. Tissue samples obtained from the main descending artery were classified as large while tissue samples obtained from branches of this artery were classified as small. Only arteries located from the midline to the apex of the hearts were used. The classification of these arteries corresponds to that of Harder _et al_. (1979). The arteries were cut into strips about 2 cm wide and 10 cm long with a piece of thread attached at one end.

The arterial strips were then submerged in test tubes containing 5 ml physiological salt solution and oxygenated at 15 min intervals for 60 min. The experiment was initiated by adding adenosine at various concentrations to half the arterial strips with the other half serving as controls. After 10 min the strips were removed from the test tubes and immediately frozen by clamping them between brass tongs precooled in liquid nitrogen (cf. McKenzie, thesis). The frozen strips were placed in 1.5 ml centrifuge tubes (Eppendorf) and stored in liquid nitrogen until homogenization.

2.2.2 PREPARATION OF SAMPLE FOR CYCLIC AMP MEASUREMENTS

The homogenization procedure used was essentially identical to that described by McKenzie (1975, thesis) for longitudinal muscle of rabbit small intestine. Briefly, the frozen arterial strip, 0.3 ml 5% TCA (trichloroacetate) and a tungsten carbide ball were placed into a Teflon capsule and cooled in liquid nitrogen before being vibrated in a Mikro-Dismembrator (Braun Melsungen) to pulverize the tissue. The vibration parameters were 60 Hz with an amplitude of about 5 mm for 30 seconds. The resulting powder was transferred to 1.5 ml centrifuge tubes (Eppendorf), capped and placed in liquid nitrogen for storage prior to measuring tissue cyclic AMP levels.

The homogenate was thawed and $[^{3}H]$-cyclic AMP (adenosine 3':5'-$[^{3}H]$-cyclic monophosphate) (0.1 pmole, approximately 2000 cmp, specific activity of 24 Ci/mmol) was added for an estimation of recovery of cyclic AMP. Aliquots from the supernatant of the neutralized TCA extract (see below) were added to Aquasol and counted in a Beckman scintillation counter. The values were extrapolated from a quench correction curve and the recovery of $[^{3}H]$-cyclic AMP was greater than 80%. The homogenate was then centrifuged for 1 min in an Eppendorf 3200 bench centrifuge and the pellet and supernatant were separated. The pellet was washed one time with 0.1 ml 5% TCA and the supernatants pooled. It was solubilized with 0.2 ml of 0.5 N NaOH and used for protein determination by the method of Lowry et al. (1951) while cyclic AMP levels were determined in the supernatant.

The TCA extract was first neutralized by addition of small amounts of calcium carbonate and centrifugation according to the method

outlined by Tihon et al. (1977). Calcium carbonate powder was added to each tube in small amounts until further addition did not produce bubbling of the solution (cf. Tihon et al., 1977). Aliquots were then taken from the supernatant and used for the measurement of cyclic AMP by radioimmunoassay (RIA) according to the method of Steiner et al. (1972).

2.2.3 PREPARATION OF [^{125}I]-2'-O-SUCCINYL CYCLIC AMP TYROSINE METHYL ESTER ([^{125}I]-TME-ScAMP)

The radioactive cyclic AMP derivative [^{125}I]-TME-ScAMP was prepared according to the method of Hunter and Greenwood (1962) using chloramine-T and Na-metabisulphite. The product was purified according to the method of Steiner et al. (1972) by applying the reaction mixture to a Sephadex G-10 column (1x25 cm). The eluting buffer was 0.2 mM Na-acetate, pH 5.8. The fractions (4-5 ml) were collected by a Golden Retriever Fraction Collector (Model 1200 PUP). Aliquots (10 μl) were taken from each fraction and scanned for [^{125}I] in a gamma counter (Beckman ModelGAM 8000) after each iodination and separation. A typical elution profile of radioactivity is shown in figure 2. As can be seen three peaks of [^{125}I] are present. According to Steiner et al., (1972), what is contained in peak one is unknown while peak two contains free [^{125}I] and peak three contains [^{125}I]-TME-ScAMP. The contents of three fractions from peak three ie., fractions 56, 57 and 58, were pooled and stored frozen for use in the RIA of cyclic AMP.

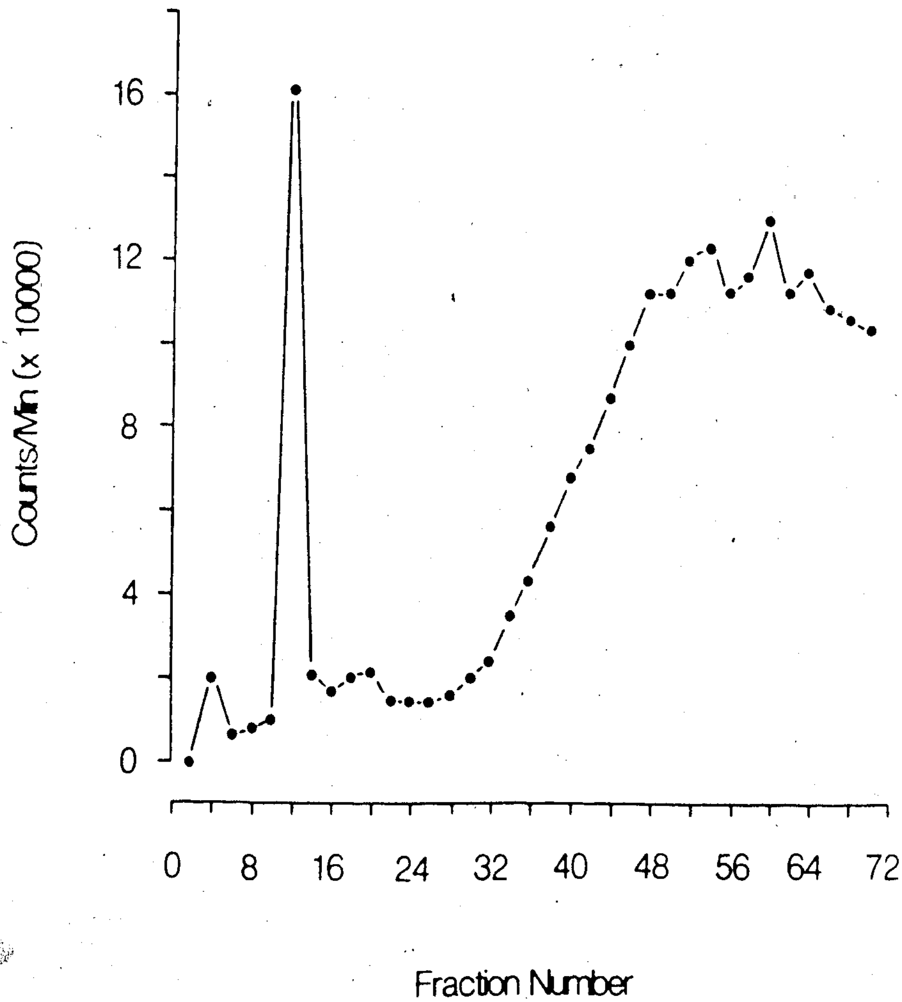


FIG. 2 Representation plot of radioactive counts/min in 10 μl aliquot vs Fraction number. Each Fraction was 4-5 ml. Three peaks of activity are shown containing products of the radio-iodination of TME-ScAMP. The three peaks are found in fractions 4, 12 and 50-64. Each point represents a single determination.

2.2.4 IMMUNOASSAY PROCEDURE

Cyclic AMP immunoassay was performed in a final concentration of 50 mM Na-acetate buffer, pH 4.75. Each tube (Eppendorf 1.5 ml capacity) contained 100 μl of 100 mM Na-acetate buffer, pH 4.75, 80 μl of sample (or water or cyclic AMP or acetylated cyclic AMP), 20 μl of [^{125}I]-TME-ScAMP (about 10,000 cpm) and 100 μl of antiserum which was diluted in 50 mM Na-acetate buffer, pH 4.75, with 30 mg/ml of bovine serum albumin (BSA). The dilution and specificity of the rabbit antisera to cyclic AMP as well as the optimal incubation period are discussed below. Routinely the immunoassay mixtures were incubated at 4 degrees C overnight before separation of the free and bound label which was accomplished by adding 100 μl of pig-gamma-globulin (5 mg/ml) in 100 mM Na-phosphate buffer, pH 7.0 and 400 μl of 25% polyethyleneglycol (PEG) 6000 in the same buffer to each tube. The tubes were then centrifuged for four min at full speed. The supernatant was decanted and the pellet washed with 200 μl of PEG at least once which was found to be sufficient. The pellet was then counted in a gamma counter.

2.2.5 ANTISERA TO CYCLIC AMP

Sera containing antibodies against TME-ScAMP were obtained from 3 rabbits by Dr. Baer according to standard immunological procedures.

The antibody titre was checked in the antisera from the 3 rabbits and is shown in figure 3. From these results, 1/1000 dilution of the antisera appeared to give about 40-50% binding of [^{125}I]-TME-ScAMP which is optimal for RIA purposes.

A study was also performed to find the optimal time of incubation

FIG. 3 Plot of antigen radioactivity ([^{125}I]-TME-ScAMP) bound vs dilution of antisera from three different rabbits (A, B and C). Results are expressed as the mean of triplicate determinations. Standard error of the means (S.E.M.) of the triplicate determinations are not shown. They are less than 10% of the mean values shown.

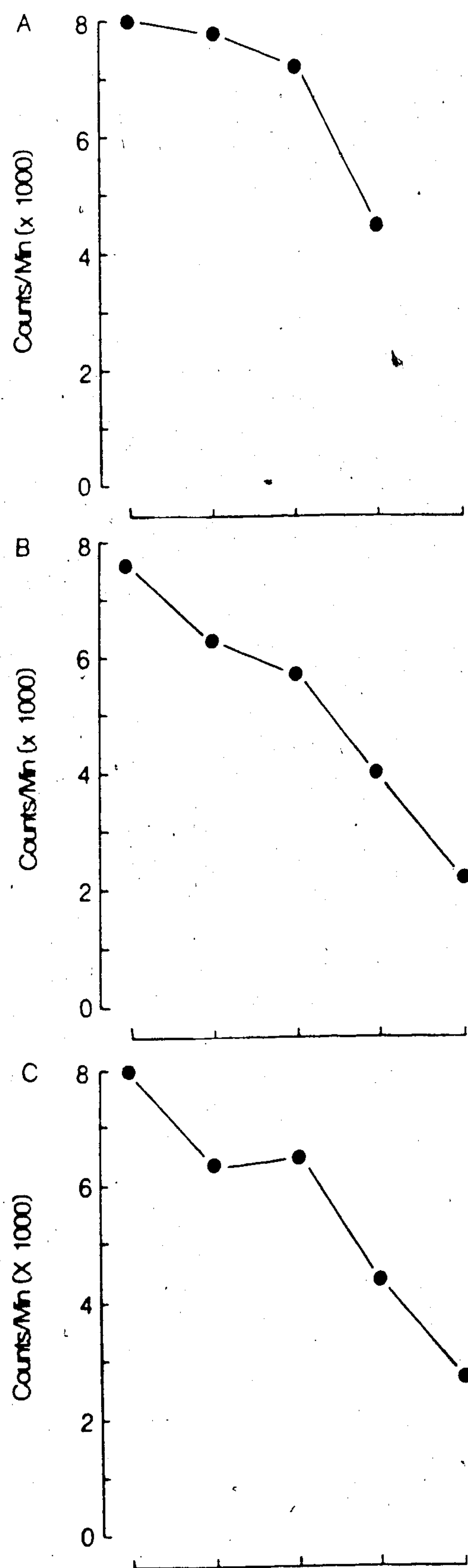


FIG. 3

for the RIA. As can be seen in figure 4, overnight incubation of the reaction mixture at 4 degrees C resulted in optimal binding of [^{125}I]-TME-ScAMP to the antisera. Therefore, this period of incubation was routinely used for RIA of cyclic AMP in tissue samples.

The specificity of the antisera was also tested by examining the ability of ATP, ADP, AMP and adenosine as compared to cyclic AMP to displace [^{125}I]-TME-ScAMP and is shown in figure 5. Antisera from rabbits 1 and 3 were about 100,000 times more sensitive to cold cyclic AMP than to adenosine or the adenine nucleotides. Antisera from rabbit 2 were greater than 10,000 times more sensitive to cyclic AMP than to the other compounds. Antisera from rabbits 1 and 3 were also found to be greater than 50,000 times more sensitive to cyclic AMP than to cyclic GMP while antisera from rabbit 2 were only 5,000 times more sensitive (data not shown). Therefore, the antisera were all selective for cyclic AMP and could be used for RIA purposes.

2.2.6 EFFECT OF ACETYLATION ON SENSITIVITY OF RIA FOR CYCLIC AMP

Acetylation of cold cyclic AMP standards and tissue samples has been shown by Harper and Brooker (1975) to greatly increase the sensitivity of the RIA for cyclic AMP. Triethylamine and then acetic anhydride are added to the purified tissue sample or to the cold cyclic AMP standard in a ratio of 10 μl/5 μl/500 μl. Acetic anhydride was added immediately after triethylamine as suggested by the authors to minimize the time that the sample was exposed to basic conditions. As can be seen in figure 6, acetylation of the cold cyclic AMP standards resulted in a 10 fold shift to the left of the dose-response curves indicating an increase in sensitivity of the immunoassay. Cyclic AMP

FIG. 4 Histogram of the amount of radioactive antigen ([^{125}I]-TME-ScAMP) bound to antisera from three different rabbits (A, B and C) with increasing time of incubation of the assay. Results are expressed as the mean of triplicate determinations. S.E.M. of the triplicate determinations are not shown. They are less than 10% of the means shown.

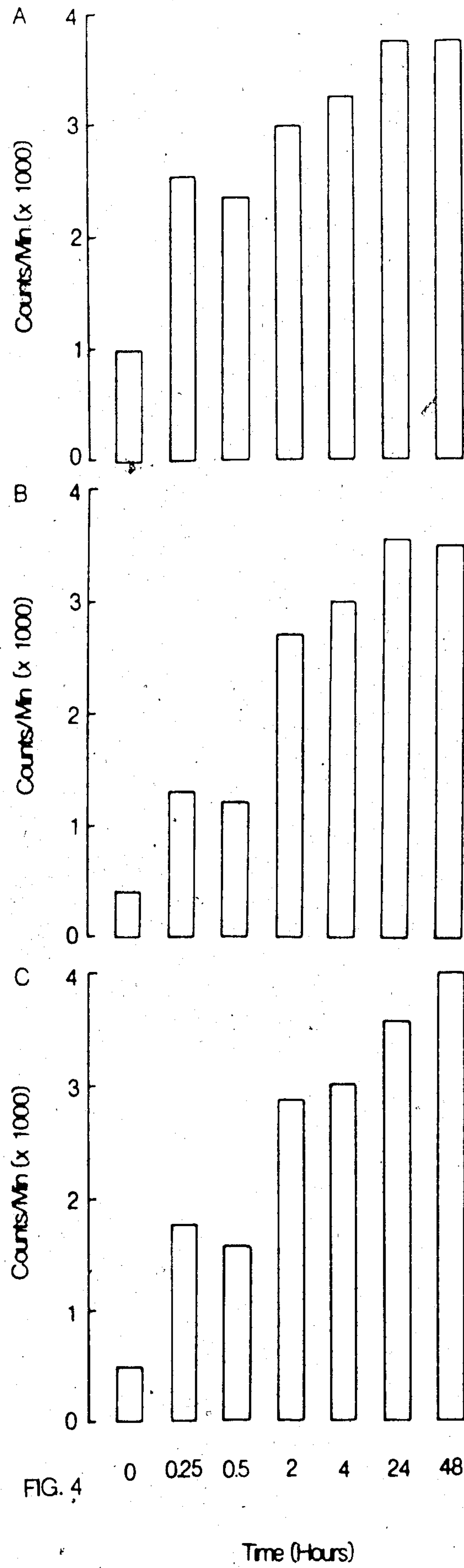


FIG. 4

FIG. 5 Study of the specificity of antisera from three different rabbits (A, B and C) to radioactive antigen ([^{125}I]-TME-ScAMP). Competition curves between radioactive antigen and cold ATP (○) , ADP (■) , AMP (□) , adenosine (▲) and cyclic AMP (●) are shown. Results are expressed as the mean of triplicate determinations. S.E.M. are not shown. They are less than 10% of the mean values shown.

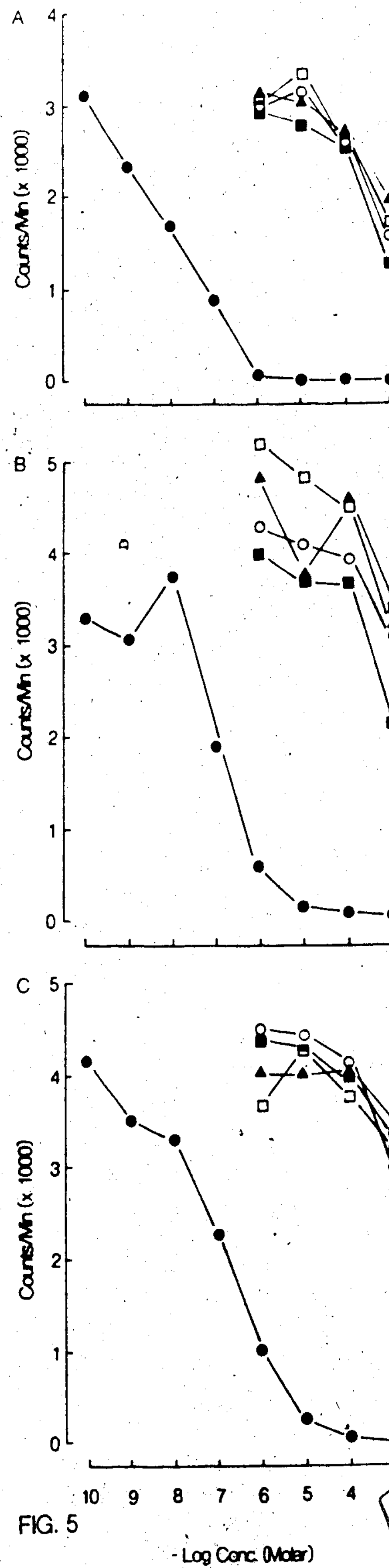
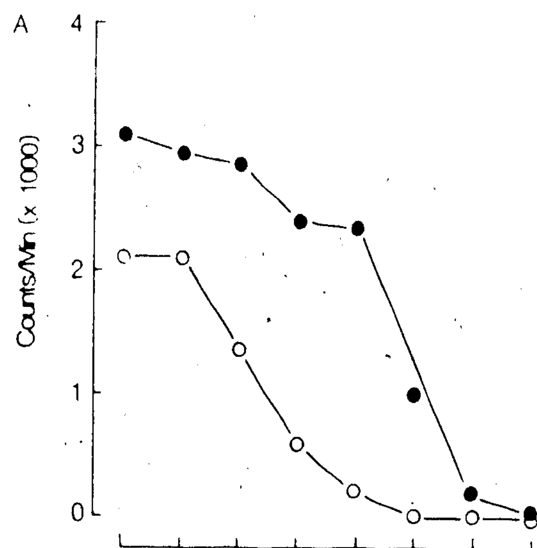
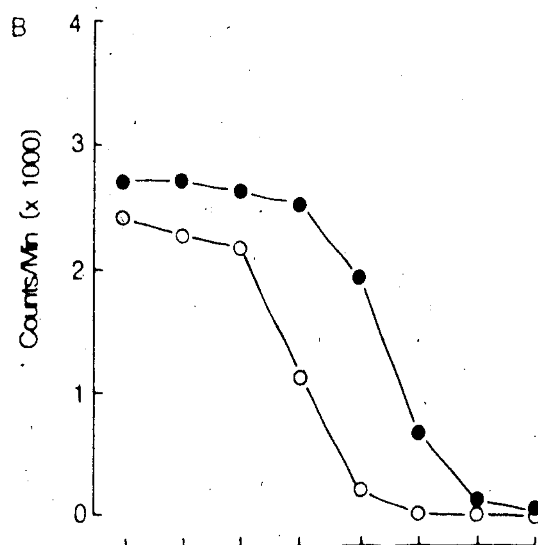


FIG. 5

FIG. 6 Effect of acetylation of cold cyclic AMP (○) on the standard RIA curve to cyclic AMP (●). The curves show competition between radioactive antigen ([^{125}I]-TME-ScAMP) and cold cyclic AMP or acetyl-cAMP for antisera from three different rabbits (A, B and C). Results are expressed as the mean of triplicate determinations. S.E.M. are not shown. They are less than 10% of the mean values shown.





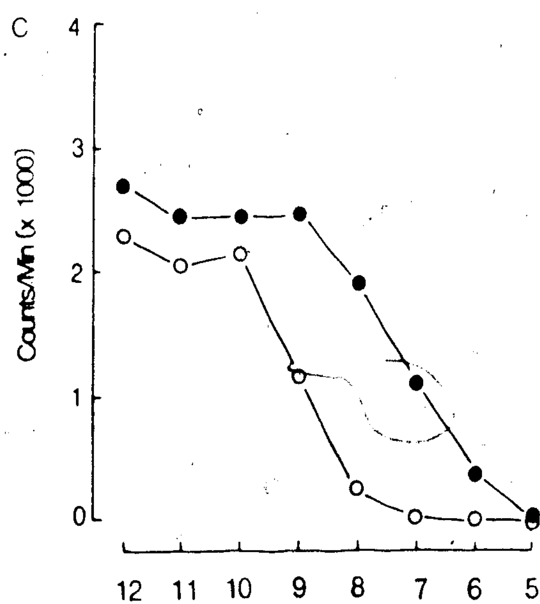


FIG. 6

- Log Conc. (Molar)

standards and cyclic AMP in tissue extracts were thus routinely acetylated when measuring cyclic AMP levels in beef coronary arteries.

The data dealing with measurement of cyclic AMP in beef coronary artery strips was expressed as the mean ± standard error of the mean (S.E.M.), n=3. Significance levels for the difference between adenosine treated and control tissues were estimated using Student's paired "t"-test and the difference between groups was considered different when $P < 0.05$.

2.3 MEASUREMENT OF ADENYLATE CYCLASE ACTIVITY IN SMOOTH MUSCLE

2.3.1 SMOOTH MUSCLE PREPARATIONS EMPLOYED

Güinea pig taenia caeci and rabbit small intestine were obtained as described above. The former was used as such while only the longitudinal muscle of the rabbit small intestine was used for adenylate cyclase studies. The longitudinal muscle was obtained by inserting a 5 ml pipette through the lumen of a segment of small intestine and lightly passing a scalpel blade along the length of the tissue segment. The longitudinal muscle was then teased away from the circular muscle with a cotten-tipped applicator stick. To assure muscle viability, the muscle was frequently soaked in physiological salt solution. In this way, about 1 g of longitudinal muscle was obtained from the small intestine of one rabbit while 5-6 guinea pigs were required to obtain about 1 g of taenia caeci.

2.3.2 PREPARATION OF ADENYLATE CYCLASE FROM THE 10,000 g PELLET OF SMOOTH MUSCLE

The longitudinal muscle of the small intestine from 1 rabbit or the taenia caeci from 6 guinea pigs were minced finely with scissors and homogenized in ten volumes of 20 mM TRIS-HCl (Tris(hydroxymethyl)-aminomethane) pH 7.5, and containing 1 mM $MgCl_2$ at 4 degrees C using a glass homogenizer and a motor-driven teflon pestle. The homogenate was filtered through glass wool and cheese cloth and then centrifuged in a Sorvall (RC2-B) centrifuge at 4 degrees C for 20 min at 10,000 x g. The resulting pellet was washed and resuspended in a small volume of the same buffer. Aliquots of 200 µl were frozen and stored under liquid nitrogen until use. Protein determinations were carried out by the method of Lowry et al. (1951).

2.3.3 FRACTIONATION OF THE LONGITUDINAL MUSCLE FROM RABBIT SMALL INTESTINE

In some experiments, the longitudinal muscle of the rabbit small intestine was prepared by a differential centrifugation method described by Kidwai (1975), see figure 7. The tissue was minced finely with scissors and then homogenized in ten volumes of 0.25 M sucrose solution at 4 degrees C using a Polytron generator PT10 at a setting of 6 for three 10 second bursts. The homogenate was filtered through glass wool and cheese cloth and centrifuged in the Sorvall at 4 degrees C for 10 min at 1,200 x g. The pellet (P-1) was resuspended in buffer and 200 µl aliquots were stored under liquid nitrogen. The supernatant (S-1) was centrifuged in the Sorvall at 4 degrees C for 15 min at

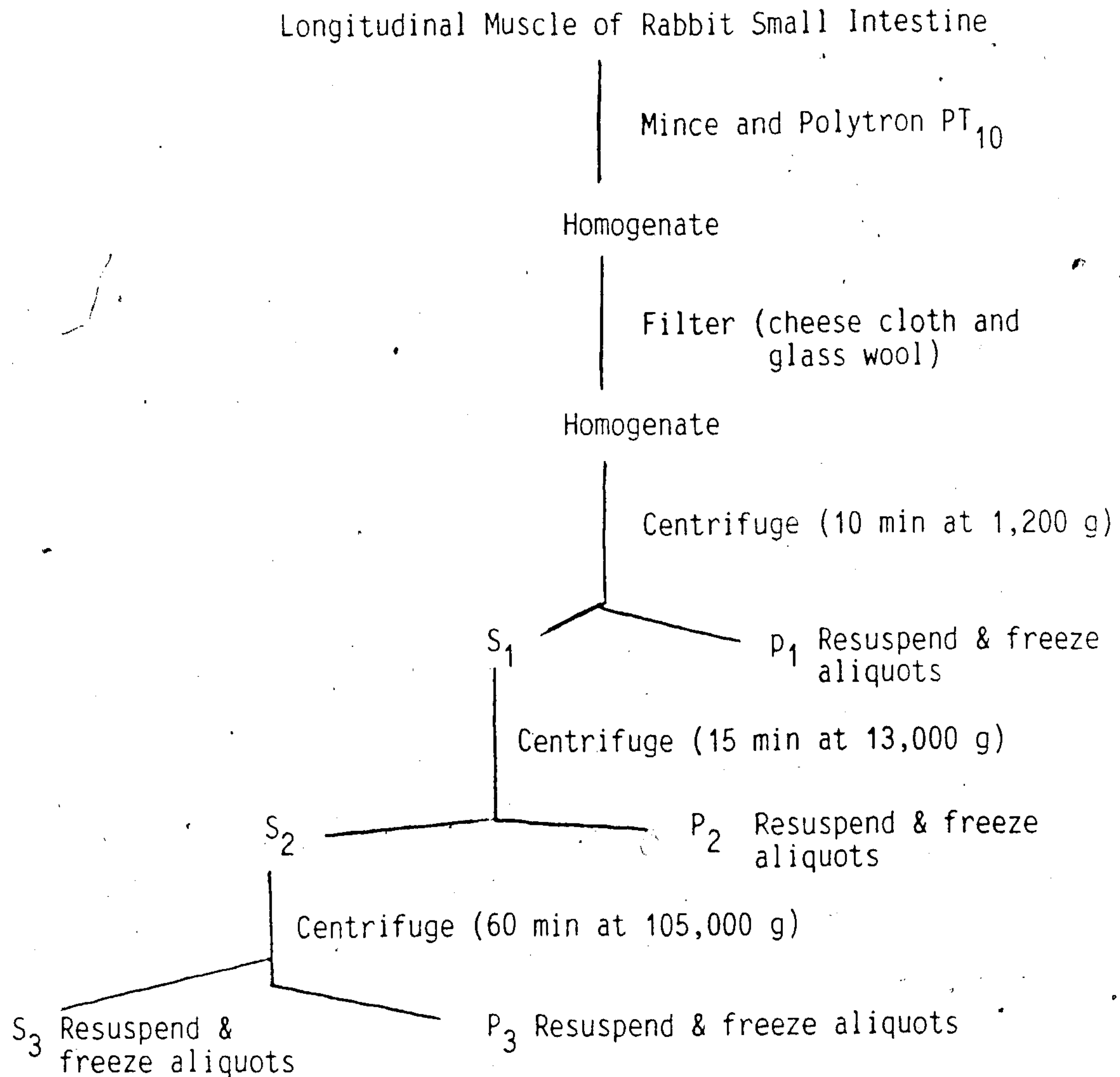


FIG. 7 Schematic representation of fractionation of the longitudinal muscle from rabbit small intestine by a differential centrifugation method described by Kidwai (1975). P-1, P-2, P-3 and S-3 denote resuspended pellets from Fractions 1, 2 and 3 and the final supernanant, respectively.

13,000 x g. The resulting pellet was resuspended in buffer and 200 μl aliquots stored under liquid nitrogen. The supernatant (S-2) was centrifuged in a Beckman (Model L3-40) ultracentrifuge at 4 degrees C for 60 min at 105,000 x g. Both the supernatant (S-3) and the pellet (P-3), which was resuspended in a small volume of buffer, were stored under liquid nitrogen in 500 μl and 200 μl aliquots, respectively. All protein determinations were by the method of Lowry et al. (1951). Adenylate cyclase activity was determined in the various fractions. According to Kidwai (1975), P-1 contains sedimental nuclei and some unbroken cells and connective tissue. P-2 contains sedimental mitochondria and P-3 contains microsomes which consists of smooth and rough endoplasmic reticulum and fragments of plasma membrane. S-3 supposedly is the soluble fraction consisting of the cytoplasmic components. Any other variations in the method of broken cell preparation will be specified.

2.3.4 ASSAY OF ADENYLATE CYCLASE

The assay of adenylate cyclase was carried out according to the method described by Baer (1975). The total volume of the assay mixtures was 0.05 ml and contained the following: 25 mM sodium N-2-hydroxymethylpiperazine-N-2-ethanesulphonate (pH 8), 5 mM $MgCl_2$, 1 mM sodium cyclic AMP, 0.2 mg/ml sodium creatine kinase, 20 mM sodium creatine phosphate, 0.1 mM alpha-[^{32}P]-ATP (about 500,000 cpm, specific activity of 25 Ci / mmol), enzyme and other additions as indicated. For example, in some experiments EDTA (ethylenediamine tetraacetic acid) and DTT (dithiotheiotol) were added to the reaction

mixture to complex divalent cations and stabilize disulfide bonds, respectively (cf. Anand-Srivastava et al., 1983). Incubations were carried out at 30 degrees C and for 20 min unless otherwise specified. The assay was initiated by addition of either enzyme or alpha-[^{32}P]-ATP and was terminated by addition of a "STOP" solution at 4 degrees C (125 mM EDTA and 25 mM of the following: ATP, 5'-AMP and cyclic AMP with the pH adjusted to 7 with sodium bicarbonate). [^{32}P]-cyclic AMP was isolated from the reaction mixture by chromatography on prewashed polyethyleneimine-impregnated cellulose thin layer plates 10x20 cm from Macherey-Nagel/Brinkman. About 5 μl of each reaction mixture was applied (along a 1.5 mm line) to the plate which was then developed in 0.25 mM LiCl. Spots containing ATP plus 5'-AMP and cyclic AMP were visualized and isolated under ultraviolet light, placed into scintillation vials and counted in 10 ml toluene containing PPO (2,5-diphenyloxazole) (4g/1) and POPOP (1,4-bis[2-(5-phenyl-oxazolyl)] benzene) (0.2g/l). The ratio of counts/min in the two spots (calculated by computer) provided a measure of the percentage conversion of ATP to cyclic AMP.

Results were expressed as the mean pmoles cyclic AMP/mg protein/min plus and minus the S.E.M. of triplicate determinations from one broken cell preparation. In all cases the experiments shown were performed in at least 3 different broken cell preparations. Thus, one broken cell preparation consisted of the resuspended 10,000 g pellet obtained from the tissues of 6 guinea pigs or 1 rabbit. Whether the results shown are representative of those obtained in the 3 different broken cell preparations is stated in the text of the

results section. 10 mM NaF was used in each experiment as a control agent to test the responsiveness of the preparation (cf. Perkins, 1973). It invariably stimulated adenylate cyclase activity.

Statistics were not performed on this portion of the thesis which deals with broken cell preparations. Statisical analysis of data dealing with evaluation of the means of a small number of observations has two basic assumptions (cf. Hill, 1971). Neither of these assumptions are valid with respect to this section. The first assumption states that we have some appreciation of the standard deviation that occurs in the real universe. However, there is a lack of reproducibility of some results in this section which is real but the reason(s) for this remain unknown (see section 4.2). The second assumption states that the differences between means of samples are distributed in the shape of the normal curve etc. Again, the lack of reproducibility of some results in this section precludes our making such an assumption. Therefore, the results in this section are necessarily descriptive in nature.

2.4 MATERIALS

The physiological salt solution had the following composition: 116 mM NaCl, 5.4 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM $MgCl_2$, 1.2 mM NaH_2PO_4, 22 mM $NaHCO_3$ and 11.2 mM glucose. The buffer solution was aerated with 95% O_2 / 5% CO_2 and kept at 37 degrees C. These chemicals were obtained from Fisher Scientific Co., Edmonton Alberta or Sigma, St. Louis MO. USA.

The remaining chemicals used in this study and their sources were

as follows: Acetic anhydride from J.T. Baker Chemical Co., N.J. USA; adenosine, adenosine 3':5'-cyclic monophosphate (cyclic AMP), adenosine deaminase, adenosine 5'-diphosphate (ADP), adenosine 5'-monophosphate (AMP) and adenosine 5'-triphosphate (ATP) from Sigma, USA; Aquasol, New England Nuclear, Boston, Mass., USA; bovine serum albumin (BSA), Sigma, USA; 4-(3-Butoxy-4-methoxybenzyl)-2-imidazolidine (Ro 20-1724) from Hoffman-LaRoche Inc., calcium carbonate ($CaCO_3$) from J.T. Baker Co., USA; Chloramine T from Eastman Kodak Co., Rochester, N.J., USA; 2-chloroadenosine from Sigma, USA; copper sulphate ($CuSO_4.5H_2O$) from Fisher Scientific Co., USA; creatine kinase and creatine phosphatate (sodium salts) from Boehringer Mannheim BmbH, W-Germany; 2'-deoxy-adenosine 3':5'-cyclic monophosphate (deoxy-cyclic AMP) and 2'-deoxy-adenosine 5'-monophosphate (deoxy-5'-AMP) and 2'-deoxyadenosine 5'-triphosphate (deoxy-ATP) from Sigma, USA; dithiotheiotol (DTT) from Calbiochem-Behring San Diego C.A., USA; ethylenediamine tetraacetate acid (disodium salt) (EDTA) from Fisher Scientific Co., USA; forskolin from Calbiochem-Behring, USA; folin-ciocaltean (phenol reagent) from Fisher Scientific Co., USA; 1-methyl-3-isobutylxanthine (MIX) from Aldrich Co., Milwaukee, WI. USA; 5'-N-ethylcarboxamide adenosine (NECA) from Dr. Schotensack; N-2-hydroxyethylpiperazine-N'-2-ethane-sulphonic acid (HEPES) A grade from Calbiochem, USA; N-6-(L-2-phenyl-isopropyl)-adenosine (PIA) from Research Biochemicals Inc., Wagland M. A., USA: Polyethylene Glycol 6000 (PEG) from J.T. Baker Chemical Co., USA; POPOP (1,4-bis[2-(5-phenyloxazolyl)] benzene) from Amersham/ Searle Corp., Des Plaines Ill., PPO (2,5-diphenyloxazole) from Eastman Kodak Co., USA; Sephadex G-10 from Pharmacia, Upsala, Sweden; sodium

acetate, sodium fluoride (NaF) and sodium hydroxide from Fisher Scientific Co., USA; sodium metabisulphite from J.J. Baker Chemical Co., USA; sodium nitrate, sodium/potassium tartrate and sodium phosphate from Fisher Scientific Co., USA; toluene and trichloroacetic acid (TCA) from Fisher Scientific Co., USA; TRIS(HCl) (Tris(hydroxymethyl)-aminomethane) from Sigma, USA; tyrosine-methyl-ester of succinyl-cyclic AMP (TME-ScAMP) from Boehringer-Mannheim, W-Germany; Verapamil (Isoptin) Knoll Co., Ludwigshafen, FRG.

All chemicals were of reagent grade and dissolved in deionized distilled water except forskolin and Ro 20-1724. Stock solutions of these compounds were prepared in dimethylsulfoxide (DMSO) and stored at -20 degrees C. Subsequent dilutions were made with deionized distilled water. DMSO alone, in the concentrations resulting from drug addition, did not alter any of the parameters studied.

The radiochemicals employed and their sources were as follows: [^{3}H]-adenosine 3':5'-cyclic monophosphate (25 Ci/mmole) from Amersham International, UK; [^{125}I]-Na from Edmonton Radiopharmaceutical Co., Edmonton, Canada; alpha-[^{32}P]-adenosine 5'-triphosphate and alpha [^{32}P]-2'-deoxyadenosine 5'-triphosphate both at (25 Ci/mmole) from ICN, Irvine C.A., USA.

2.5 ABBREVIATIONS

The abbreviations which were used in this thesis are as follows:

alpha-[^{32}P]-ATP	alpha-[^{32}P]-adenosine 5'-triphosphate
5'-AMP	adenosine 5'-monophosphate
ATP	adenosine 5'-triphosphate
BSA	bovine serum albumin
C	catalytic unit
Ci/mmol	curie/millimole
cm	centimeter
cpm	counts per minute
cyclic AMP	adenosine 3':5'-cyclic monophosphate
degrees C	degrees celsius
deoxy-alpha-[^{32}P]-ATP	deoxy-alpha-[^{32}P]-adenosine 5'-triphosphate
DMSO	dimethylsulfoxide
DTT	dithiotheiotol
ED_{50}	concentration causing half maximal effect
EDTA	ethylenediamine tetraacetate
g	gram
GDP	guanosine 5'-diphosphate
Gpp(NH)p	guanosine 5'-imidodiphosphate
GTP	guanosine 5'-triphosphate
[^{3}H]-cyclic AMP	[^{3}H]-adenosine 3':5'-cyclic monophosphate
HEPES	N-2-hydroxyethyl-piperazine-N'-2'-ethanesuphonic acid
[^{125}I]-TME-ScAMP	[^{125}I]-tyrosine methyl ester of succinyl adenosine 3':5'-cyclic monophosphate

mg/ml	milligram/milliliter
min	minute
MIX	1-methyl-3-isobutylxanthine
ml	milliliter
mm	millimeter
mM	millimolar
mv	millivolts
N	guanine nucleotide binging site
NECA	5'-N-ethylcarboxamide adenosine
Ni	inhibitory guanine nucleotide binding site
nM	nanomolar
Ns	stimulatory guanine nucleotide binding site
$[^{32}P]$-cyclic AMP	$[^{32}P]$-adenosine 3':5'-cyclic monophosphate
PEG	polyethylene glycol 6000
%	percent
PIA	phenylisopropyladenosine
pmol	picomole
POPOP	1,4-bis[2-(5-phenyloxazolyl)] benzene
PPi	inorganic diphosphate
PPO	2,5-diphenyl-oxazole
R	receptor
Ra	stimulatory adenosine receptor
Ri	inhibitory adenosine receptor
RIA	radioimmunoassay
Ro 20-1724	4-(3-butoxy-4-methoxybenzyl)-2-imidazolidine

S.E.M.	standard error of the mean
TCA	tetrachloroacetic acid
TEA	tetraethylammonium
TRIS(HCl)	Tris(hydroxymethyl)-aminomethane
µl	microliter
µM	micromolar
x g	times gravity

3 RESULTS

3.1 MEASUREMENT OF CYCLIC AMP LEVELS IN BEEF CORONARY ARTERIES: EFFECT OF ADENOSINE

Cyclic nucleotide levels were measured by the RIA technique developed by Steiner et al. (1972). Since the antisera used in the immunoassay were developed in our laboratory, control experiments to ensure proper assay conditions and antibody specificity were conducted, and are described in the methods.

Basal levels of cyclic AMP varied as much as 100 fold in tissues obtained from one beef heart. Thus strict control of experimental conditions was necessary. Each arterial segment was cut in half, parallel to the longitudinal muscle and cutting through the circular muscle of the blood vessel. The two halves either served as control tissues for measurement of basal levels of cyclic AMP or adenosine was applied to one half and the other served as a control tissue. In this way, identical paired tissues were obtained with respect to both longitudinal and circular smooth muscle. Furthermore, attempts were made to standardize procedural factors such as time and tissue handling. The variability in basal levels of cyclic AMP was not reduced despite these precautions.

The effects of adenosine (1 mM and 0.1 mM) on cyclic AMP levels in large and small beef coronary arteries are shown in figure 8 (see methods for definition of large and small coronary arteries). Adenosine (1 mM) caused a significant increase in tissue levels of cyclic AMP in large and small coronary arteries. To the contrary,

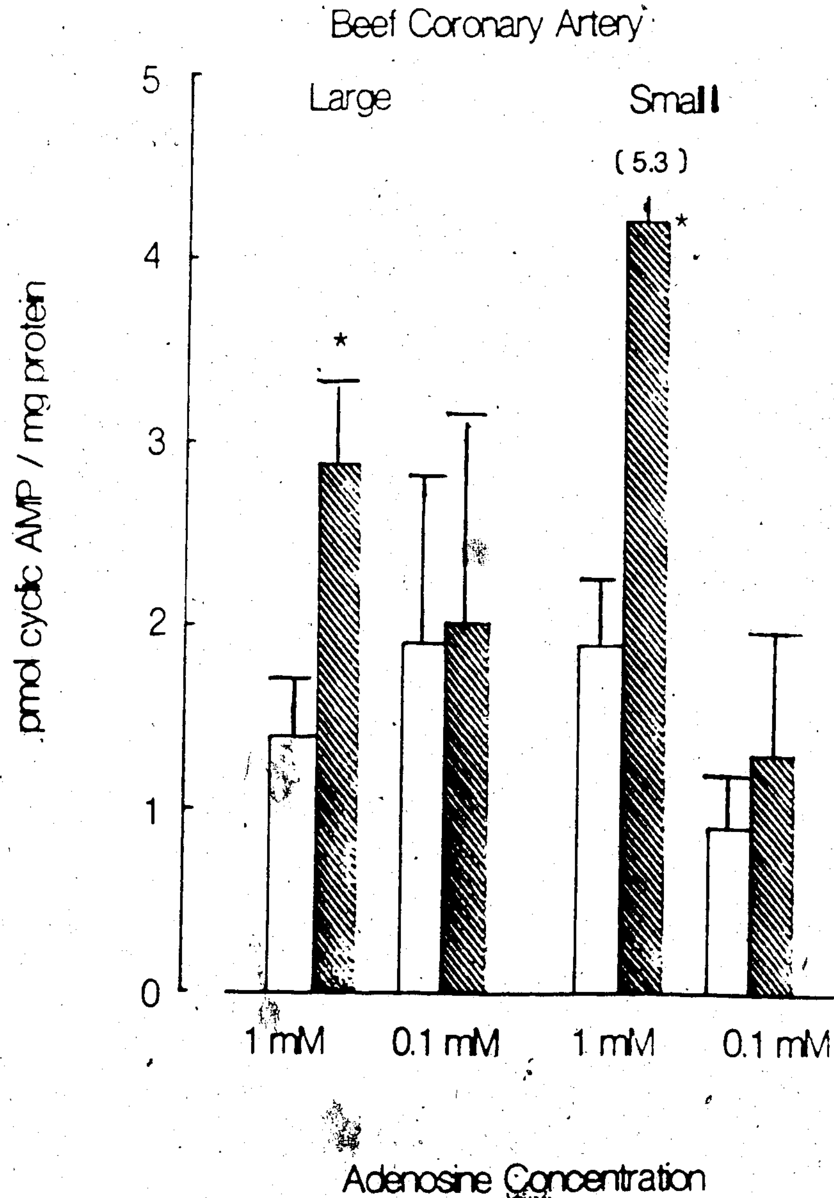


FIG. 8 Elevation of basal tissue levels of cyclic AMP (□) by 1 mM and 0.1 mM (▨) adenosine in strips of large and small beef coronary artery in 10 min. Results are expressed as the mean ± S.E.M. (n=3) of determinations from 8 - 13 tissues (coronary artery obtained from three beef hearts, n=3). In one case the S.E.M. is written, (5.3). Asterisks represent a significant difference between control and treated tissues; $P < 0.05$.

0.1 mM adenosine did not significantly increase tissue levels of cyclic AMP in the arteries. However, a trend towards higher levels of cyclic AMP was apparent in small arteries treated with 0.1 mM adenosine.

3.2 STUDIES ON ADENYLATE CYCLASE IN SMOOTH MUSCLE BROKEN CELL PREPARATIONS

3.2.1 EFFECTS OF VARIOUS COMPOUNDS OF ADENYLATE CYCLASE ACTIVITY IN GUINEA PIG TAENIA CAECI

The effect of various compounds including 10 mM NaF and 1 mM of the following, GTP, forskolin, adenosine, 2-chloroadenosine, NECA, PIA and isoproterenol was examined with respect to stimulation of adenylate cyclase activity from the 10,000 g pellet from guinea pig taenia caeci (see figure 9a and b). NaF and forskolin stimulated adenylate cyclase activity. No stimulation of enzyme activity was seen with any of the other compounds tested. These results were obtained from 1 broken cell preparation consisting of taenia caeci from 6 guinea pigs. They are representative of results obtained in 3 different broken cell preparations (see section 2.3.4).

3.2.2 EFFECT OF VARIOUS CONCENTRATIONS OF DRUGS ON ADENYLATE CYCLASE ACTIVITY IN GUINEA PIG TAENIA CAECI

The effect of various concentrations of adenosine, 2-chloroadenosine and isoproterenol with respect to adenylate cyclase activity in guinea pig taenia caeci are shown in figure 10. No stimulation

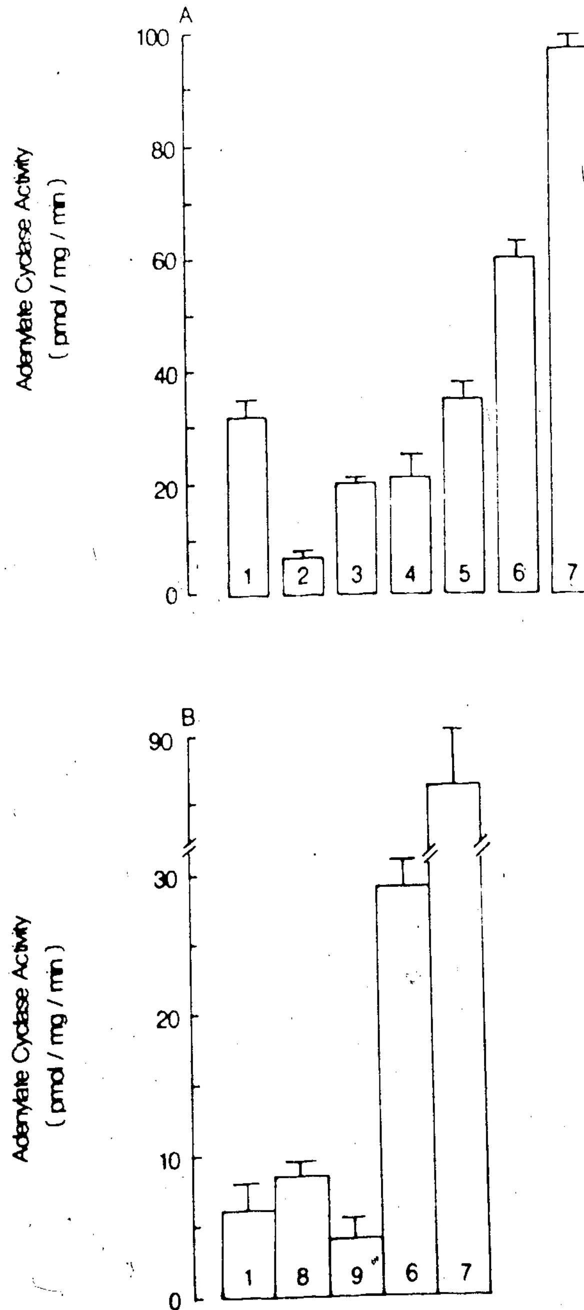


FIG. 9 Histogram of the effects of various compounds on adenylate cyclase activity in the 10,000 g pellet from guinea pig taenia caeci. Basal cyclase activity (1) and the effect of 1 mM adenosine (2), 1 mM 2-chloroadenosine (3), 1mM isoproterenol (4), 1 mM GTP (5), 1 mM forskolin (7), 1 mM NECA (8), 1 mM PIA (9), and 10 mM NaF (6) are shown. Results are expressed as mean ± S.E.M. of triplicate determinations.

FIG. 10 Lack of effect of various concentrations of adenosine (A), 2-chloroadenosine (B) and isoproterenol (C) on adenylate cyclase activity in the 10,000 g pellet from guinea pig taenia caeci. Results are expressed as mean ± S.E.M. of triplicate determinations. Error bars are not shown when the S.E.M. are contained within the symbol.

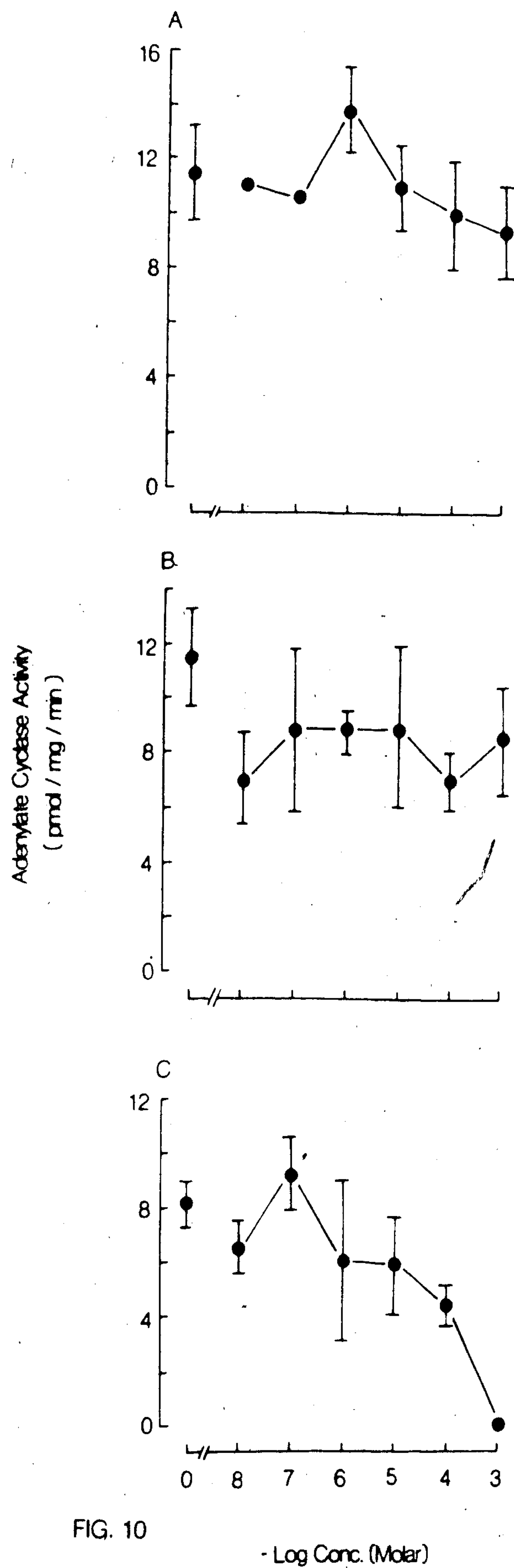


FIG. 10

over basal adenylate cyclase activity was observed with these compounds at any concentration tested. The results shown in figure 10 were obtained from 1 broken cell preparation and they are representative of results obtained in 3 different broken cell preparations.

3.2.3 EFFECT OF VARIOUS CONCENTRATIONS OF FORSKOLIN ON ADENYLATE CYCLASE ACTIVITY IN GUINEA PIG TAENIA CAECI AND THE LONGITUDINAL MUSCLE OF THE RABBIT SMALL INTESTINE

The effect of various concentrations of forskolin on adenylate cyclase activity from guinea pig taenia caeci and the longitudinal muscle of the rabbit small intestine are shown in figure 11. In both broken cell preparations, forskolin in the range 1 μM to 1 mM caused a concentration-dependent stimulation of adenylate cyclase activity. These results complement the contractility studies concerning the mechanism of forskolin-induced relaxation of smooth muscle (see below). In both preparations forskolin caused greater stimulation of the enzyme than did NaF. Thus, 10 mM NaF caused 670 and 158% stimulation, and 1 mM forskolin 1085 and 458% stimulation, in the guinea pig taenia caeci and rabbit small intestine, respectively. The results shown in figure 11 were obtained from 1 broken cell preparation of longitudinal muscle of the rabbit small intestine and 1 broken cell preparation of guinea pig taenia caeci. They are representative of those obtained from 3 different broken cell preparations in each case.

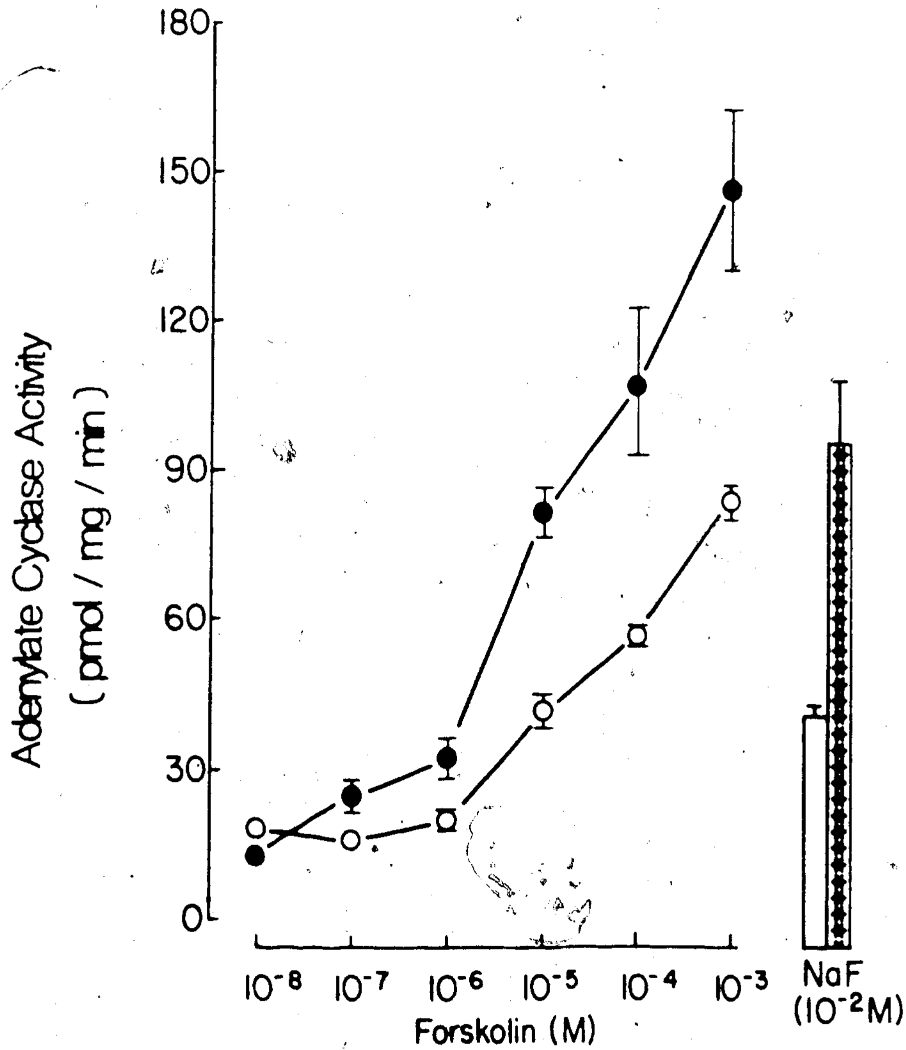


FIG. 11 Stimulation by increasing concentrations of forskolin (●, ○) and 10 mM NaF (□, ⊠) of adenylate cyclase activity in the 10,000 g pellet from guinea pig taenia caeci (●, ⊠) and the longitudinal muscle [illegible] rabbit small intestine (○, □). Results [illegible] expressed as mean ± S.E.M. of triplicate determinations. Error bars are not shown when the S.E.M. are contained within the symbol.

3.2.4 EFFECT OF VARIOUS CONCENTRATIONS OF GTP AND Gpp(NH)p ON ADENYLATE CYCLASE ACTIVITY IN THE LONGITUDINAL MUSCLE OF THE RABBIT SMALL INTESTINE AND THE GUINEA PIG TAENIA CAECI

The effect of various concentrations of GTP on adenylate cyclase activity from guinea pig taenia caeci is shown in figure 12. GTP displayed a bell-shaped effect causing concentration-dependent stimulation of adenylate cyclase activity between 1 μM and 100 μM. However, 1 mM GTP caused less stimulation of the enzyme than a 10 fold lower concentration (figure 12). Similar results were obtained with Gpp(NH)p, a stable analog of GTP (figure 12). Adenosine deaminase (5 U/ml, see below) did not alter the concentration-response curve to the GTP analog. Thus, the stimulation of adenylate cyclase by Gpp(NH)p was not altered by endogenous adenosine. The maximum increase over basal adenylate cyclase activity was 143 and 561% by GTP and Gpp(NH)p, respectively. The results shown in figure 12 were obtained in 1 broken cell preparation and they are representative of those obtained in 3 different preparations.

The concentration effect of Gpp(NH)p on adenylate cyclase from a 10,000 g pellet of the longitudinal muscle of the rabbit small intestine is shown in figure 13. In this preparation, Gpp(NH)p in the range of 100 nM to 100 μM caused a concentration-dependent stimulation of adenylate cyclase. A 10 fold higher concentration of Gpp(NH)p did not cause further stimulation of the enzyme. The results shown in figure 13 were obtained from 1 broken cell preparation and they are representative of those obtained in 3 different

FIG. 12 Concentration-effects, of GTP (A) and Gpp(NH)p (B) in the presence ( ) and absence ( ) of adenosine deaminase, (5 U/ml) on adenylate cyclase activity in the 10,000 g pellet from guinea pig taenia caeci. Results are expressed as mean ± S.E.M. of triplicate determinations. Error bars are not shown when the S.E.M. are contained within the symbol.

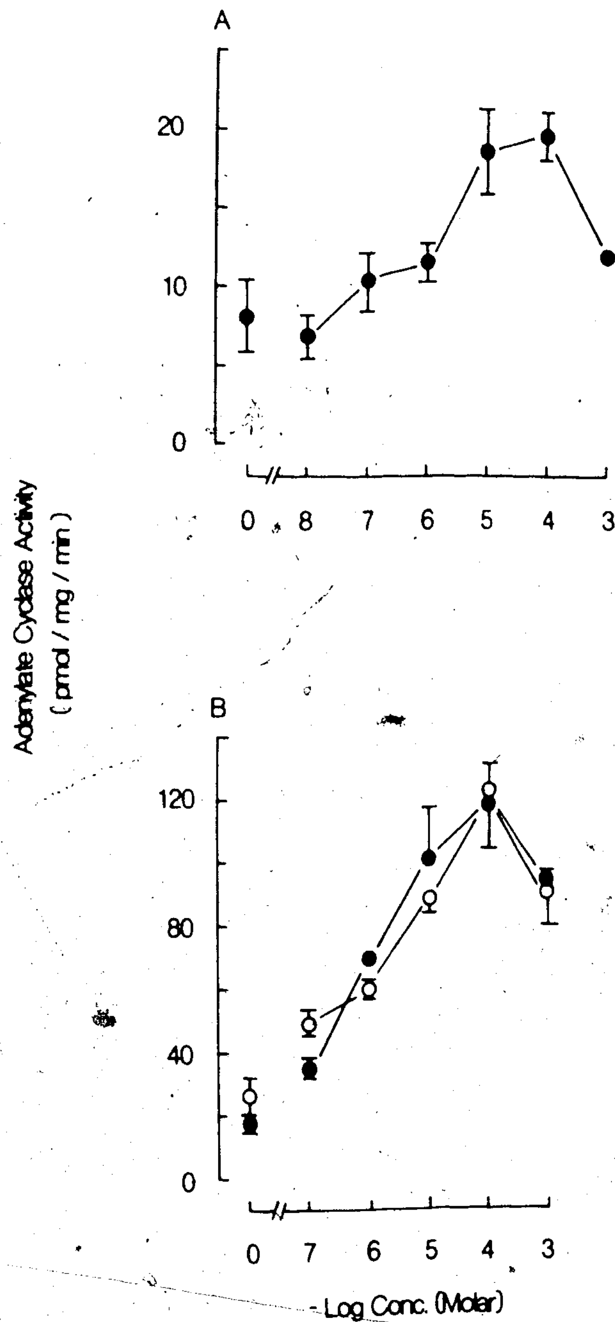


FIG. 12 Concentration-effects, of GTP (A) and Gpp(NH)p (B) in the presence (○) and absence (●) of adenosine deaminase, (5 U/ml) on adenylate cyclase activity in the 10,000 g pellet from guinea pig taenia caeci. Results are expressed as mean ± S.E.M. of triplicate determinations. Error bars are not shown when the S.E.M. are contained within the symbol.

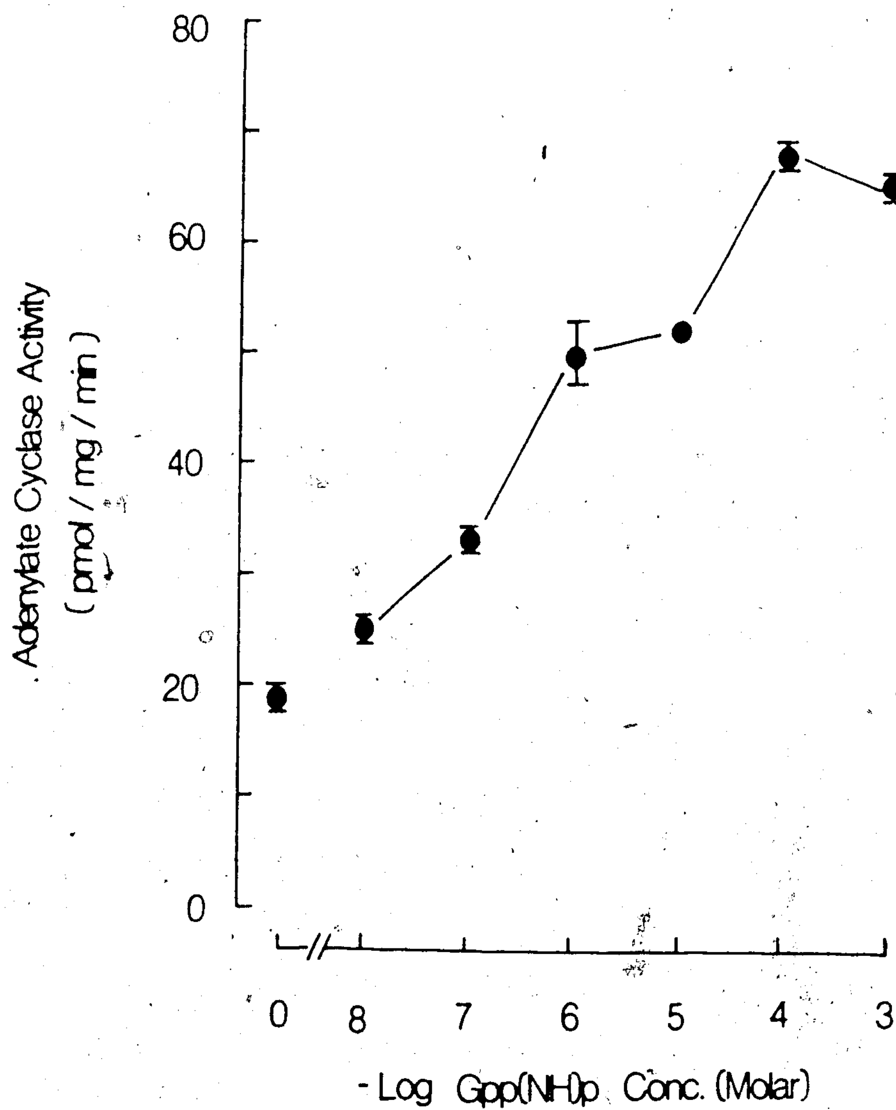


FIG. 13 Concentration-effects of Gpp(NH)p (●) on adenylate cyclase activity in the 10,000 g pellet from the longitudinal muscle of the rabbit small intestine. Results are expressed as mean ± S.E.M. of triplicate determinations. Error bars are not shown when the S.E.M. is contained within the symbol.

preparations.

3.2.5 SUBSTITUTION OF DEOXY-alpha-[^{32}P]-ATP FOR alpha-[^{32}P]-ATP IN THE ADENYLATE CYCLASE ASSAY IN GUINEA PIG TAENIA CAECI

Investigators have eliminated the possibility of endogenous adenosine obscuring hormone-mediated stimulation of adenylate cyclase in a broken cell preparation in the following two ways: substitution of deoxy-alpha-[^{32}P]-ATP for alpha [^{32}P]-ATP in the cyclase assay and hydrolysis of adenosine to inosine, which is inactive at adenosine receptors, by addition of adenosine deaminase to the enzyme preparation. In this manner, adenosine-mediated stimulation of adenylate cyclase has been demonstrated in a number of different cell types (Londos et al., 1981).

The effect of substituting deoxy-alpha-[^{32}P]-ATP for alpha-[^{32}P]-ATP in the assay of adenylate cyclase from guinea pig taenia caeci with respect to stimulation of adenylate cyclase activity by various agents is shown in figure 14. NaF (10 mM) stimulated the enzyme by 451 and 489% in the deoxy-alpha-[^{32}P]-ATP and alpha-[^{32}P]-ATP assays, respectively. Forskolin (1 mM) stimulated the enzyme by 1,013 and 1,008% in the deoxy-alpha-[^{32}P]-ATP and alpha-[^{32}P]-ATP assays, respectively. Thus NaF and forskolin stimulated adenylate cyclase from guinea pig taenia caeci to the same extent whether deoxy-alpha-[^{32}P]-ATP or alpha-[^{32}P]-ATP was used as substrate in the assay. Also, no stimulation of adenylate cyclase activity was seen in this preparation with 2-chloroadenosine or isoproterenol when either deoxy-alpha-[^{32}P]-ATP or alpha-[^{32}P]-ATP were used as substrate.

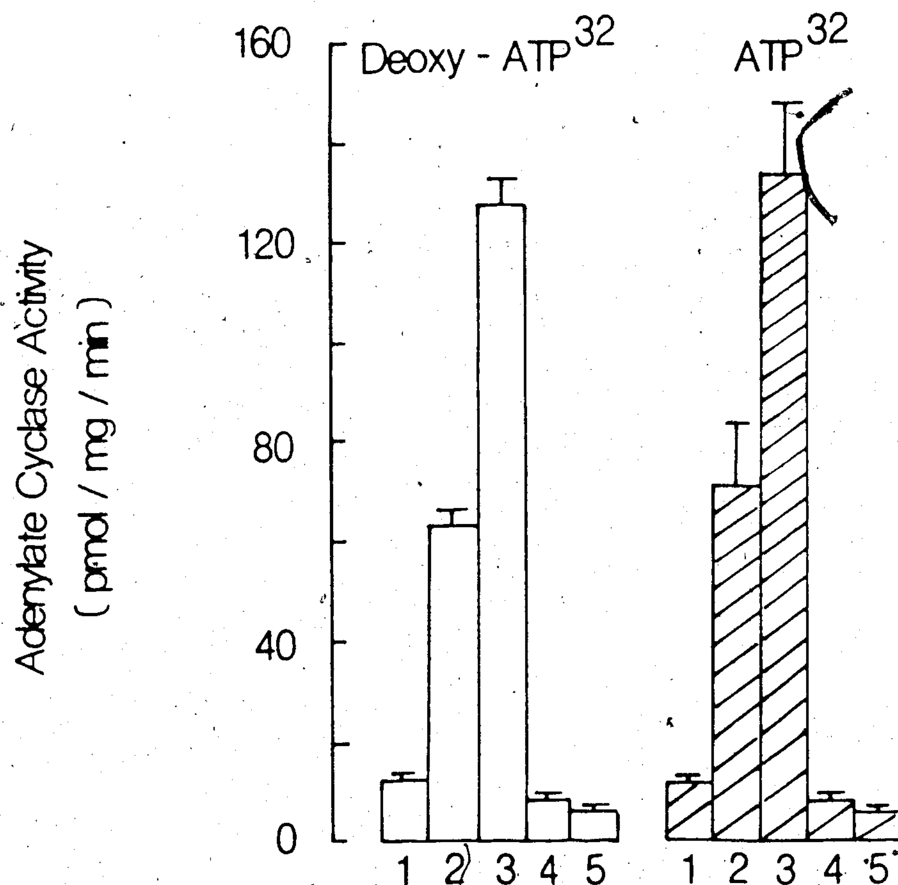


FIG. 14 Effect on basal (1) adenylate cyclase activity in the 10,000 g pellet from guinea pig taenia caeci of 1 mM forskolin (3), 1 mM 2-chloroadenosine (4), 1 mM isoproterenol (5) and 10 mM NaF (2) using alpha-[^{32}P]-ATP (▨) or deoxy-alpha-[^{32}P]-ATP (□) as substrate in the assay system. Results are shown as mean ± S.E.M. of triplicate determinations.

We also attempted to stimulate adenylate cyclase from guinea pig taenia caeci with adenosine in the absence and presence of GTP(100 μM) or forskolin (100 μM) and using deoxy-alpha-[^{32}P]-ATP instead of alpha-[^{32}P]-ATP in the assay system. The concentration-effect curves for adenosine under these conditions are shown in figure 15. No stimulation of adenylate cyclase activity was found at any concentration of adenosine tested. In the presence of forskolin, adenosine inhibited adenylate cyclase activity. 1 μM and 10 μM forskolin stimulated the enzyme by 128 and 536%, respectively (see figure 16). Under these conditions, no further stimulation of the enzyme was seen with NECA at any concentration tested (see figure 16). Thus, receptor-mediated stimulation of adenylate cyclase in smooth muscle was not observed under any condition tested using deoxy-alpha-[^{32}P]-ATP as substrate in the enzyme assay. The results shown in figures 14, 15 and 16 were obtained from 1 broken cell preparation of guinea pig taenia caeci and they are representative of those obtained in 3 different enzyme preparations.

3.2.6 EFFECTS OF GTP AND ADENOSINE DEAMINASE ON THE ACTION OF NECA ON ADENYLATE CYCLASE ACTIVITY IN THE LONGITUDINAL MUSCLE OF THE RABBIT SMALL INTESTINE AND GUINEA PIG TAENIA CAECI

The effects of concentrations of NECA with respect to adenylate cyclase activity from guinea pig taenia caeci and the longitudinal muscle from rabbit small intestine in the presence of adenosine deaminase (5 U/ml) and GTP (50 μM) are shown in figures 17 and 18, respectively. This amount of adenosine deaminase will metabolize

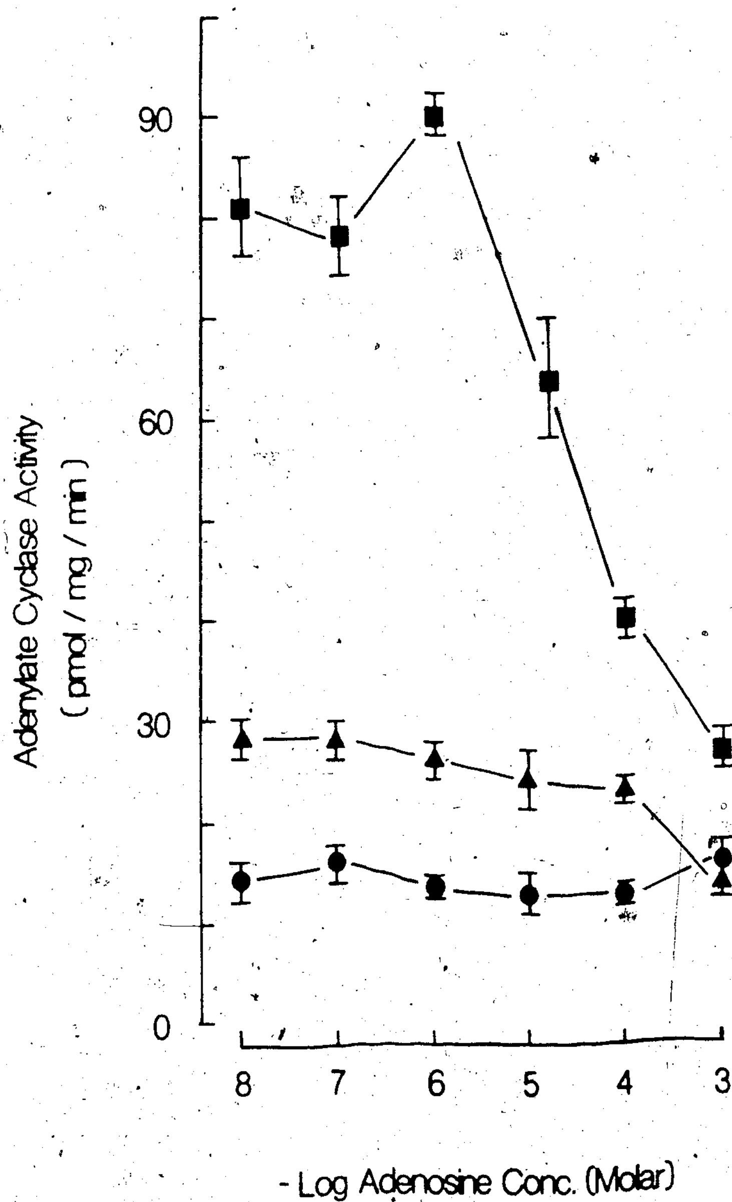


FIG. 15 Concentration-effect of adenosine on basal (●) adenylate cyclase activity in the 10,000 g pellet from guinea pig taenia caeci or on adenylate cyclase activity treated with 100 μM GTP (▲) or 1 mM forskolin (■) using deoxy-alpha-[^{32}P]-ATP as substrate in the assay system. Results are expressed as mean ± S.E.M. of triplicate determinations. Error bars are not shown in some cases for clarity.

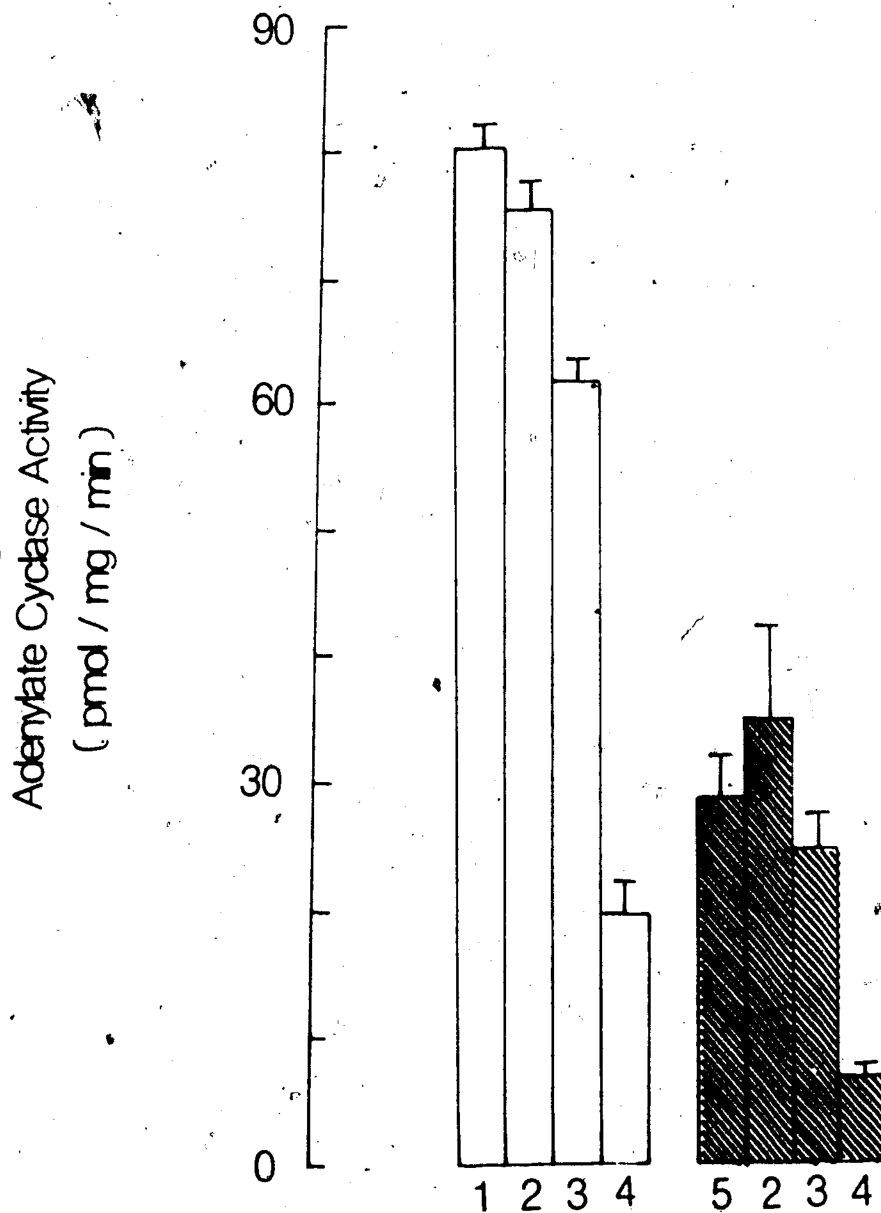


FIG.16 Concentration-effect of NECA (10 μM (2), 100 μM (3) and 1 mM (4)) on adenylate cyclase activity in the 10,000 g pellet from guinea pig taenia caeci treated with 10 μM (□) or 1 μM (▧) (1.5) forskolin using deoxy-alpha-[^{32}P]-ATP as substrate in the assay system. Results are expressed as mean ± S.E.M. of triplicate determinations.

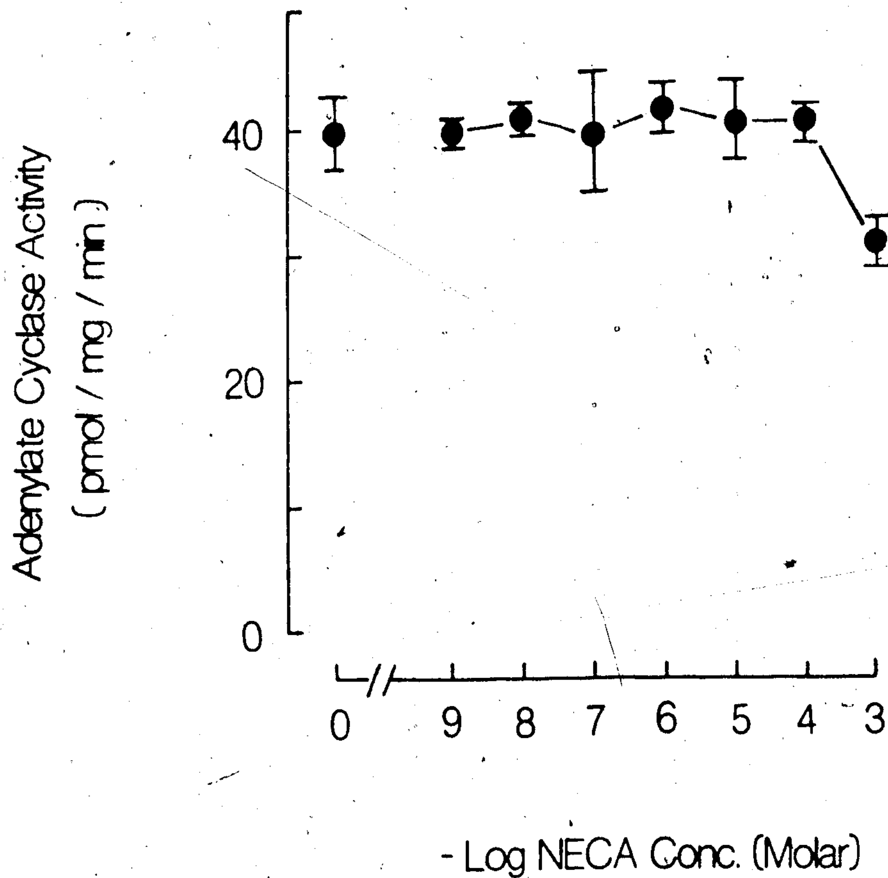


FIG. 17 Lack of stimulation by various concentrations of NECA (●) of adenylate cyclase activity in the 10,000 g pellet from guinea pig taenia caeci. The adenylate cyclase preparation was treated with 50 μM GTP and adenosine deaminase. Results are expressed as mean ± S.E.M. of triplicate determinations.

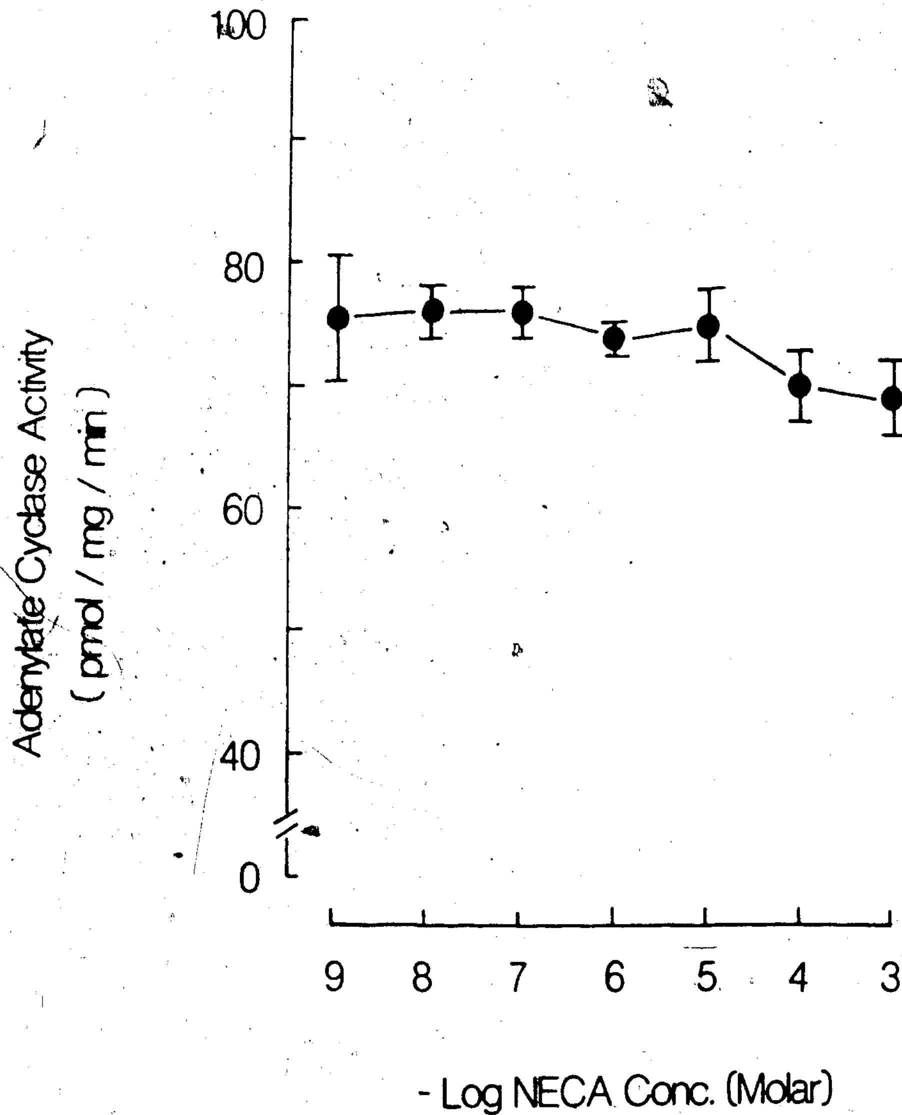


FIG. 18 Lack of stimulation by various concentrations of NECA (●) of adenylate cyclase activity in the 10,000 g pellet from the longitudinal muscle of rabbit small intestine. The adenylate cyclase preparation was treated with 50 μM GTP and adenosine deaminase. Results are expressed as mean ± S.E.M. of triplicate determinations.

5 μmoles of adenosine per min at 37 degrees C and is sufficient for use in tissues with high phosphorylase activity and low adenylate cyclase activity (Londos et al., 1981). Under these conditions, where endogenous adenosine is eliminated and the guanine nucleotide is present, no stimulation of adenylate cyclase was seen with NECA in concentration range from 1 nM to 1 mM. Thus, the absence of GTP and/or the presence of endogenous adenosine in the system do not appear to explain the lack of sensitivity of adenylate cyclase from these smooth muscles to drugs. The results shown in figures 17 and 18, were obtained in 1 broken cell preparation and they are representative of results obtained from 3 different preparations.

3.2.7 EFFECT OF Gpp(NH)p AND ADENOSINE DEAMINASE ON THE ACTION OF NECA ON ADENYLATE CYCLASE ACTIVITY IN THE LONGITUDINAL MUSCLE OF THE RABBIT SMALL INTESTINE

Adenylate cyclase from the longitudinal muscle from rabbit small intestine was treated with 0.1 μM or 1 mM Gpp(NH)p plus adenosine deaminase and the effect of NECA on enzyme activity was examined (figure 19). No stimulation of adenylate cyclase was observed in this preparation by NECA in the concentration range from 1 nM to 1 mM, under the conditions where endogenous adenosine was eliminated and the stable GTP analog was present. The results shown in figure 19 were obtained in 1 broken cell preparation and are representative of those obtained in 4 different enzyme preparations.

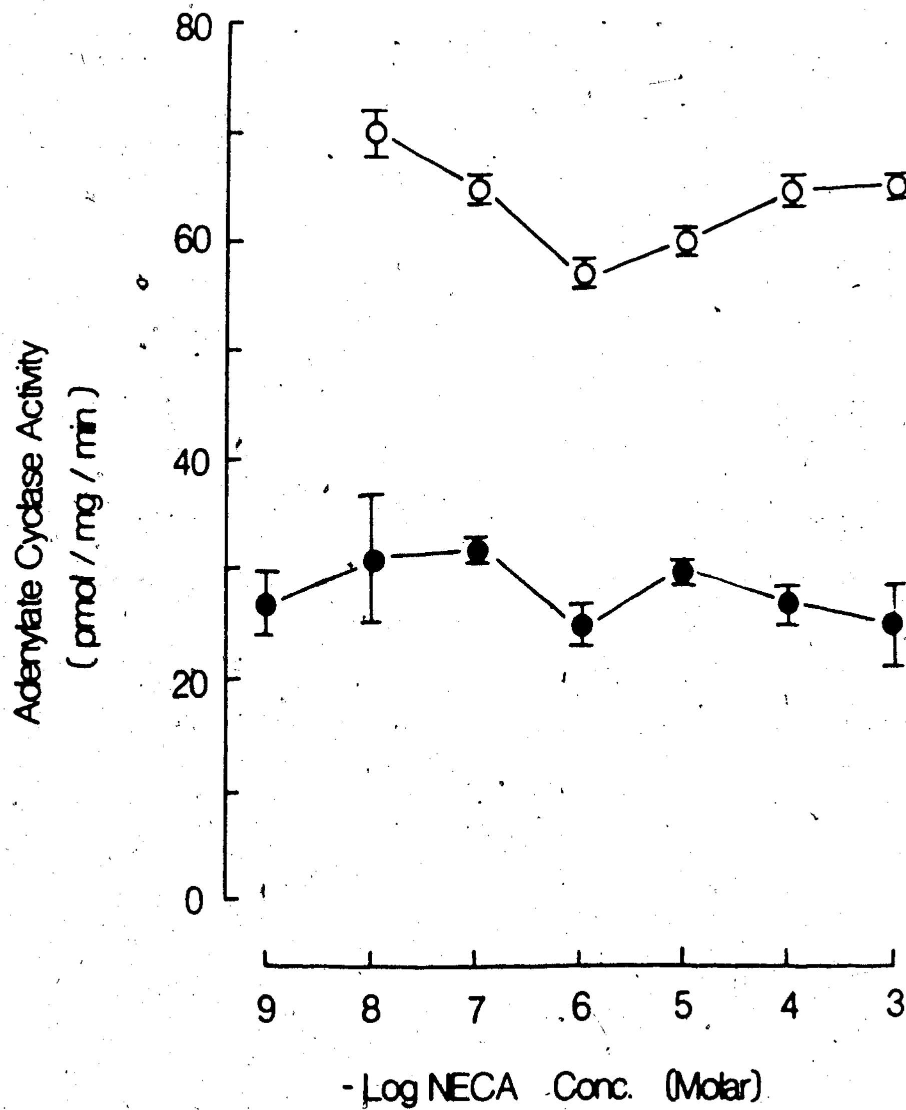


FIG. 19 Lack of stimulation by various concentrations of NECA (●○) of adenylate cyclase in the 10,000 g pellet from the longitudinal muscle of rabbit small intestine treated with 0.1 μM (●) or 1 mM (○) Gpp(NH)p and adenosine deaminase. Results are expressed as mean ± S.E.M. of triplicate determinations.

3.2.8 EFFECT OF ISOPROTERENOL ON Gpp(NH)p-INDUCED STIMULATION OF SMOOTH MUSCLE ADENYLATE CYCLASE ACTIVITY IN GUINEA PIG TAENIA CAECI TREATED WITH ADENOSINE DEAMINASE

To test the possibility that receptor-mediated stimulation of adenylate cyclase could not be demonstrated because an optimal concentration of guanine nucleotide was not being used, the effect of 100 μM isoproterenol on Gpp(NH)p-induced stimulation of adenosine deaminase treated adenylate cyclase from guinea pig taenia caeci was examined. The results are shown in figure 20. Gpp(NH)p caused stimulation of adenylate cyclase in a concentration-dependent manner up to 10 μM. A further increase in the concentration of Gpp(NH)p to 100 μM caused submaximal stimulation of the enzyme. Treatment of the preparation with 100 μM isoproterenol appeared to prevent the decrease in stimulation of adenylate cyclase by 100 μM Gpp(NH)p while not altering the action of the GTP analog at any other concentration. Although not as pronounced, this effect was observed in another broken cell preparation of guinea pig taenia caeci. In a third broken cell preparation 100 μM isoproterenol did not alter the effect of Gpp(NH)p on adenylate cyclase activity. Therefore, this effect of isoproterenol was not reproducible.

Stimulation of adenylate cyclase from guinea pig taenia caeci by 100 μM Gpp(NH)p in the absence and presence of 100 μM isoproterenol was examined over the 9 min time course of an assay (see figure 21). Gpp(NH)p stimulated the enzyme on an average by 154% and it plus isoproterenol stimulated the enzyme on an average by 150%. Thus, isoproterenol did not alter stimulation of adenylate cyclase obtained

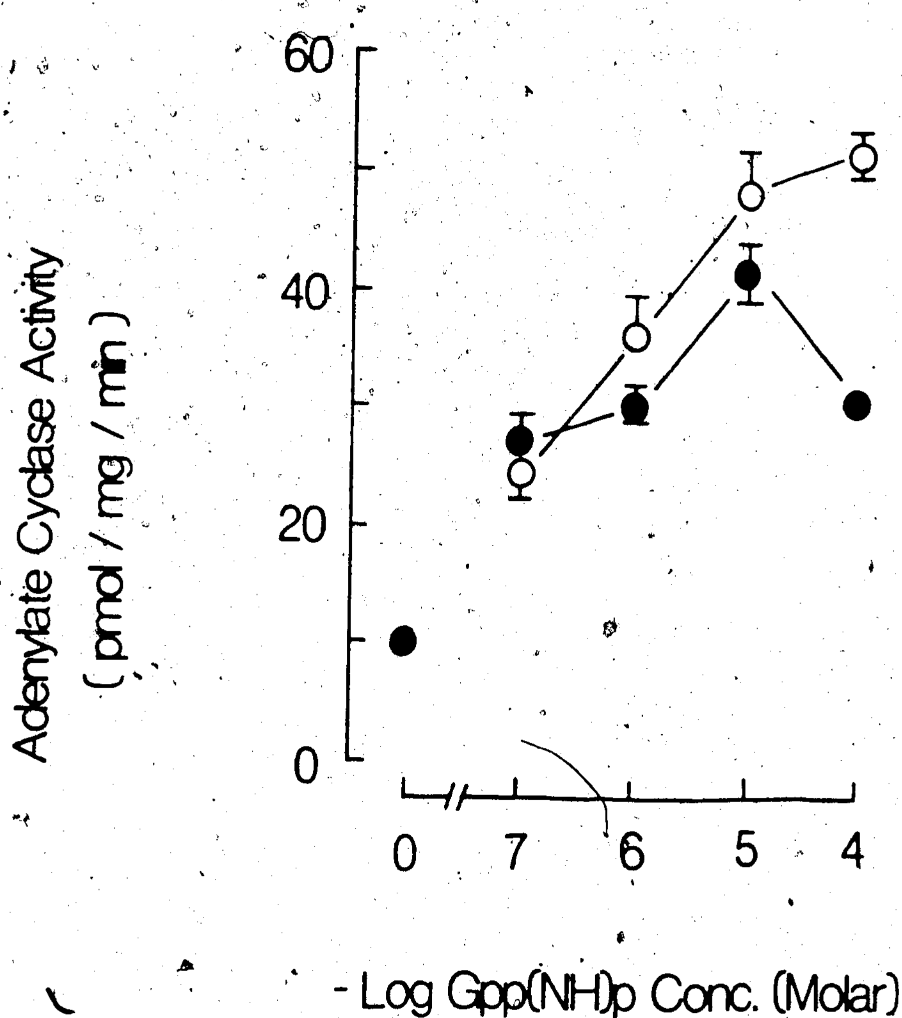


FIG. 20 Concentration-dependent stimulation of adenylate cyclase activity in the 10,000 g pellet from guinea pig taenia caeci (treated with adenosine deaminase) by Gpp(NH)p in the absence (●) or presence (○) of 100 μM isoproterenol. The assay mixture was incubated for 9 min. Results are expressed as the mean ± S.E.M. of triplicate determinations. Error bars are not shown when the S.E.M. are contained within the symbol.

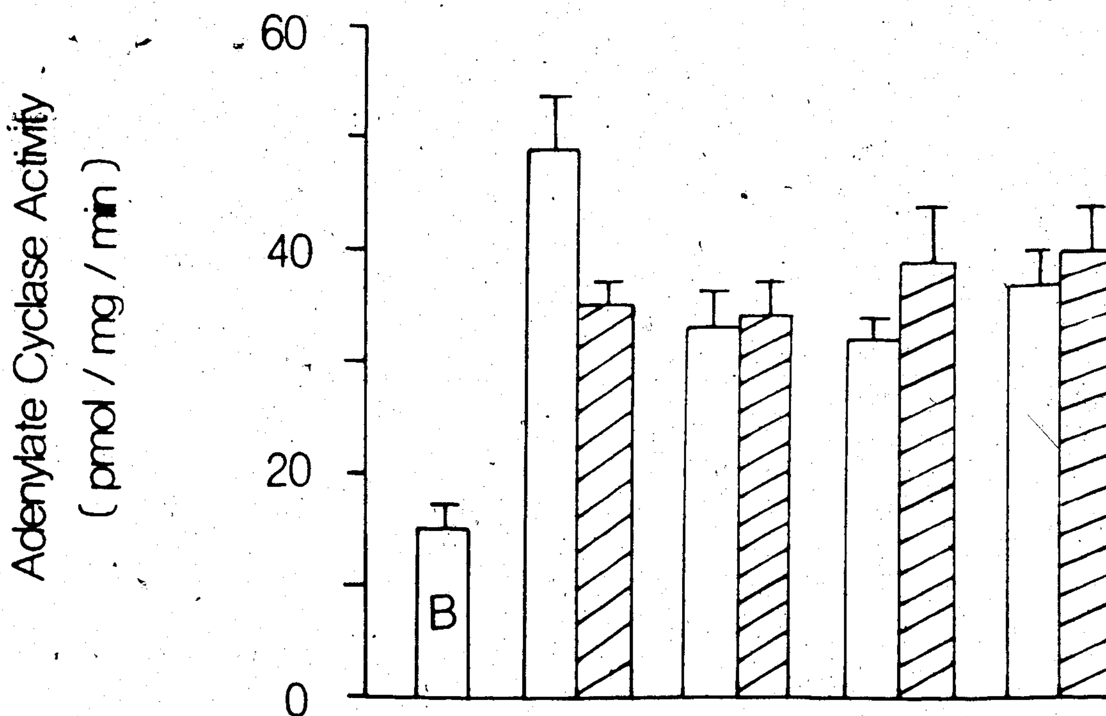


FIG. 21 Repeated stimulation of basal (B) adenylate cyclase activity in the 10,000 g pellet from guinea pig taenia caeci (treated with adenosine deaminase) by 100 μM Gpp(NH)p in the absence (☐) or presence (▨) of 100 μM isoproterenol. The assay mixture was incubated for 9 min. Results are expressed as mean ± S.E.M. of triplicate determinations.

with Gpp(NH)p alone. Furthermore, at no time was the enzyme stimulated to a greater extent by the combination of these agents over the guanine nucleotide analog alone. The results shown in figure 21 were obtained from 1 broken cell preparation and are representative of 2 preparations.

3.2.9 TIME COURSE OF STIMULATION OF ADENYLATE CYCLASE ACTIVITY FROM GUINEA PIG TAENIA CAECI WITH Gpp(NH)p IN THE ABSENCE AND PRESENCE OF ISOPROTERENOL

The time course of Gpp(NH)p-induced stimulation of adenylate cyclase from guinea pig taenia caeci in the presence and absence of isoproterenol (100 μM) is shown in figure 22. Gpp(NH)p alone or with isoproterenol were added to the assay at 16 min. The basal rate of adenylate cyclase activity before addition of drug was about 2 pmol/mg/min. After 4 min of addition of drug the rate of enzyme activity had not changed appreciably. However, it increased to 8 pmol/mg/min, thereafter. As can be seen in figure 22, isoproterenol had no effect on the stimulation of adenylate cyclase at any time point examined. In another broken cell preparation of guinea pig taenia caeci isoproterenol decreased the Gpp(NH)p-induced stimulation of adenylate cyclase activity. In a third broken cell preparation results similar to those shown in figure 22 were obtained.

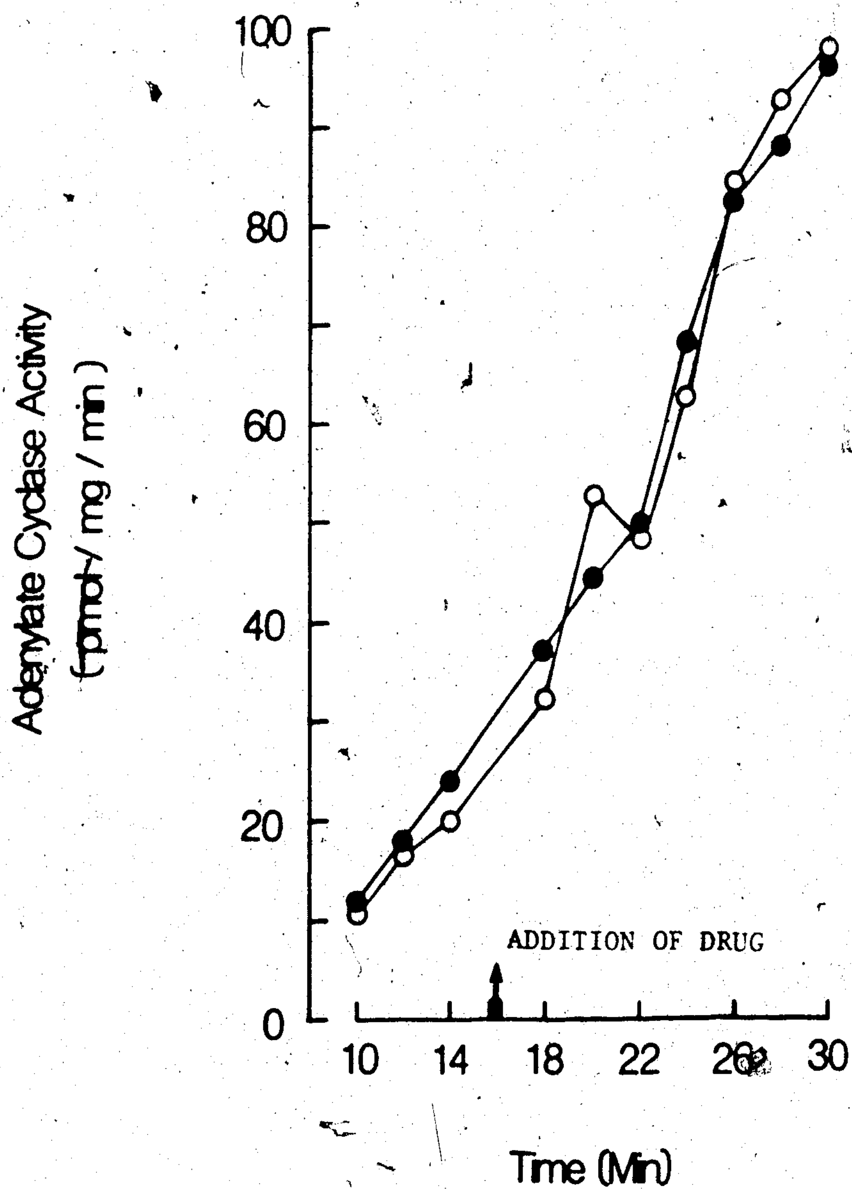


FIG. 22 Time-dependent stimulation of adenylate cyclase activity in the 10,000 g pellet from guinea pig taenia caeci (treated with adenosine deaminase) by 100 μM Gpp(NH)p in the absence (●) or presence (○) of 100 μM isoproterenol. Results are single determinations.

3.2.10 EFFECT OF Gpp(NH)p ON THE ACTION OF ISOPROTERENOL ON GUINEA PIG TAENIA CAECI ADENYLATE CYCLASE TREATED WITH ADENOSINE DEAMINASE

The effect of 1 μM and 100 μM Gpp(NH)p on the action of isoproterenol (1 μM to 1 mM) on adenylate cyclase from guinea pig taenia caeci in the presence of adenosine deaminase is shown in figure 23. No stimulation of adenylate cyclase was seen with isoproterenol at any concentration tested in either of the Gpp(NH)p treated enzyme preparations. The results shown in figure 23 were obtained in 1 broken cell preparation and they are representative of results obtained in 3 different preparations.

3.2.11 EFFECT OF THE REGENERATING SYSTEM IN THE ADENYLATE CYCLASE ASSAY OF ENZYME ACTIVITY IN GUINEA PIG TAENIA CAECI

The above experiment was performed with two distinctive features. Firstly, the regenerating system consisting of creatine phosphate and creatine kinase was eliminated from the adenylate cyclase assay. Secondly, the enzyme was preincubated with drug for 30 min prior to initiating the assay by addition of the substrate, alpha-[^{32}P]-ATP. Isoproterenol-induced stimulation of adenylate cyclase from vascular smooth muscle has been demonstrated utilizing this protocol by other investigators (Hamet et al., 1978). The concentration-response effect of isoproterenol on adenylate cyclase activity in guinea pig taenia caeci in the presence or absence of 1 or 100 μM Gpp(NH)p is shown in figure 24. There was no stimulation of adenylate cyclase activity. These results suggest that the regenerating system was not obscuring

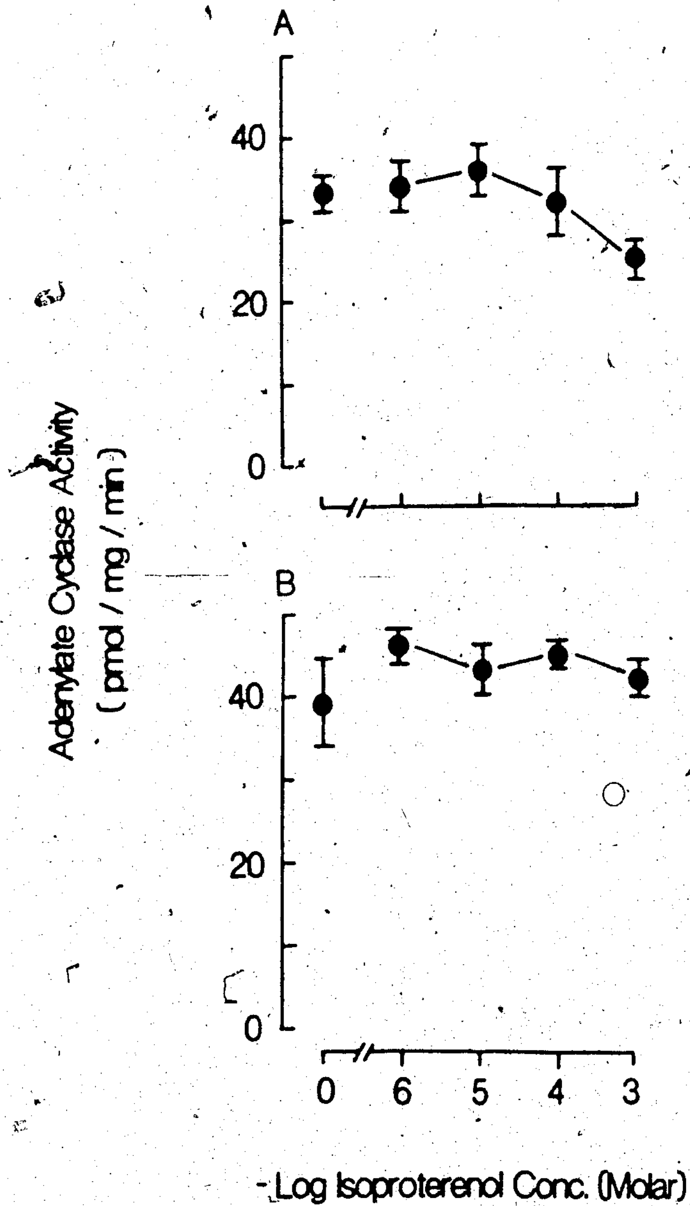


FIG. 23 Lack of stimulation of adenylate cyclase activity in the 10,000 g pellet from guinea pig taenia caeci (treated with adenosine deaminase) by various concentrations of isoproterenol in the presence of 1 μM (A) or 100 μM (B) Gpp(NH)p. The assay mixture was incubated for 9 min and contained EDTA (1 mM) and DTT (1 mM). Results are expressed as mean ± S.E.M. of triplicate determinations.

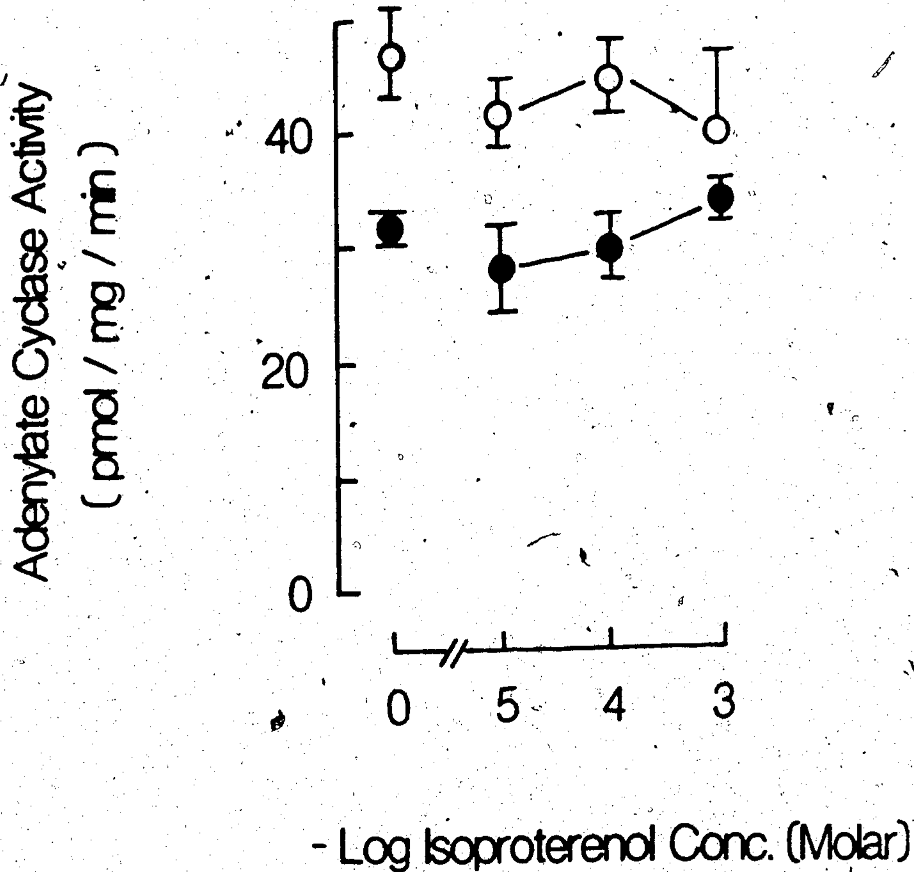


FIG. 24 Lack of stimulation of adenylate cyclase activity in the 10,000 g pellet from guinea pig taenia caeci (treated with adenosine deaminase) by various concentrations of isoproterenol in the presence of 1 μM (●) or 100 μM (○) Gpp(NH)p. The assay mixture was incubated for 9 min and contained EDTA (1 mM) and DTT (1 mM). Furthermore, it was devoid of a regenerating system and adenylate cyclase was treated with isoproterenol and Gpp(NH)p for 30 min prior to initiating the assay by addition of the substrate, alpha-[^{32}P]-ATP. Results are expressed as mean ± S.E.M. of triplicate determinations.

enzyme stimulation in this preparation. These results were obtained in one broken cell preparation and they are representative of results obtained in three different preparations.

3.2.12 EFFECT OF VARIOUS COMPOUNDS ON ADENYLATE CYCLASE ACTIVITY FROM MEMBRANE FRACTIONS OF LONGITUDINAL MUSCLE OF RABBIT SMALL INTESTINE

As described in the methods, the longitudinal muscle of the rabbit small intestine was fractionated into the 1,200 g, 13,000 g and 105,000 g pellets and the final supernanant, and termed P-1, P-2, P-3 and S-3, respectively. The effects of a variety of agents on adenylate cyclase activity in these fractions were determined alone and in combination with Gpp(NH)p. The results from 1 broken cell preparation are depicted in form of histograms (see figure 25) and in tabular form (see table 1). Isoproterenol did not increase basal adenylate cyclase activity in P-2, P-1 and S-3. On the other hand, isoproterenol stimulated the enzyme activity by 44% in fraction P-3. Similarily, NECA did not increase adenylate cyclase activity in fractions P-1 and S-3. However 55 and 29% stimulation of the enzyme activity was seen with NECA in fractions P-3 and P-2, respectively. These results are representative of 2 broken cell preparations of longitudinal muscle from rabbit small intestine. In another 2 preparations no stimulation of adenylate cyclase activity was observed with isoproterenol or NECA.

Gpp(NH)p stimulated adenylate cyclase activity in all the fractions tested except S-3. Gpp(NH)p in combination with isoproterenol caused no further stimulation in fractions P-1, P-2 and S-3.

FIG. 25 Effect on basal (1) adenylate cyclase activity in fraction S-3 (A), P-1 (B), P-2 (C) and P-3 (D) from the longitudinal muscle of rabbit small intestine (treated with adenosine deaminase) of the following: 100 μM isoproterenol and 100 μM NECA in the absence (2,3 respectively) or presence (5,6 respectively) of 100 μM Gpp(NH)p, 100 μM Gpp(NH)p alone (4), 10 mM NaF (7) and 1 mM forskolin (8). Note that the scales for adenylate cyclase activity differ in the various fractions. The assay mixture was incubated for 12 min and contained EDTA (1 mM) and DTT (1 mM). Results are expressed as mean ± S.E.M. of triplicate determinations. In panel D number 8, 190 (8) represents the mean ± (S.E.M.).

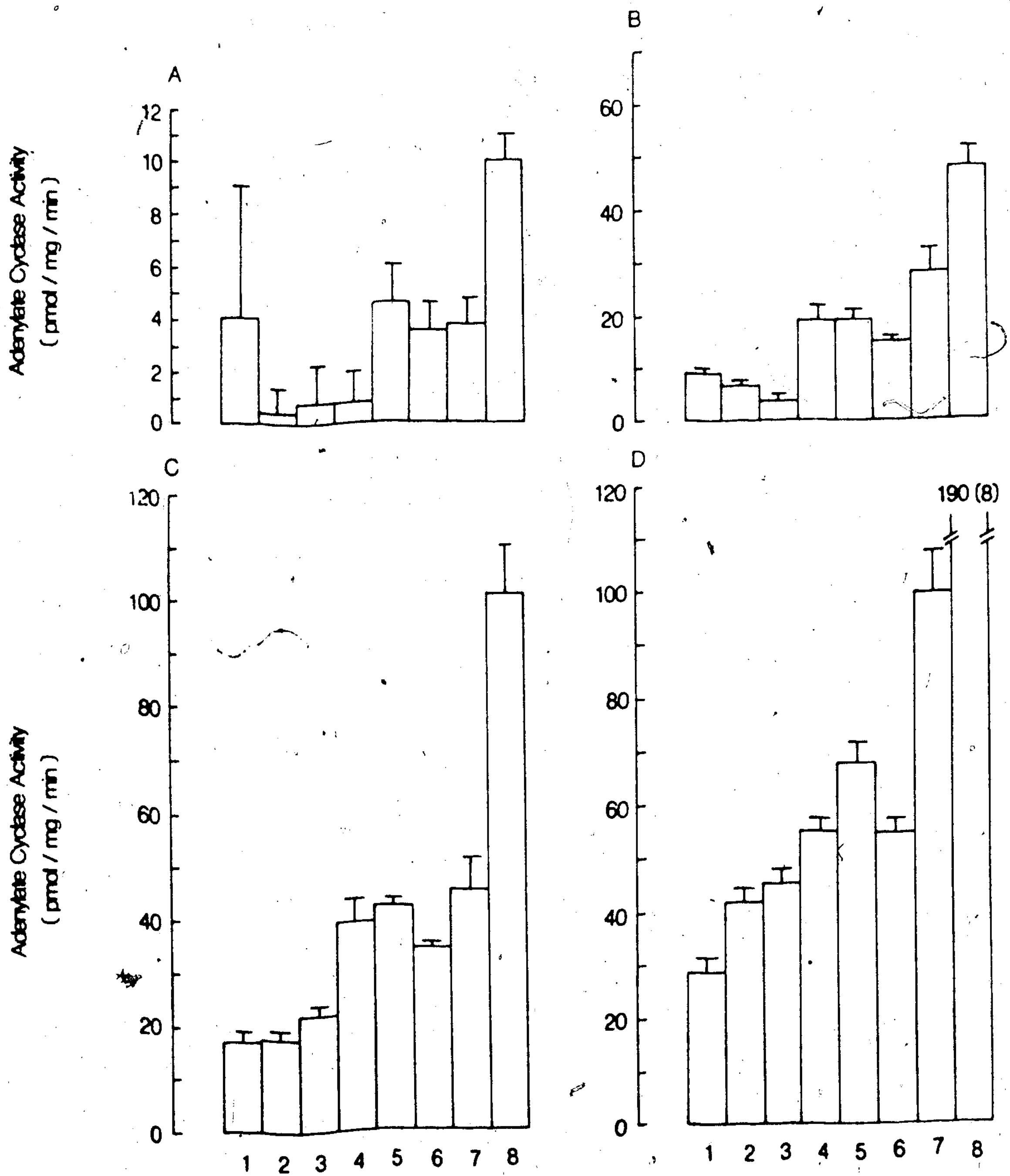


FIG. 25

TABLE 1. Effect on basal adenylate cyclase activity in various fractions from the longitudinal muscle of rabbit small intestine of a variety of agents.

Drug Treatment (Concentration)	Percent basal activity (± S.E.M.) in Fractions			
	P-1	P-2	P-3	S-3
Isoproterenol (100 μM)	(—)	3 (9)	44 (6)	(—)
NECA (100 μM)	(—)	29 (8)	55 (7)	(—)
Gpp(NH)p (100 μM)	115 (15)	132 (10)	87 (4)	(—)
Gpp(NH)p (100 μM) plus Isoproterenol (100 μM)	118 (13)	152 (1)	132 (7)	15 (32)
Gpp(NH)p (100 μM) plus NECA (100 μM)	65 (5)	104 (2)	89 (5)	(—)
NaF (10 mM)	215 (17)	165 (16)	245 (8)	(—)
Forskolin (1 mM)	437 (9)	494 (12)	550 (5)	146 (14)

For other details of these results, see the legend to Figure 26 and the text.

(—) designates no effect.

S.E.M. of triplicate determinations are shown in brackets.

These results are representative of data obtained from 4 broken cell preparations. In one broken cell preparation, 52% additional stimulation of enzyme activity was seen with the drug in combination with the GTP analog in fraction P-3. No such effect was observed in 3 other broken cell preparations. Gpp(NH)p in combination with NECA showed no stimulation of adenylate cyclase activity in any fraction above that found with the guanine nucleotide analog alone. This result is representative of that found in 3 other broken cell preparations.

The effect of NaF and forskolin on adenylate cyclase activity were also tested in all fractions. NaF stimulated enzyme activity in all fractions except S-3. Forskolin was the most potent stimulator of adenylate cyclase activity in all fractions. This result is representative of that found in 4 different broken cell preparations.

3.2.13 EFFECT OF VARIOUS CONCENTRATIONS OF ISOPROTERENOL AND NECA IN THE PRESENCE AND ABSENCE OF Gpp(NH)p ON ADENYLATE CYCLASE ACTIVITY FROM FRACTION P-3 OF THE LONGITUDINAL MUSCLE OF THE RABBIT SMALL INTESTINE

Effect of various concentrations of isoproterenol and NECA were determined in fraction P-3 from the longitudinal muscle of the rabbit small intestine. The effect of various concentrations of the drugs on basal enzyme activity is shown in figure 26. Neither NECA nor isoproterenol caused stimulation of adenylate cyclase activity at any concentration tested. These results were obtained from a broken cell preparation and they are representative of results obtained in three

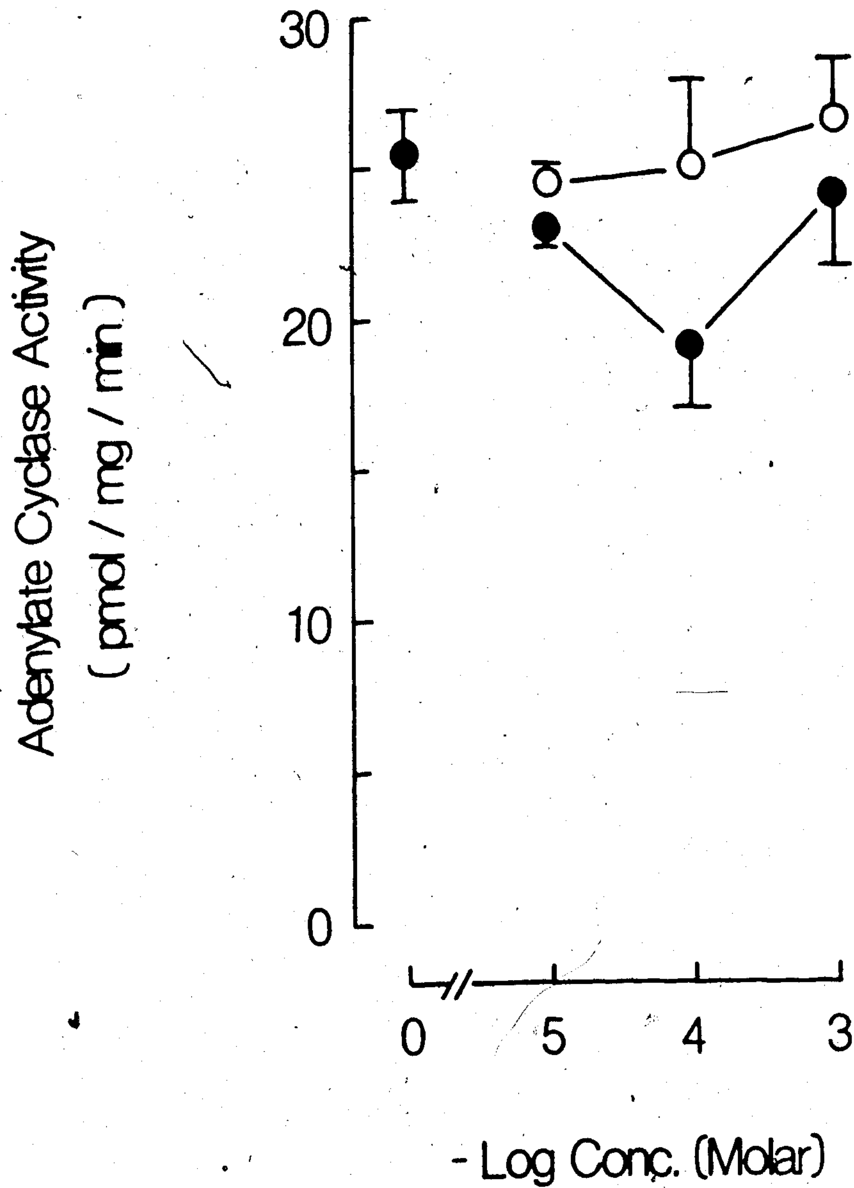


FIG.26 Lack of stimulation of basal adenylate cyclase activity in fraction P-3 from the longitudinal muscle of rabbit small intestine (treated with adenosine deaminase) by various concentrations of isoproterenol (○) and NECA (●). The assay mixture was incubated for 12 min and contained EDTA (1 mM) and DTT (1 mM). Results are expressed as mean ± S.E.M. of triplicate determinations. Error bars are not shown in some cases for clarity.

different preparations.

The effect of Gpp(NH)p on isoproterenol-induced stimulation of adenylate cyclase activity in the P-3 fraction from the longitudinal muscle of the rabbit small intestine was also examined in more detail. The effect of various concentrations of isoproterenol in the absence and presence of 100 μM Gpp(NH)p is shown in figure 27. Once again, isoproterenol did not alter basal adenylate cyclase activity in this preparation at any concentration tested. Thus, our attempts to show stimulation of adenylate cyclase activity with isoproterenol and NECA in fraction P-3 from the rabbit small intestine preparation were unsuccessful both in the absence and presence of Gpp(NH)p. These results were obtained from 1 broken cell preparation and they are representative of results obtained in 3 different preparations.

3.3 CONTRACTILITY STUDIES

3.3.1 EFFECT OF FORSKOLIN ON SMOOTH MUSCLE CONTRACTILITY

Forskolin caused concentration-dependent relaxation in rat aorta, beef and dog coronary artery, guinea pig taenia caeci and inhibition of spontaneous activity in rabbit small intestine. Typical relaxations by forskolin in rabbit small intestine and beef coronary artery are shown in figure 28. Cumulative concentration-response curves for forskolin in these smooth muscles are shown in figure 29. From these concentration-response curves the concentrations causing half-maximal relaxation (ED_{50}) and the 95% confidence limits were calculated (see table 2). It is evident that vascular smooth muscle

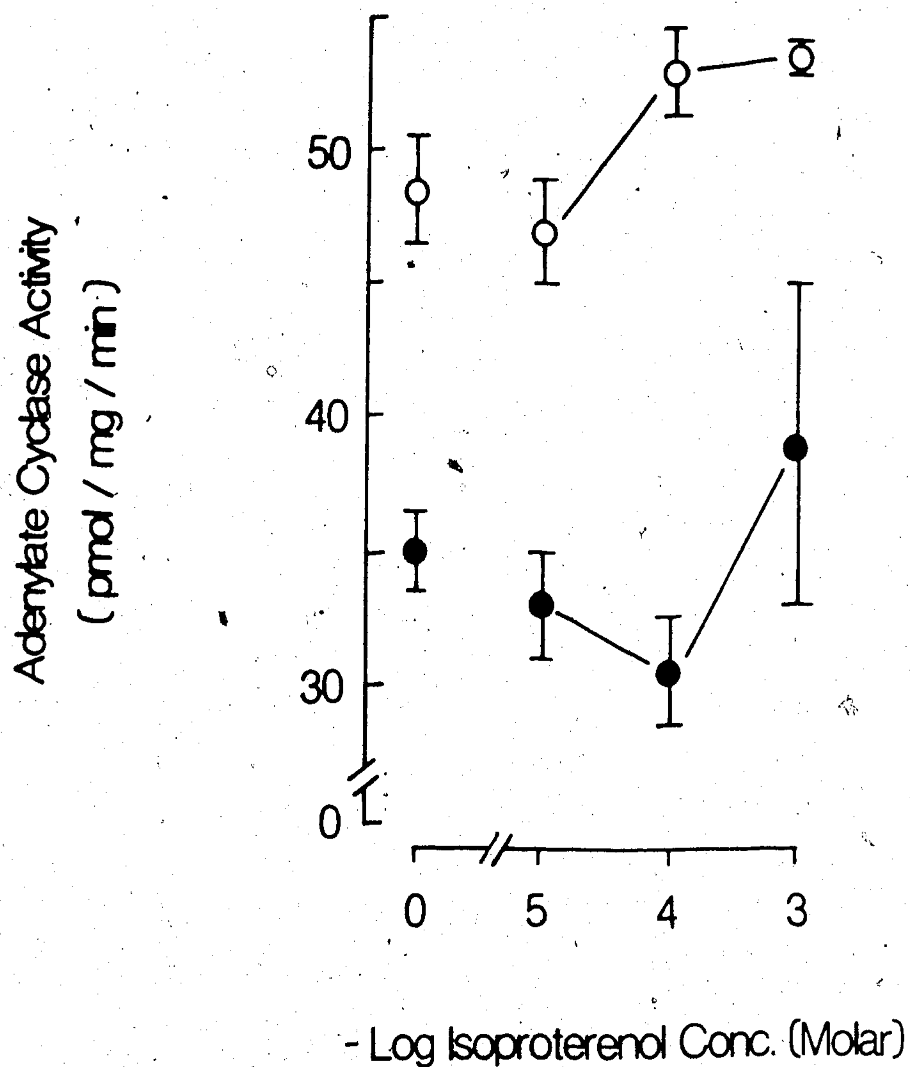


FIG. 27 Lack of stimulation of adenylate cyclase activity in fraction P-3 from the longitudinal muscle of rabbit small intestine (treated with adenosine deaminase) by various concentrations of isoproterenol in the absence (●) or presence (○) of 100 μM Gpp(NH)p. The assay mixture was incubated for 12 min and contained EDTA (1 mM) and DTT (1 mM). Results are expressed as mean ± S.E.M. of triplicate determinations.

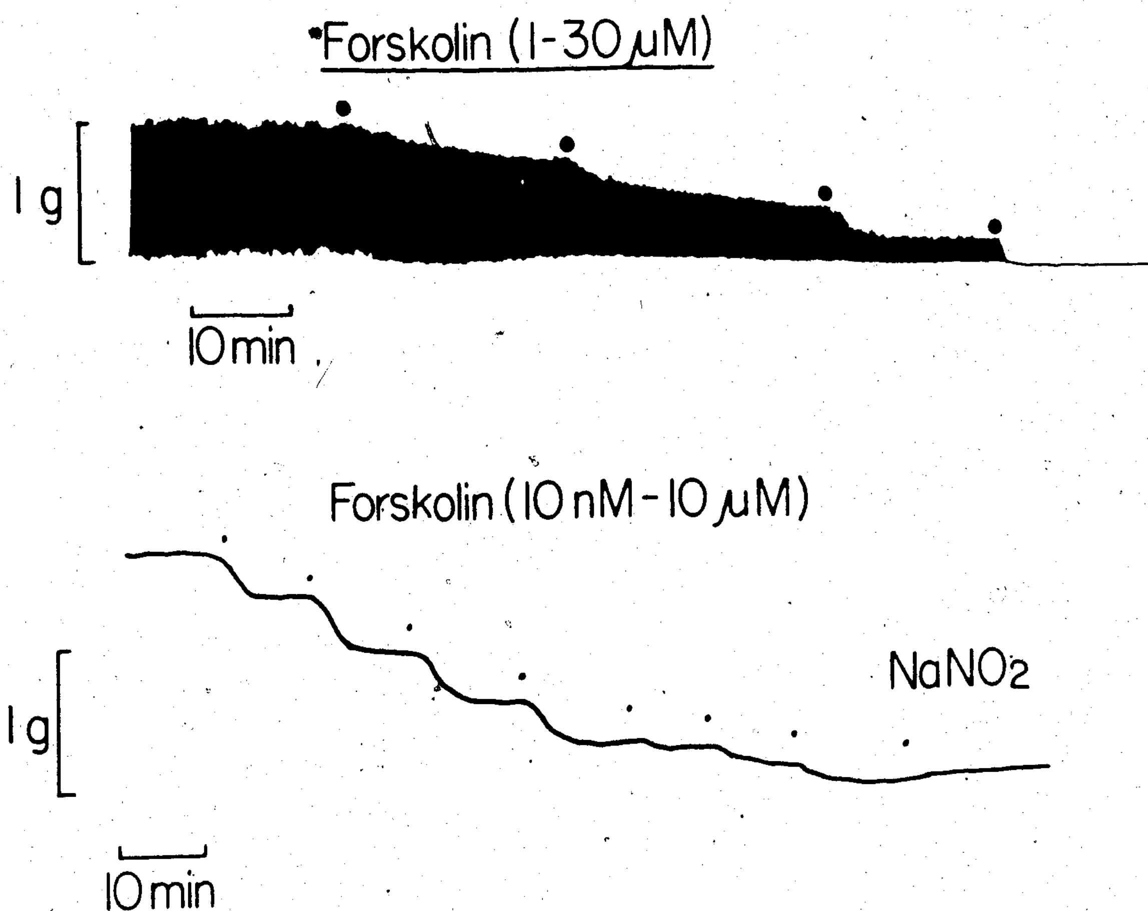


FIG. 28 Representative traces of cumulative inhibition of spontaneous activity in rabbit small intestine (upper tracing) and cumulative relaxation of beef coronary artery (contracted with 20 mM KCl - lower tracing) by forskolin.

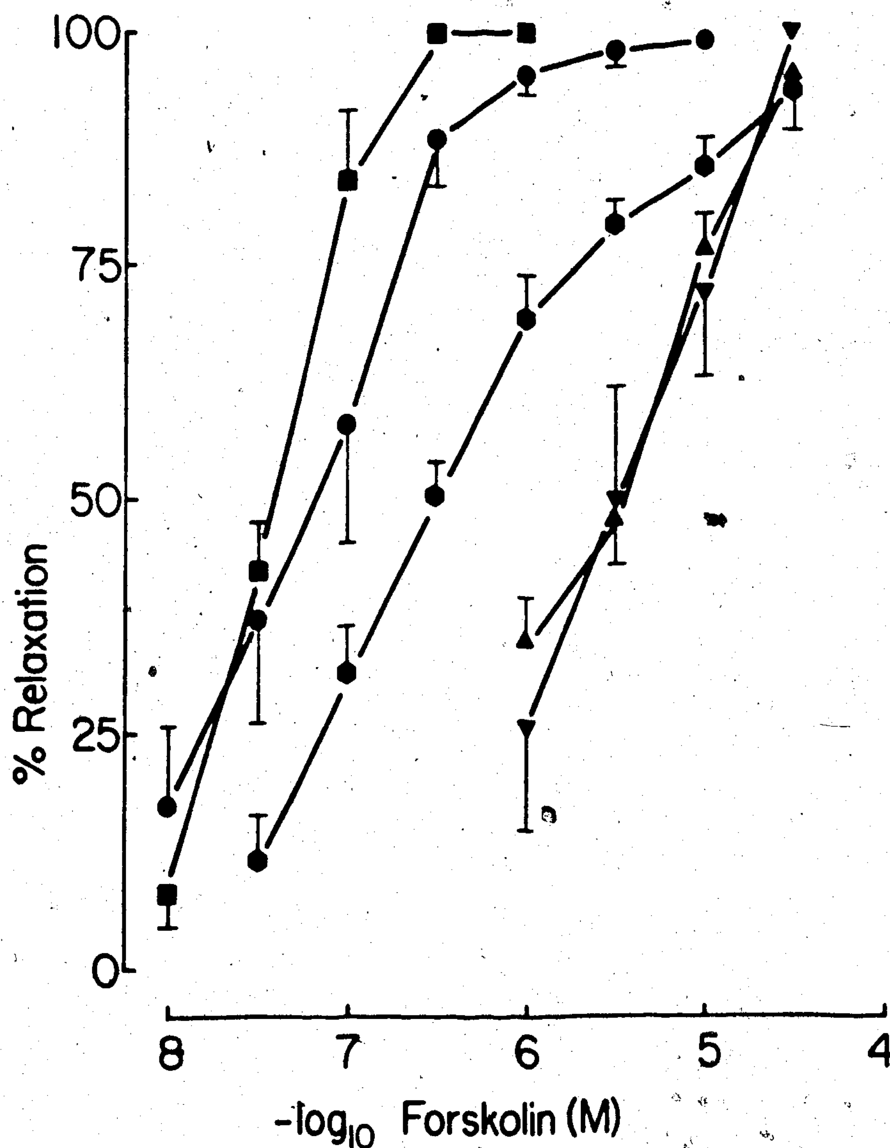


FIG. 29 Cumulative concentration-response curves for forskolin-mediated relaxation or inhibition of spontaneous activity in rat aorta (■), beef coronary artery (●), dog coronary artery (⬧) , guinea pig taenia caeci (▲) , and rabbit small intestine (▼) . Results are expressed as mean ± S.E.M., n=4-6. Only one error bar is shown in some cases for clarity. Also, error bars are not shown when the S.E.M. are contained within the symbol.

TABLE 2. Estimations from cumulative concentration-response curves of concentrations of forskolin causing half-maximal relaxation (ED_{50}) and 95 percent confidence limits in vascular and non-vascular smooth muscle.

Tissue Preparation	ED_{50}	(95% confidence limits)
Rat Aorta	37 nM	(29 nM - 46 nM)
Beef Coronary Artery	51 nM	(44 nM - 57 nM)
Dog Coronary Artery	0.3 μM	(0.18 μM - 0.51 μM)
Guinea Pig Taenia Caeci	2.8 μM	(1.8 μM - 4.3 μM)
Rabbit Small Intestine	2.8 μM	(0.92 μM - 8.6 μM)

was about 10 to 100 fold more sensitive to the relaxant action of forskolin than guinea pig taenia caeci or rabbit small intestine. Furthermore, dog coronary artery appeared to be less sensitive to the action of forskolin than the other vascular smooth muscles.

3.3.2 EFFECT OF MIX AND Ro 20-1724 ON SMOOTH MUSCLE CONTRACTILITY

The phosphodiesterase inhibitors caused "direct" relaxation of the smooth muscles examined in this study, thus, before measuring the effects of MIX or Ro 20-1724 on the relaxant action of forskolin, the inhibitory concentrations causing relaxation were determined. This was done by adding increasing concentrations (in 10 fold concentration increments) to the muscle strips from 2-3 preparations until noticable relaxation was observed. In the concentration-response curve studies with forskolin, one tenth of these concentrations were used. In beef coronary artery, guinea pig taenia caeci and rabbit small intestine, MIX was used at 0.1 μM, 1 μM and 10 μM, and Ro 20-1724 was used at 10 nM, 0.1 μM and 1 μM, respectively. Therefore, the phosphodiesterase inhibitors were employed in concentrations which were devoid of "direct" relaxant or inhibitory effects.

3.3.3 EFFECT OF MIX AND Ro 20-1724 ON RELAXANT OR INHIBITORY EFFECTS OF FORSKOLIN IN SMOOTH MUSCLE

Cumulative concentration-response curves, to forskolin-mediated relaxation in guinea pig taenia caeci measured in the presence or absence of 1 μM MIX or 0.1 μM Ro 20-1724, are shown in figure 30.

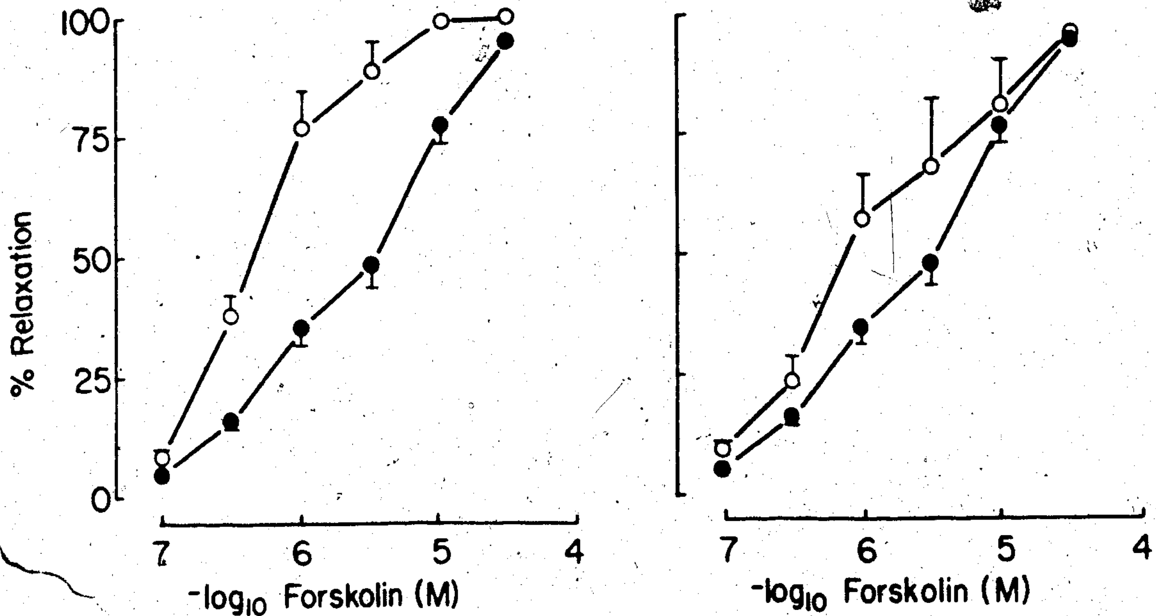


FIG. 30 Cumulative concentration-response curves for forskolin-mediated relaxation of guinea pig taenia caeci. Control curves (●) and curves from tissues treated (○) with Ro 20-1724 (0.1 μM) (left panel) or MIX (1 μM) (right panel) are shown. Results are expressed as mean ± S.E.M., n=6. Some error bars are not shown either when the S.E.M. are contained within the symbol or for the sake of clarity. The ED_{50} of the control curve was significantly different from the ED_{50} of the curve from tissues treated with Ro 20-1724 ($P < 0.05$) but not with MIX.

The concentration-response curves in the presence of the phosphodiesterase inhibitors were shifted to the left compared to a control curve. However, the shift was statistically significant only in the Ro 20-1724 treated tissues. This potentiation of the relaxant effects of forskolin by treatment of the tissue with phosphodiesterase inhibitors suggests that cyclic AMP may be involved in forskolin-mediated relaxation in the guinea pig taenia caeci. The ED_{50} values were obtained as means of values from individual strips and the 95% confidence limits are shown in table 3.

Cumulative concentration-response curves, to forskolin-mediated inhibition of spontaneous activity in rabbit small intestine measured in the presence or absence of 10 μM MIX or 1 μM Ro 20-1724, are shown in figure 31. In this case, the concentration-response curves in the presence of both phosphodiesterase inhibitors were statistically significantly shifted to the left compared to the control curves. Thus, treatment of the tissue with either MIX or Ro 20-1724 potentiated the inhibitory effects of forskolin suggesting an involvement of cyclic AMP in the action of the diterpene. Again the ED_{50} values obtained from these curves and the 95% confidence limits are shown in table 3.

Beef coronary artery proved to be very sensitive to the relaxant action of the phosphodiesterase inhibitors. The concentration-response curves to forskolin-mediated relaxation of this preparation both in the presence and absence of 100 nM MIX and 10 nM Ro 20-1724 are shown in figure 32. The concentration-response curves to forskolin in beef coronary artery in the presence of the phosphodiesterase

TABLE 3. Estimations from cumulative concentration-response curves of concentrations of forskolin causing half-maximal relaxation (ED_{50}) and the 95 percent confidence limits in smooth muscle: Effect of phosphodiesterase inhibitors.

Tissue Preparation	Forskolin ED_{50} (μM) (95% confidence limits (μM))		
	Conditions		
	Control	MIX	Ro 20-1724
Guinea Pig Taenia Caeci	3.0 (1.9 - 4.7)	1.5 (0.48 - 4.5)	0.5* (0.33 - 0.75)
Rabbit Small Intestine	2.9 (0.68 - 12)	0.41* (0.085 - 2.0)	0.22* (0.079 - 0.59)

For further details of these results see Methods and Results

Asterisks (*) represent a significant difference between the ED_{50} values of the control and treated tissues: $P < 0.05$.

FIG. 31 Cumulative concentration-response curves for forskolin-mediated inhibition of rabbit small intestine. The control curve (●) and curves from tissues treated (▽□) with Ro 20-1724 (1 μM) (□) or MIX (10 μM) (△) are shown. Results are expressed as mean ± S.E.M.; n=4. Some error bars are not shown either when the S.E.M. are contained within the symbol or for the sake of clarity. The ED_{50} of the control curve was significantly different from the ED_{50} of the curves from tissues treated with Ro 20-1724 or MIX; $P < 0.05$.

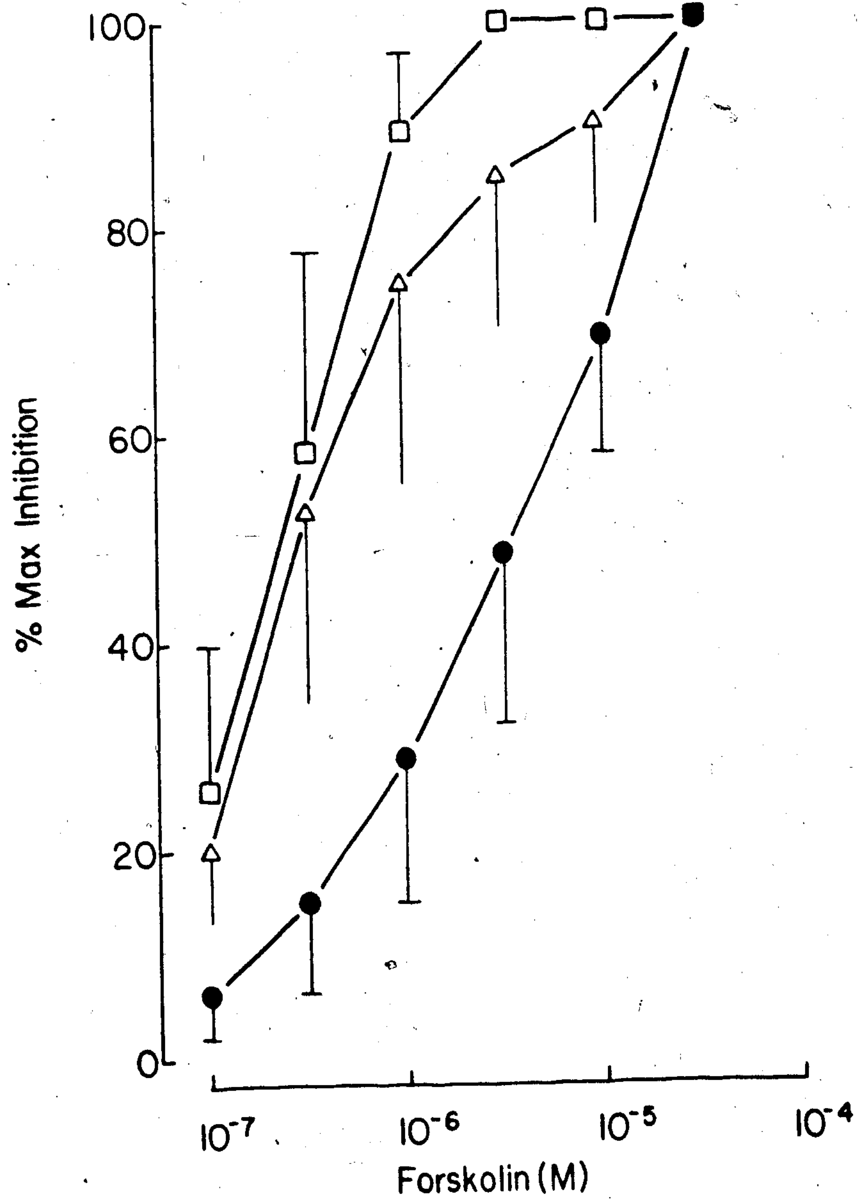


FIG. 31

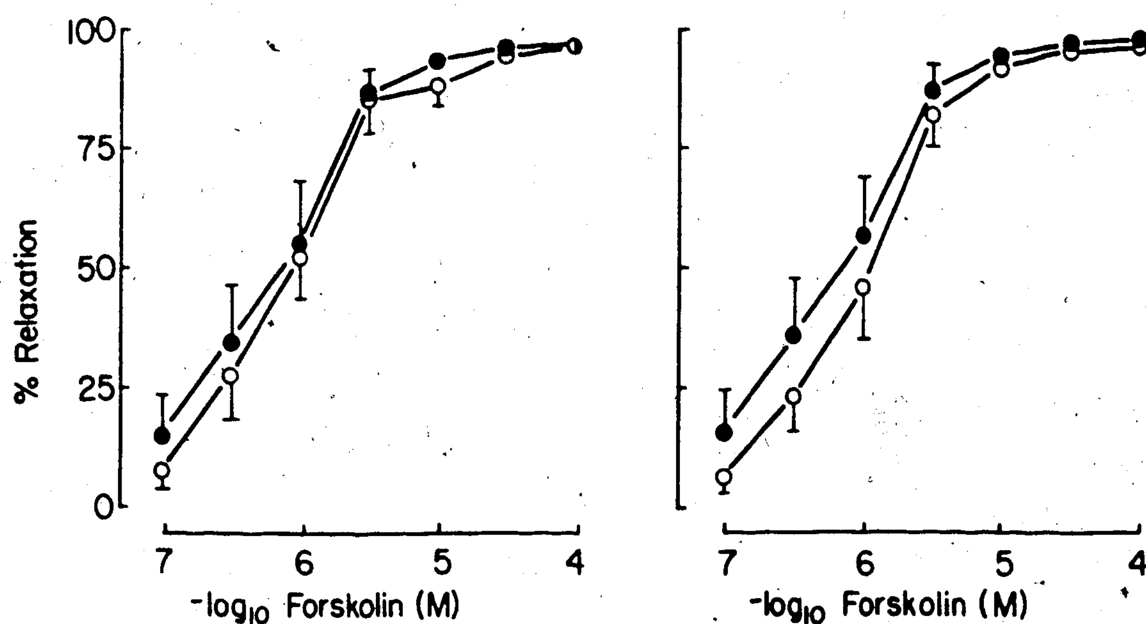


FIG. 32 Cumulative concentration-response curves for forskolin-mediated relaxation of beef coronary artery. Control curves (●) and curves from tissues treated (○) with Ro 20-1724 (10 nM) (left panel) or MIX (0.1 μM) (right panel) are shown. Results are expressed as mean ± S.E.M., n=6. Some error bars are not shown either when the S.E.M. are contained within the symbol or for the sake of clarity. The ED_{50} of the control curves was not significantly different from the ED_{50} of the curves from treated tissues; $P < 0.05$.

inhibitors were not shifted compared to a control curve. These results suggest that cyclic AMP may not be involved in forskolin-mediated relaxation of vascular smooth muscle.

3.3.4 EFFECT OF Ro 20-1724 ON THE INHIBITORY ACTIONS OF ISOPROTERENOL, VERAPAMIL AND 2-CHLOROADENOSINE IN RABBIT SMALL INTESTINE

Isoproterenol, verapamil and 2-chloroadenosine caused concentration-dependent inhibition of spontaneous activity in the rabbit small intestine. Control cumulative concentration-response curves for these compounds and curves obtained in the presence of 1 μM Ro 20-1724 are shown in figures 33, 34 and 35. Only the curve to isoproterenol in the presence of the phosphodiesterase inhibitor was shifted to the left significantly compared to the control curve. Ro 20-1724 did not alter the inhibition of spontaneous activity by the other agents. These results suggest that, in contrast to the action of verapamil or 2-chloroadenosine, the action of isoproterenol might involve a cyclic AMP mechanism (see section 4.3).

3.3.5 EFFECT OF ADENOSINE ANALOGS ON SMOOTH MUSCLE CONTRACTILITY

Various adenosine analogs have been used to classify adenosine receptors as Ra and Ri by Londos and coworkers (1981) (see introduction). NECA, PIA and adenosine or 2-chloroadenosine are thought to act preferentially on Ra, Ri and both receptors, respectively. Thus, we examined the relaxant effects of these compounds in rabbit small intestine and beef coronary artery.

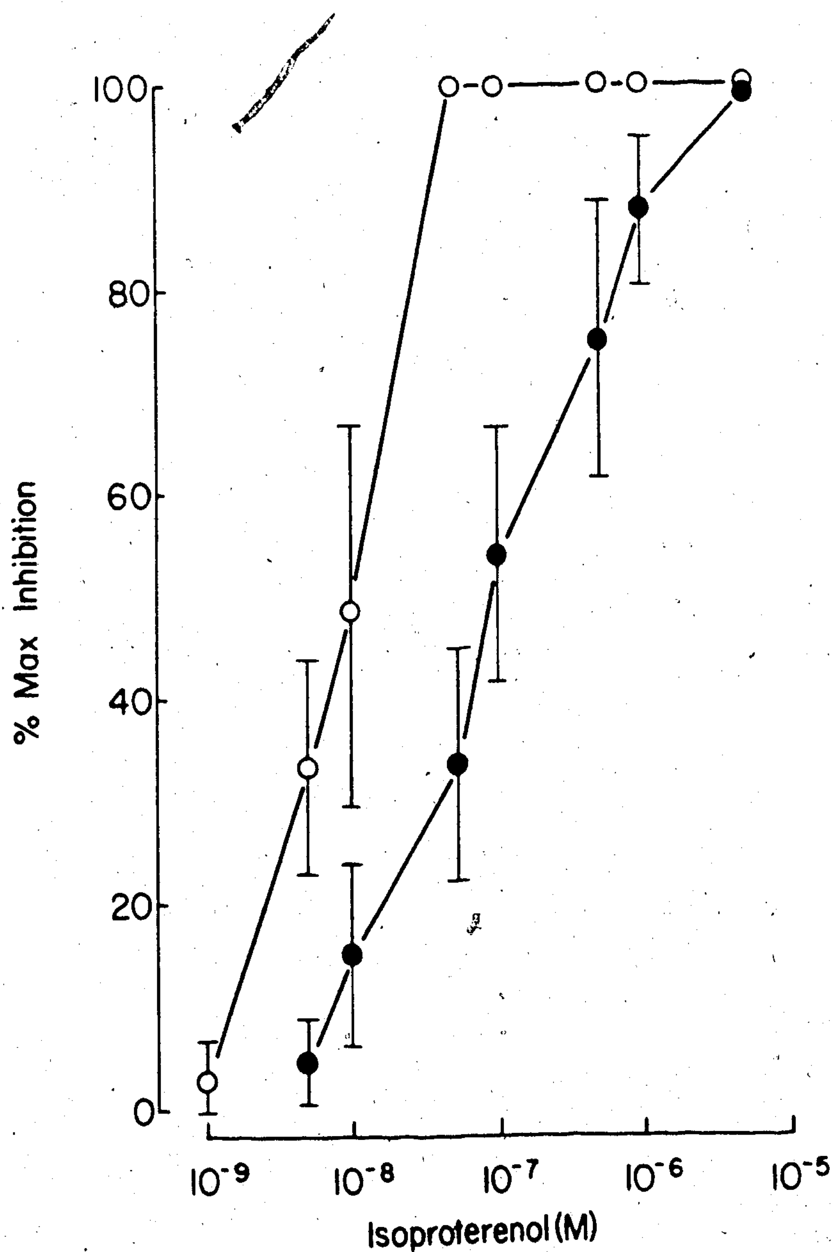


FIG. 33 Cumulative concentration-response curves for isoproterenol-mediated inhibition of rabbit small intestine. The control curve (●) and the curve from tissue treated (○) with Ro 20-1724 (.1 μM) are shown. Results are expressed as mean ± S.E.M., n=4. Error bars are not shown when the S.E.M. are contained within the symbol. The ED_{50} of the control curve was significantly different from the ED_{50} of the curve from the treated tissues; $P < 0.05$.

FIG. 34 Cumulative concentration-response curves for verapamil-mediated inhibition of rabbit small intestine. The control curve (●) and the curve from tissues treated (○) with Ro 20-1724 (1 μM) are shown. Results are expressed as mean ± S.E.M., n=4. Some error bars are not shown either when S.E.M. are contained within the symbol or for the sake of clarity. The ED_{50} of the control curve was not significantly different from the ED_{50} of the curves from treated tissues; $P < 0.05$.

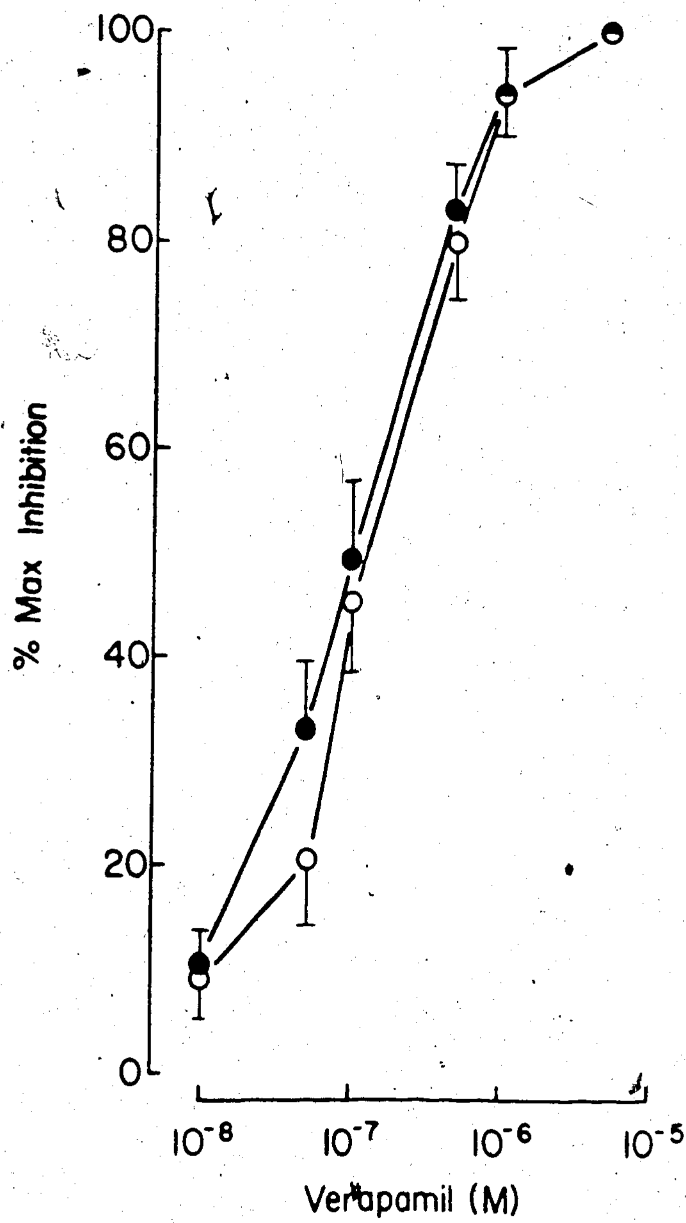


FIG. 34

FIG. 35 Cumulative concentration-response curves for 2-chloro-adenosine-mediated inhibition of rabbit small intestine. The control curve (●) and the curve from tissues treated (○) with Ro 20-1724 (1 uM) are shown. Results are expressed as mean ± S.E.M., n=4. Some error bars are not shown either when the S.E.M. are contained within the symbol or for the sake of clarity. The ED_{50} of the control curve was not significantly different from the ED_{50} of the curves from treated tissues; $P < 0.05$.

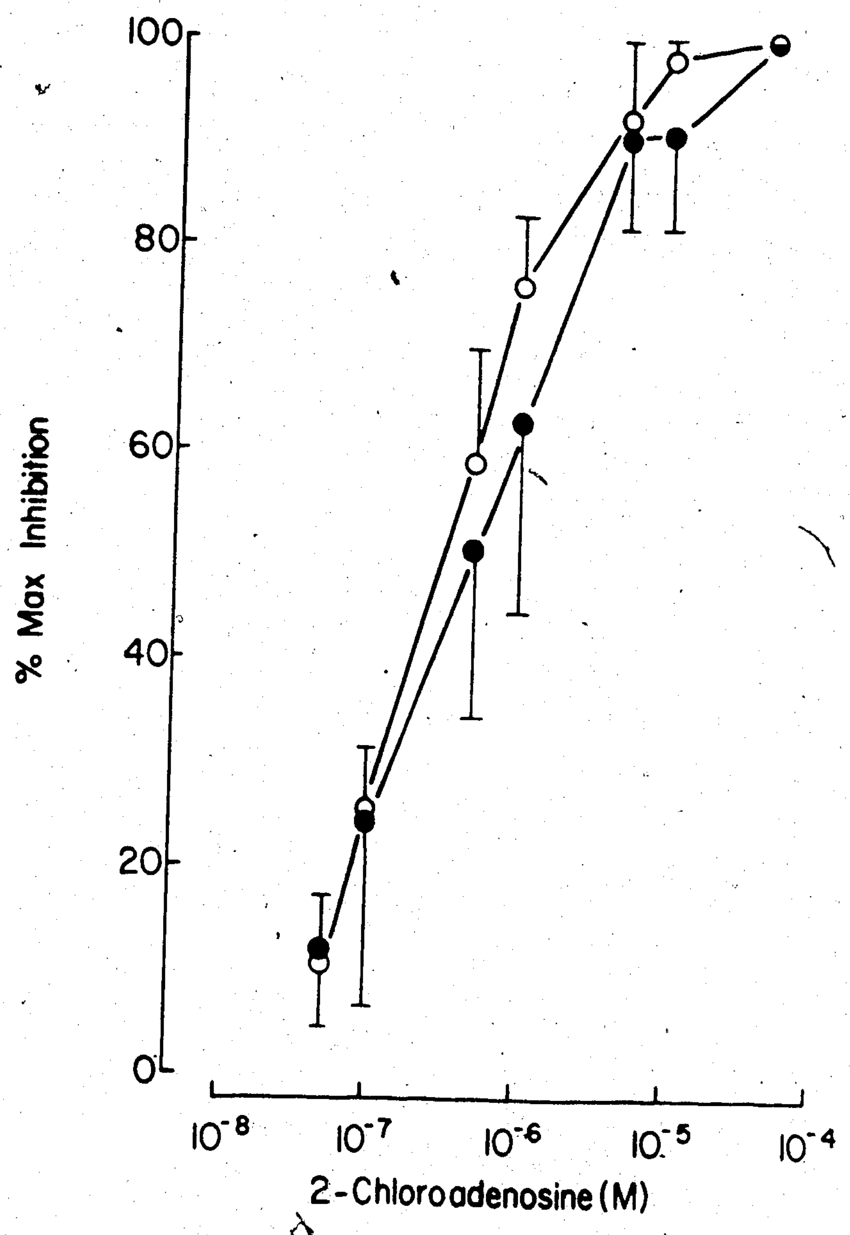


FIG. 35

Cumulative concentration-response curves for the adenosine analogs are shown in figures 36 and 37. In both smooth muscle preparations, the following order of potency was obtained: NECA = PIA > 2-chloroadenosine. If Ri receptors were present in these preparations, one would expect PIA to be the most potent and NECA to be the least potent relaxant agent. On the other hand, if Ra receptors were present, NECA should be more potent than PIA. Since neither of these sequences of potency were observed, these compounds may not be useful for classification of adenosine receptors mediating smooth muscle relaxation. Furthermore, our results may suggest that adenosine receptors in smooth muscle differ from those coupled to adenylate cyclase in other cell types.

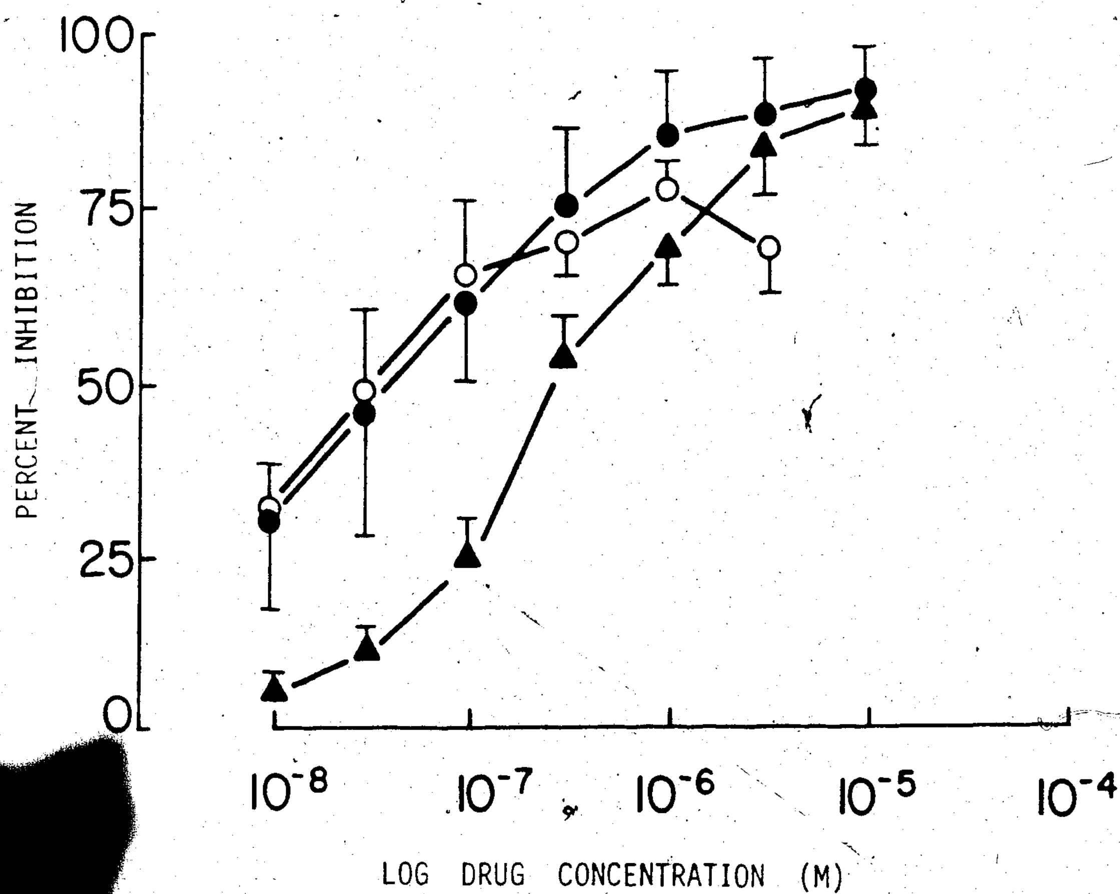


FIG. 36 Cumulative concentration-response curves for inhibition by NECA (●), PIA (○) and 2-chloroadenosine (▲) of rabbit small intestine. Results are expressed as mean ± S.E.M., n=6.

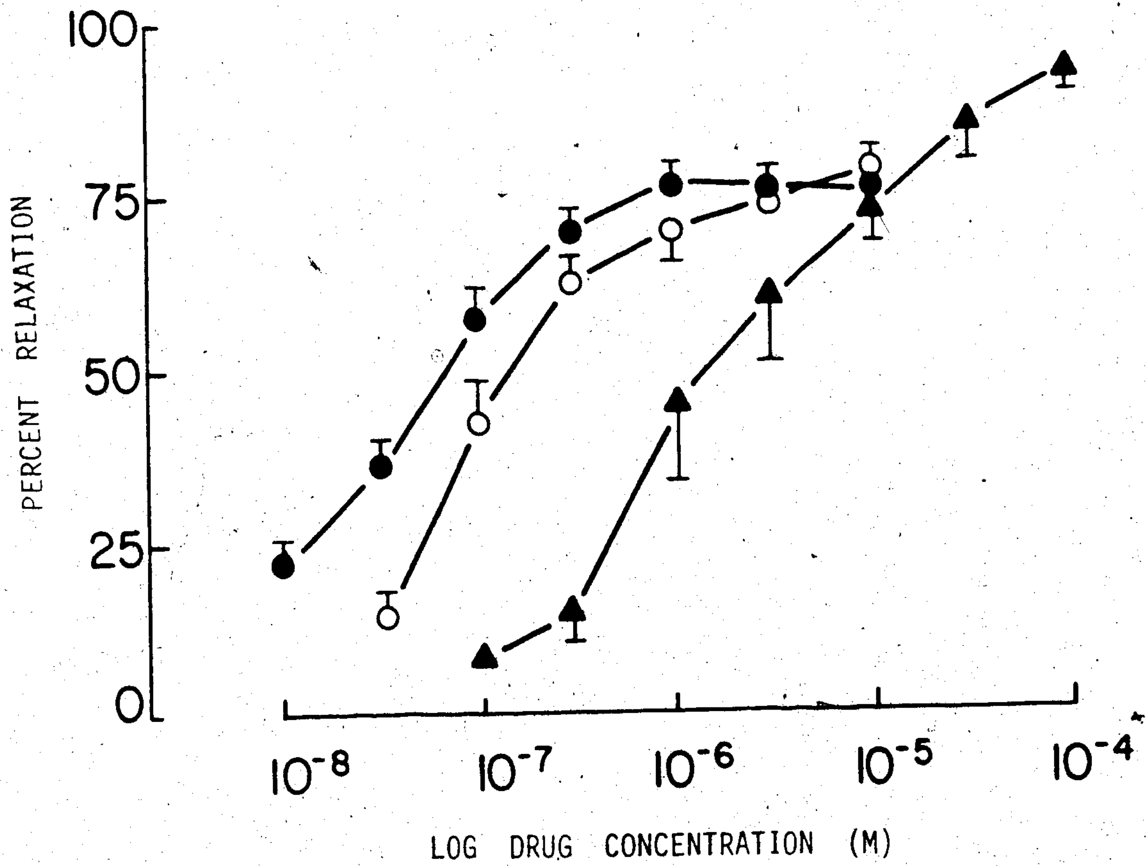


FIG. 37 Cumulative concentration-response curves for relaxation by NECA (●) , PIA (○) and 2-chloroadenosine (▲) of beef coronary artery. Results are expressed as mean ± S.E.M., n=6. Some error bars are not shown either when the S.E.M. are contained within the symbol or for the sake of clarity.

4 DISCUSSION

4.1 EFFECT OF ADENOSINE ON TISSUE LEVELS OF CYCLIC AMP IN SMOOTH MUSCLE

Simultaneously and independently, four groups addressed themselves to the hypothesis that cyclic AMP is involved in adenosine-mediated smooth muscle relaxation. Kukovetz and coworkers (for review see Kukovetz et al., 1979) found that adenosine increased tissue levels of cyclic AMP in a dose- and time-dependent manner, which was correlated with the smooth muscle relaxant effect of this nucleoside in beef and hog coronary arteries. In contrast, results from this laboratory (McKenzie et al., 1977) and from other laboratories (Herlihy et al., 1976; Verhaeghe, 1977) indicated that tissue levels of cyclic AMP were not altered by adenosine in intestinal and vascular smooth muscles. Kukovetz and coworkers (1979) concluded that cyclic AMP mediates smooth muscle relaxation by adenosine and isoproterenol. The opposite conclusion was drawn by the other investigators. Since no obvious explanation existed for the positive and negative findings reported by the various groups, the hypothesis invoking cyclic AMP involvement in smooth muscle relaxation by adenosine remained controversial in nature. Thus, we decided to re-examine the effects of adenosine on tissue levels of cyclic AMP in beef coronary artery.

The RIA for cyclic AMP, which was developed by Steiner et al. (1972) and modified by Harper and Brooker (1976), was employed in the present study to measure tissue levels of cyclic AMP. Other methods

were available at the time for measuring cyclic AMP levels in intact tissues. However, most are tedious and not as sensitive as the RIA (for review see Baer, 1974). Thus, the main advantages of the RIA are the sensitivity of the assay, the rapidity of processing samples and the reproducibility of the results. In addition, the RIA was used by Kukovetz and coworkers to demonstrate adenosine-mediated increases in tissue levels of cyclic AMP and we wanted to duplicate their experimental conditions as closely as possible.

Initially, up to 100 fold variation was noted in basal levels of cyclic AMP in strips from beef coronary arteries which were obtained from a single beef heart. As discussed in the results, our attempts to minimize this variability were unsuccessful. McKenzie et al. (1977) made a similar observation concerning the variability of basal levels of cyclic AMP in the longitudinal muscle of the rabbit small intestine. Thus, although this phenomenon may apply to many smooth muscles, it has been ignored in the literature by other investigators. The reason(s) for this variability of basal levels of cyclic AMP in intact tissues is unknown. Possibly, the contribution of different cell types is important. In this regard, recently, endothelial cells have been shown to be important to the pharmacological actions of drugs (Furchgott, 1981). Thus, the total amounts of basal levels of measured cyclic AMP in vascular tissue would depend on the integrity of the endothelial cell layer. Damage to endothelial cells is unavoidable in blood vessels despite our efforts to minimize tissue handling. Therefore, it may be erroneous to expect that the basal levels of cyclic AMP measured in two different strips of smooth muscle

would be identical. Assuming that the problems associated with tissue handling could be overcome, other complications may cause the variability in basal levels of cyclic AMP which we have observed.

It is possible that basal levels of cyclic AMP are variable because of the nature of the adenylate cyclase complex itself. The ultimate measureable levels of cyclic AMP depends on extracellular, intracellular and intramembrane factors. Thus, neurotransmitters, neuromodulators, hormones, calmodulin, ions and GTP all affect basal adenylate cyclase activity (see Introduction). In addition, unknown factors may be involved. Thus, the complexity of the control of adenylate cyclase activity may preclude our expectations that the basal levels of cyclic AMP which we determine in two different populations of smooth muscle cells would be identical.

Tissue levels of cyclic AMP were measured in "large" and "small" beef coronary arteries. The former were arterial segments of the left descending coronary artery, and the latter were segments of branches of this artery (see Methods). Incubation of the "small" arterial strips with adenosine for 10 min caused increases in tissue levels of cyclic AMP which were similar to those found by Kukovetz and coworkers (1979). Thus, at 1 mM and 0.1 mM adenosine the levels of cyclic AMP in the "small" coronary arteries were increased. However, only the increase in cyclic AMP levels observed with 1 mM was significant. Kukovetz and colleagues (1979) evaluated the effect of adenosine on tissue levels of cyclic AMP in coronary arteries from beef hearts with n=91, and found the increase to be significant with 0.1 mM and lower concentrations of adenosine. As discussed above, the

extensive report of this group, which dealt with measurement of tissue levels of cyclic AMP in response to adenosine as well as the effects of the cyclic nucleotide on adenylate cyclase from coronary artery, provided strong evidence to support a role for cyclic AMP in adenosine-mediated relaxation of vascular smooth muscle.

Adenosine increased tissue levels of cyclic AMP in "large" beef coronary arteries less than in the arterial branches. Interestingly, various investigators have found that adenosine is a more potent relaxant of the latter preparation (Winbury et al., 1969; Schnarr and Sparks, 1972; Cohen and Kirk, 1973).

Herlihy et al. (1976) claimed that tissue levels of cyclic AMP in hog carotid artery were not altered by adenosine except at high concentrations of the nucleoside. These authors found that 1 mM and 0.1 mM adeonsine elevated cyclic AMP levels to an extent similar to that found by Kukovetz's group. The failure by Herlihy and colleagues to observe significant changes in cyclic AMP levels with adenosine at lower concentrations may be a result of the small number of observations this group made.

Verhaeghe (1977) was unable to demonstrate elevation of cyclic AMP by 10 μM adenosine in dog saphenous vein. This concentration of the nucleoside relaxed the preparation by 43%. Verhaeghe (1977) discussed the possibility that the effect of adenosine on tissue levels of cyclic AMP could be masked by the agent used to contract the smooth muscle. Alternatively, the mechanism of adenosine-receptor mediated events may differ depending on the species examined. In support of this possibility, no elevation of cyclic AMP levels was found

in response to 100 μM adenosine by Herlihy and coworkers (1976) in dog coronary artery. Another suggestion which might explain this discrepancy is that the mechanism of adenosine action differs in arteries and veins. The negative findings by McKenzie et al. (1977) with respect to alteration of cyclic AMP levels by adenosine in the longitudinal muscle of the rabbit small intestine support the possibility that considering smooth muscle as a group could be erroneous when discussing the mechanism of action of adenosine, and that differences in mechanism of action may exist between different smooth muscles.

Recent studies have raised the possibility that an alteration of total levels of cyclic AMP by a compound cannot be used as an indicator of the mechanism of action of that compound. Thus, the relevance of the original four criteria of Sutherland and Rall (1960) has been questioned. In this regard, measurement of the amount of cyclic AMP bound to the catalytic unit of protein kinase appears to be more relevant than the total levels of cyclic AMP present when concerned with steroid production (Dufau et al., 1977). In heart (Corbin et al., 1977; Keely, 1977; Hayes et al., 1979) and uterine smooth muscle (Harbon and Clauser, 1971; Harbon et al., 1976) specific pools of cyclic AMP may ultimately control functional aspects of agonist induced activity. An example of cell heterogeneity complicating the issue in smooth muscle is KCl-induced contraction of rat myometrium with an increase in cyclic AMP levels (Diamond and Holmes, 1975). This was later found to result from release of endogenous noradrenaline from noradrenergic nerve terminals by Kroeger (1979) utilizing the unilateral gravid term pregnant rat model to evaluate

the contribution of different cell types to the levels of cyclic AMP measured.

In light of the difficulties which arise when trying to assess the relevance of data obtained by measuring tissue levels of cyclic AMP in response to hormones discussed above and our findings concerning the variability of basal levels of cyclic AMP, this experimental approach was discontinued. To further investigate the role of cyclic AMP in hormone-mediated relaxation of smooth muscle, the demonstration of adenosine- and/or isoproterenol-mediated stimulation of adenylate cyclase activity in broken cell preparations was attempted.

4.2 EFFECT OF DRUGS ON ADENYLATE CYCLASE ACTIVITY IN BROKEN CELL PREPARATIONS FROM SMOOTH MUSCLE

Recent progress in the understanding of the adenylate cyclase complex - the existence of a guanine nucleotide binding protein and its involvement as a coupling factor between hormone-receptors and the catalytic unit of cyclase (Rodbell, 1975 and 1980) - has revolutionized our thinking about transmembrane transduction of information via adenylate cyclase. Also, recently, various complications have been realized which occur when measuring hormone-responsiveness of adenylate cyclase in response to adenosine in broken cell preparations ie., importance of endogenous adenosine (cf. Londos _et al_., 1981). Therefore, it was felt that with this knowledge the demonstration of hormone-mediated stimulation of adenylate cyclase in broken cell preparations would be possible, assuming such receptor-mediated events occur physiologically.

The first experiments examined the effects of a variety of agents on adenylate cyclase activity from the 10,000 g pellet of the guinea pig taenia caeci and the longitudinal muscle from rabbit small intestine. The latter was chosen because it was studied by McKenzie _et al_. (1977) in this laboratory and it has a high smooth muscle content. The former was chosen because it has been studied with respect to the purinergic nerve hypothesis (Burnstock, 1979). Thus, the presence and functional integrity of the components of the adenylate cyclase complex consisting of hormone receptors (R), guanine nucleotide binding site (N) and the catalytic site (C) were investigated. Evidence for the existence of these components of the adenylate cyclase complex and models of their interaction has been discussed in the Introduction of this thesis.

Forskolin caused a concentration-dependent stimulation of adenylate cyclase activity in the 10,000 g pellet from guinea pig taenia caeci and the longitudinal muscle from the rabbit small intestine. Therefore, C units appeared to be present and functional in these preparations. The magnitude of the effect of forskolin should provide information concerning the relative functional states of C units in different adenylate cyclases from a particular smooth muscle preparation with a number of assumptions. First, a fixed number of C units exist per mg protein in cyclase preparations. Second, the action of forskolin is not influenced by such factors as the presence of endogenous hormones or the coupling of N to C, ie. the C units are the site of action of forskolin.

Stimulaton of adenylate cyclase activity from the smooth muscle

preparations used in this study was also achieved invariably with 10 mM NaF. For some time now, NaF has been used routinely by investigators to check the viability of the enzyme preparations being investigated (Perkins, 1973). Evidence has accumulated to suggest that flouride stimulates adenylate cyclase via an action on the N unit (Ross and Gilman, 1980). Thus our results indicate that N units were present in the adenylate cyclase preparations from guinea pig taenia caeci and the longitudinal muscle of the rabbit small intestine. Furthermore, these N units could be coupled to C units by NaF.

Interestingly, in both smooth muscle enzyme preparations the maximal observable stimulation of adenylate cyclase activity by NaF was less than seen with forskolin. Seamon et al. (1981) found that in some cell types adenylate cyclase was more sensitive to the action of NaF than to forskolin and the opposite was observed in other cell types. These authors grouped cell types according to their relative sensitivities to fluoride and the diterpene. These experiments were performed in crude enzyme preparations. The relevance of such a classification is unknown.

The concentration-effect curve for GTP-mediated stimulation of adenylate cyclase activity from guinea pig taenia caeci was bell-shaped in nature. The guanine nucleotide stimulated the enzyme preparations in concentrations between 1 and 100 μM. A 10 fold higher concentration of GTP caused a reduction in the observable stimulation of adenylate cyclase. However, enzyme activity was still above basal. This bell-shaped stimulatory and inhibitory effect of

GTP may result from the coexistence of Ns and Ni units in the preparation and the differential affinities of these units for GTP. Since GTP presumably acts through the N unit (Rodbell, 1975 and 1980), stimulation of adenylate cyclase with the guanine nucleotide provides an indication of the functional coupling between N units and C units.

Gpp(NH)p also displayed a bell-shaped concentration-effect curve with respect to stimulation of adenylate cyclase activity in the guinea pig taenia caeci preparations with maximal stimulation occurring at a concentration of 100 μM. However, in the longitudinal muscle of the rabbit small intestine maximal stimulation was maintained with 1 mM of Gpp(NH)p. This nucleotide analog is thought to act in a manner similar to that of GTP (Harwood et al., 1973). We found that the magnitude of maximal stimulation of adenylate cyclase obtained with Gpp(NH)p was greater than that obtained with GTP. Similar observations have been observed by investigators in other adenylate cyclase systems (Londos et al., 1974). This effect of Gpp(NH)p is attributed to its stable nature and thus the absence of GDP formation (Selinger and Cassel, 1981).

Isoproterenol and adenosine as well as a number of nucleoside analogs including 2-chloroadenosine, NECA and PIA were tested for their ability to stimulate adenylate cyclase activity in broken cell preparations of guinea pig taenia caeci. No stimulation of the enzyme over basal activity was observed with any of these compounds which cause smooth muscle relaxation via stimulation of receptors. Many attempts in the past have failed to demonstrate adenylate cyclase from smooth muscle which could be stimulated by adenosine or

isoproterenol (Triner et al., 1971; Schonhoffer et al., 1971; Volicer and Hynie, 1971; Triner et al., 1972; Triner et al., 1975; Cohen et al., 1977; Hardman et al., 1977; McKenzie et al., 1977; Hamet et al., 1978). The most common reason forwarded for the failures in smooth muscle has been the possible disruption of hormone-receptors by the homogenization procedures used to procure broken cell preparations containing adenylate cyclase. It would be desirable to demonstrate the presence or absence of hormone receptors by direct ligand-binding experiments. To our knowledge, ligand-binding studies to adenosine receptors in these smooth muscles have not been reported.

To further investigate the hypothesis that isoproterenol and/or adenosine mediated smooth muscle relaxation via a mechanism involving cyclic AMP, the adenylate cyclase assay conditions were manipulated so as to optimize conditions in an attempt to demonstrate receptor-mediated enzyme stimulation. The following experimental manipulations were employed: 1) Substitution of deoxy-alpha-[^{32}P]-ATP for alpha-[^{32}P]-ATP as substrate in the cyclase assay. 2) Treatment of enzyme preparations with adenosine deaminase. 3) Addition of GTP or the non-hydrolizable analog Gpp(NH)p to the cyclase assay. 4) Fractionation of smooth muscle. 5) Use of adenosine analogs which show specificity towards Ra and Ri receptors for adenosine. Data obtained from such experiments will be described and discussed below.

Deoxy-alpha-[^{32}P]-ATP has been used by Londos and coworkers (Cooper and Londos, 1979; Londos et al., 1980; Londos et al., 1981) as a substitute for alpha-[^{32}P]-ATP in the assay of adenylate cyclase from fat cells. Normally, high concentrations of ATP are used as

substrate for adenylate cyclase. The presence of non-specific phosphatases combined with 5'-nucleotidase may result in the production of adenosine despite the ATP regenerating system employed in the assay. Thus, the uncontrolable production of adenosine would mask any stimulation of the enzyme by additional application of the nucleoside. On the other hand, deoxy-alpha-[^{32}P]-ATP is hydrolyzed to deoxy-adenosine which has little activity at adenosine receptors, and thus this problem is circumvented.

We have attempted to demonstrate receptor-mediated stimulation of adenylate cyclase from guinea pig taenia caeci using deoxy-alpha-[^{32}P]-ATP as substrate in the assay, and compared the results with data obtained with ATP. NaF and forskolin caused identical stimulation and neither isoproterenol nor 2-chloroadenosine caused any stimulation of adenylate cyclase activity regardless of whether alpha-[^{32}P]-ATP or deoxy-alpha-[^{32}P]-ATP served as substrate in the assay system. These results suggest that receptor-mediated stimulation of adenylate cyclase was not being masked by the production of adenosine from the substrate ATP. Furthermore, adenosine did not stimulate cyclase activity in the presence or absence of GTP or forskolin when deoxy-alpha-[^{32}P]-ATP was used as substrate. However, adenosine caused a concentration-dependent inhibition of adenylate cyclase activity in the presence of forskolin. A similar observation was made with NECA and forskolin. However, inhibition only occurred at 1 mM NECA with lower concentrations of the nucleoside analog not altering enzyme activity. Forskolin has been shown to potentiate receptor-mediated increases in cyclic AMP in intact

tissues (Seamon et al., 1981). If we assume that isoproterenol and adenosine stimulate adenylate cyclase in broken cell preparations then our results suggest that forskolin is not useful as a potentiating agent with respect to either isoproterenol or adenosine mediated-stimulation of adenylate cyclase activity in these broken cell preparations. Londos et al. (1981) have claimed that NECA has no action on the "P site". However, the concentration range was much lower. Our results suggest that the inhibition of adenylate cyclase activity by adenosine and NECA in preparations treated with forskolin may be an action on the "P site" rather than a receptor-mediated event, since with NECA inhibition of activity occurred only at a concentration of 1 mM in the presence of 1 μM forskolin.

The study of nucleoside metabolism has been an active area of research over the past two decades and extensive reviews on the subject are available (Arch and Newsholme, 1978). Briefly, the existence of intracellular concentrations of adenosine above 1 μM is thought to be uncommon and three enzymes are responsible for maintaining adenosine levels at a minimum: adenosine kinase, adenosine deaminase and S-adenosylhomocysteinase. Thus, adenosine is either phosphorylated to 5'-AMP by adenosine kinase and ultimately enters the metabolic pool of adenine nucleotides or deaminated to inosine (cf. Arch and Newsholme, 1978), which is inactive at adenosine receptors (Sattin and Rall, 1971; Huang et al., 1972; Blume et al., 1973; Maguire et al., 1975; Huang and Drummond, 1976; Green and Stanberry, 1977; Londos and Wolff, 1977; Wolberg et al., 1978). Recently, the "methylation pathway" via S-adenosylhomocysteinase

has [illegible]ized as a possible source of adenosine as well as a mecha[illegible]by intracellular levels of the nucleoside are maintained [illegible]t a [illegible]l (Zimmerman et al., 1979; Hoffman et al., 1980). We [illegible]ve t[illegible]ted adenylate cyclase from smooth muscle with adenosine [illegible]ami[illegible]e in an attempt to maintain endogenous levels of adenosine a[illegible]nimum. This would eliminate the possibility that receptor-me[illegible]ted stimulation of the enzyme was being masked by inherent ade[illegible]late cyclase stimulation by the nucleoside. This approach has bee[illegible]tilized by other investigators, leading to the demonstration of nucl[illegible]side-sensitive adenylate cyclase which otherwise was not stimulated by adenosine (Premont et al., 1977; Londos et al., 1978). The majority of our experiments were conducted on enzyme preparations which [illegible] routinely treated with adenosine deaminase. It will become appar[illegible] that the presence of endogenous adenosine was not obscuring receptor-mediated stimulation of adenylate cyclase activity.

GTP is an essential activator of adenylate cyclase and is required for receptor-mediated stimulation of adenylate cyclase activity (Rodbell et al., 1971; Rodbell, Lin and Salomon, 1974; Rodbell, Lin, Salomon et al., 1974; Londos et al., 1974; Rodbell, 1975; Rodbell, 1980). GDP is formed by the hydrolysis of GTP by GTPase and serves as a potent inhibitor of adenylate cyclase activity via tight binding to Ns units (Cassel et al., 1977). Treatment of broken cell preparations with hormone plus a guanine nucleotide apparently causes release of endogenously bound GDP (Eckstein et al., 1979; Lad et al., 1980 a and b). Gpp(NH)p, a non-hydrolizable GTP analog (Harwood et al., 1973), has also been used to demonstrate hormone effects on

adenylate cyclase activity (Lad *et al*., 1980 a and b). Furthermore, adenylate cyclase activation by Gpp(NH)p is of a permanent nature in many cell types (Cuatrecasac *et al*., 1975; Jacobs *et al*., 1975; Pfeuffer and Helmreich, 1975; Schramm and Rodbell, 1975; Levitzki *et al*., 1976; Spiegel *et al*., 1976) including smooth muscle (Krall and Korenman, 1979). Adenosine and guanine nucleotides have been shown to act synergistically to activate adenylate cyclase from fibroblasts (Clark and Seney, 1976) and from Leydig I-10 tumor cell membranes (Wolff and Cook, 1977). Furthermore, adenosine and Gpp(NH)p act to form a stable active form of turkey erythrocyte adenylate cyclase (Servilla *et al*., 1977). An absolute requirement for GTP in the activation of adenylate cyclase by adenosine has been demonstrated by Cooper and Londos (1979) in hepatic membranes. As well, isoproterenol-mediated stimulation of adenylate cyclase has an absolute requirement for exogenous guanine nucleotides in rat uterus (Krall and Korenman, 1979 and 1980) and in rabbit myometrium (Roberts *et al*., 1977).

We have examined the possibility that the coupling mechanism in the adenylate cyclase complex interferred in some manner with the demonstration of receptor-mediated stimulation of adenylate cyclase activity in smooth muscle. In broken cell preparations from guinea pig taenia caeci and the longitudinal muscle of the rabbit small intestine treated with adenosine deaminase, GTP did not alter the concentration-effect of NECA, ie., no stimulation of adenylate cyclase activity was observed with any concentration of NECA tested. Furthermore, NECA-induced stimulation of adenylate cyclase activity was not

observed when Gpp(NH)p was added to the system. Similar negative results concerning the effect of Gpp(NH)p were also obtained with the concentration-effect on adenylate cyclase activity of isoproterenol in guinea pig taenia caeci. These results suggest that addition of guanine nucleotides to adenylate cyclase from smooth muscle, which is thought to couple R and C units of the complex via activation of N units, is not sufficient to enable the demonstration of receptor-mediated stimulation of the enzyme system.

The above experiment was also performed in the reverse manner; concentration-effect curves to Gpp(NH)p in the guinea pig taenia caeci preparation were constructed in the presence and absence of isoproterenol. Such experimentation yielded an interesting result. Isoproterenol prevented the decline in stimulation of adenylate cyclase activity normally seen with high concentrations of the non-hydrolizable GTP analog. However, this effect was not reproducible. The question of the reproducibility of results occurrs twice in this thesis and is discussed below.

The effect of Gpp(NH)p in the presence and absence of isoproterenol was monitored repeatedly throughout the experiment. Also a time-course study was performed in which activation of adenylate cyclase from guinea pig taenia caeci by Gpp(NH)p or Gpp(NH)p plus isoproterenol was monitored every 2 min. Gpp(NH)p-induced activation of adenylate cyclase activity was not altered by isoproterenol in these experiments.

From the time-course study, a lag-time was apparent between administration of Gpp(NH)p and stimulation of adenylate cyclase activity by the guanine nucleotide analog. Similar results have been

obtained by Rodbell and coworkers (1981) with Gpp(NH)p in rat liver plasma membranes and by McMahon and Schimmel (1982) in hamster adipocyte membranes as well as other cell types (cf. Gilman and Ross, 1980; Rodbell, 1975 and 1980). The lag period is thought to result from slow replacement of GDP with Gpp(NH)p (Ross and Gilman, 1980) or a conformational change in N units after binding of Gpp(NH)p (Iyengar, 1981; Neer and Salter, 1981).

Receptor-mediated stimulation of adenylate cyclase activity from vascular smooth muscle has been demonstrated by elimination of the regenerating system from the assay system, which may affect the kinetic characteristics of adenylate cyclase (Garbers and Johnson, 1975), and long term treatment of the enzyme with receptor-agonist and Gpp(NH)p (Hamet et al., 1978). We attempted to repeat this experiment in a broken cell preparation from guinea pig taenia caeci. Our efforts were unsuccessful. The possibility that vascular and non-vascular smooth muscle differ in this regard cannot be ruled out.

A detailed description of the fractionation procedure utilized (cf. Kidwai, 1975) can be found in the methods. It should be noted that fractionation of membranes in this manner is only the initial step involved in membrane purification. However, it is an improvement over the crude 10,000 g pellet obtained from broken cell preparations which have been described to this point in the thesis. Further purification of the plasma membrane from smooth muscle would involve such procedures as sucrose density centrifugation and subsequent characterization of the fractions obtained by electron microscopy and biochemical techniques using enzymes as markers,

ie., adenylate cyclase, sodium-potassium ATPase and 5'-nucleotidase are thought to be bound mainly to the plasma membrane (for review see: Daniel et al., 1983).

The effects of isoproterenol and NECA in the presence and absence of Gpp(NH)p as well as NaF and forskolin were examined in the various fractions from the longitudinal muscle of the rabbit small intestine termed P-1, P-2, P-3 and S-3 to designate the 1,200 g, 13,000 g, 105,000 g pellets and the final supernatant, respectively (for description of the contents of these fractions see: Kidwai, 1975). Stimulation of adenylate cyclase activity by NaF, Gpp(NH)p and forskolin was observed in all the fractions except S-3. These results suggest that functional N and C units were present in fractions P-1 to P-3. Since S-3 is thought to be composed mainly of soluble cytoplasmic components (cf. Kidwai, 1975), these results were expected. Receptor-mediated stimulation of adenylate cyclase activity was not found in any fraction with one exception. In one instance, NECA- and isoproterenol-mediated stimulation of adenylate cyclase activity was observed in fraction P-3, which is thought to be composed of microsomes consisting of smooth and rough endoplasmic reticulum and fragments of plasma membrane. However, this effect was not reproducible.

The question of the reproducibility of a result in this thesis has occurred for the second time. We must ask the following questions: First, is this observation of any significance? Second, what does it mean?

With respect to the first question, in addition to this author

other investigators in our laboratory (K. Schmidt and S. Kulshresha) have observed occasional receptor-mediated stimulation of adenylate cyclase activity in smooth muscle preparations which could not be reproduced. This would suggest that the observation might be of some significance and not merely a consequence of some experimental artifact.

Unfortunately, the answer to the second question is not known. Two possibilities exist. It is possible that adenosine- and beta-receptors are not physiologically coupled to adenylate cyclase in smooth muscle. In this case the occasionally observed receptor-mediated stimulation of adenylate cyclase activity in the preparation is hard to explain. It might result from the occasional and uncontrollable contamination of the preparation with cell types other than smooth muscle ie., nerve cells where demonstrations of receptor-mediated stimulation of adenylate cyclase are numerous. On the other hand, it is possible that adenosine- and beta-receptors are physiologically coupled to adenylate cyclase in smooth muscle. If this is true then the majority of the data reported in this thesis, which contradict the latter hypothesis, are misleading. There are at least two possible explanations for this. First, the receptors may be altered upon disruption of the cell membrane. This could result from attack by proteolytic enzymes or release of endogenous inhibitors or even the homogenization procedure itself (cf. Hardman, 1981). In this regard, ligand-binding studies would be enlightening. Secondly, there may be hitherto "undiscovered components" of the adenylate cyclase system which are especially fragile in smooth

muscle cells compared to other cell types where receptor-mediated stimulation of the enzyme is demonstrated easily. These "undiscovered components" may be unique to smooth muscle. The involvement of "undiscovered components" of the adenylate cyclase complex in smooth muscle is speculative. However, little is known about the adenylate cyclase complex and the interaction of its components especially in smooth muscle (see section 1.2.1).

In summary, the meaning of the observation of receptor-mediated stimulation of adenylate cyclase activity in smooth muscle preparations which cannot be reproduced remains obscure. A number of possible explanations have been forwarded. A final conclusion awaits further developments in our understanding of the adenylate cyclase complex in smooth muscle and/or the development of specialized techniques ie., separation of nerve cells from smooth muscle cells, specific inhibitors of proteolytic enzymes and endogenous inhibitors.

To complement these studies on the effects of hormones on adenylate cyclase activity from smooth muscle, the relaxant effects of adenosine analogs which are considered to show specificity for Ra or Ri receptors were studied for adenosine as well as a non-selective agonist, 2-chloroadenosine (cf. Londos et al., 1981). Thus, concentration-response curves to NECA, PIA and 2-chloroadenosine were constructed in beef coronary artery and rabbit small intestine *in vitro*. In both vascular and non-vascular smooth muscle preparations, the relaxant action of these compounds were qualitatively similar. The order of potency was as follows: NECA = PIA > 2-chloroadenosine. In cell types other than smooth muscle where the coupling

of adenosine-receptors to adenylate cyclase have been firmly established, NECA was more potent than PIA in systems which possessed Ra receptors, and conversely, PIA was found to be more potent than NECA in systems possessing Ri receptors. The potency of 2-chloro-adenosine, an agent showing no selectivity for Ra or Ri receptors, was between that of the analogs of adenosine displaying selectivity for adenosine receptor sub-types. Thus, our results with the adenosine analogs suggest that adenosine receptors responsible for relaxation of beef coronary artery and rabbit small intestine differ in their chemical specificity from those adenosine receptors which are coupled to adenylate cyclase; adenosine receptors in these smooth muscles and other cell types may be different proteins.

Several groups have concluded that cyclic AMP is not involved in inhibition of transmitter release (Kuroda, 1978; Reddington and Schubert, 1979; Smellie et al., 1979; Dunwiddie and Hoffer, 1980). In studies, dealing with presynaptic adenosine-receptor classification, in the rat vas deferens, the rat anococcygeus muscle and central nervous tissue (Smellie et al., 1979; Paton, 1981; Brown et al., 1982; Stone, 1983) the following order of potency was found; PIA > NECA > 2-chloroadenosine > adenosine. In contrast, in guinea pig trachea where adenosine acts at a postsynaptic receptor Brown and Collis (1982) found the following order of potency; NECA > 2-chloroadenosine > PIA ≥ adenosine. Stone (1983) proposed a pre-synaptic receptor (PIA > NECA > adenosine) and a post-junctional receptor (NECA > PIA ≥ adenosine) in smooth muscle distinct from

Ra or Ri sites regulating adenylate cyclase activity. Our results on the smooth muscle relaxant effects of adenosine analogs used to classify adenosine receptors coupled to adenylate cyclase differ from those of other investigators dealing with either presynaptic or postsynaptic adenosine receptors. This could indicate that heterogeneity of adenosine receptor types exist in smooth muscle.

In summary, our attempts to demonstrate receptor-mediated stimulation of adenylate cyclase in broken cell preparations from intestinal smooth muscle were unsuccessful despite various manipulations including the use of deoxy-alpha-[^{32}P]-ATP, adenosine deaminase and GTP/Gpp(NH)p or fractionation of smooth muscle membranes. Since we did not demonstrate the presence or absence of receptors in the broken cell preparations from smooth muscle by direct ligand-binding studies, no definitive conclusions can be drawn concerning the role of cyclic AMP in receptor-mediated relaxation of intestinal smooth muscle. However, our adenylate cyclase data and results from contractility studies with analogs of adenosine suggest that adenosine-receptors and beta-receptors in the intestinal smooth muscles examined are not linked to adenylate cyclase.

4.3 CYCLIC AMP-MEDIATED RELAXATION OF SMOOTH MUSCLE: STUDIES WITH FORSKOLIN

Up to this point, we have discussed the evidence for the possible role of cyclic AMP in receptor-mediated relaxation of smooth muscle. During the course of our investigation, two groups independently found that forskolin possessed unique properties which might

render it useful as a tool in the investigation of the role of cyclic AMP in cell function (Metzger and Lindner, 1981; Seamon et al., 1981). Forskolin was found to rapidly and reversibly stimulate adenylate cyclase in many mammalian cell types (Metzger and Lindner, 1981; Seamon et al., 1981). This stimulation was specific and occurred in both intact and broken cell preparations. From studies with various mutant cell lines and soluble adenylate cyclase from rat testes, forskolin appears to act directly on the catalytic unit of the adenylate cyclase complex. This implies that hormone-receptors and the guanine nucleotide binding protein are not necessary for activity (Seamon and Daly, 1981; Insel et al., 1982).

We expected forskolin to provide some independent evidence for the role of cyclic AMP in smooth muscle relaxation. Thus, we examined the effect of forskolin on smooth muscle contractility. Forskolin relaxed a number of smooth muscles in vitro. To investigate cyclic-AMP involvement in forskolin-induced relaxation of smooth muscle, studies were undertaken with phosphodiesterase inhibitors in intact tissues and adenylate cyclase in broken cell preparations.

The effects of forskolin on the contractility of both vascular and non-vascular smooth muscle were examined in vitro. Forskolin caused concentration-dependent relaxations of rat aorta, beef and dog coronary arteries, guinea pig taenia caeci and rabbit small intestine. Forskolin-induced relaxations of these smooth muscle preparations was slow in onset and the effect lasted about 5 to 10 min, depending on the concentration, before maximal relaxation was obtained with any particular concentration. An explanation for the

time-course of forskolin-action might be that the compound requires time to diffuse through the cell membrane to reach its site of action, presumably the catalytic site of the adenylate cyclase complex (Seamon *et al*., 1981; Insel *et al*., 1982).

The concentration-response studies with forskolin suggested that the vascular smooth muscles were about 10 to 100 times more sensitive to the relaxant effects of the diterpene than the non-vascular smooth muscles which were examined. There are a number of possible explanations for *this*. For example, forskolin may have easier access to its site of action in the former preparation, ie., to the catalytic subunit of the adenylate cyclase complex. Another possibility is differential compartmentilization of cyclic AMP and cyclic AMP-dependent protein kinase, resulting in a smaller amount of the cyclic nucleotide being necessary for achieving a particular effect. Evidence suggesting compartmentilization of cyclic AMP-dependent protein kinase in heart tissue has been mentioned above (Corbin *et al*., 1977; Hayes *et al*., 1979). The differential sensitivity of the vascular and non-vascular smooth muscles may indicate that cyclic AMP serves as a particularly effective mediator of relaxation in the former preparations. In support of this, the vascular smooth muscles were also more sensitive than the intestinal preparations to the relaxant effects of the phosphodiesterase inhibitors employed in this study.

Another compound, coleonol, has been isolated from the roots of the Indian plant *Coleus forskohlii*. This compound is thought to differ from forskolin only in the configuration of the 7-acetoxy

group which is thought to be in the beta-configuration (above the ring) and in the alpha-configuration (below the ring) in forskolin and coleonol, respectively. Dubey and coworkers (1981) have conducted in vivo and in vitro experiments with coleonol in a variety of species, and found that it possesses pharmacological effects similar to those of forskolin. In vivo, coleonol caused hypotension in cats which lasted about 1 to 2 hours, tachycardia and a transient respiratory stimulation. It also possessed positive inotropic effects and increased coronary flow in isolated rabbit hearts. In vitro, coleonol relaxed a variety of smooth muscles including cat intestine and uterus and rabbit duodenum as well as antagonizing the effects of spasmogens in guinea pig ileum and rat mesenteric artery. Furthermore, the effects of isoproterenol were potentiated by this compound in rabbit duodenum and guinea pig tracheal chains. Finally, coleonol displayed low toxicity in mice. The mechanism of action of coleonol has not been determined in these studies. However, considering the similarities in structure and pharmacological effects of this compound and forskolin, stimulation of adenylate cyclase activity may be the mechanism.

The strongest evidence we found to support cyclic AMP involvement in forskolin-induced relaxation of smooth muscle was with broken cell preparations from guinea pig taenia caeci and the longitudinal muscle of the rabbit small intestine. Forskolin caused a concentration-dependent stimulation of adenylate cyclase activity in both of these smooth muscle preparations. Although the maximal stimulatory effect of forskolin was not obtainable, the stimulation obtained with

1 mM forskolin was greater than with any other compound tested. Forskolin-mediated stimulation of adenylate cyclase in broken cell preparations from smooth muscle has been demonstrated by other investigators (Seamon et al., 1981).

We used phosphodiesterase inhibitors, MIX and Ro 20-1724, as tools to investigate cyclic AMP involvement in forskolin-mediated relaxation of smooth muscle. Thus, guinea pig taenia caeci and rabbit small intestine were treated with MIX and Ro 20-1724 in concentrations which possessed no "direct" relaxant effects in the smooth muscle preparations. The concentration-response curves to forskolin in guinea pig taenia caeci and rabbit small intestine were potentiated by treatment of the preparations with the phosphodiesterase inhibitors, except for the case of MIX in the guinea pig taenia caeci. These results support the suggestion that cyclic AMP is involved in forskolin-mediated relaxation of the rabbit small intestine. The lack of potentiation by MIX of forskolin-mediated relaxation in the guinea pig taenia caeci is discussed below.

Beef coronary arteries were treated with MIX and Ro 20-1724 in lower concentrations than the non-vascular smooth muscles since the phosphodiesterase inhibitors proved to be potent relaxant agents in the arterial preparation. Contrary to the results obtained in the rabbit small intestine, there was no difference between the concentration-effect curves for forskolin the beef coronary arteries treated with MIX or RO 20-1724 and the untreated control curves. Thus treatment of the preparation with the phosphodiesterase inhibitors did not alter the smooth muscle relaxant effects of forskolin in beef coronary

arteries. These results suggest that cyclic AMP is not involved in the smooth muscle relaxant effects of forskolin in beef coronary artery. In the meantime, however, direct evidence of forskolin effects on cyclic AMP in beef coronary arteries have been presented by Holzmann (1982).

By way of review, MIX potentiated the relaxant effects of forskolin in the rabbit small intestine but not in the guinea pig taenia caeci or beef coronary artery. Ro 20-1724 potentiated the relaxant effects of forskolin in both of the intestinal smooth muscles but not in the arterial smooth muscle. Therefore, in two smooth muscles the effects of the phosphodiesterase inhibitors were consistent. However, in the guinea pig taenia caeci the effects of the phosphodiesterase inhibitors were inconsistent. In addition, in the rabbit small intestine Ro 20-1724 did not potentiate the effects of verapamil, a calcium channel blocker (Fleckenstein, 1977) which does not alter cyclic AMP levels in smooth muscle (Gagnon _et al_., 1980), or 2-chloroadenosine. However, isoproterenol-mediated relaxations of this tissue were potentiated by Ro 20-1724. This result was not expected since beta-receptors do not appear to be coupled to adenylate cyclase in this smooth muscle (see section 4.2). The phosphodiesterase inhibitors were used in this study to test the hypothesis that forskolin-mediated relaxation of smooth muscle occurs via a mechanism involving cyclic AMP. The inconsistencies and the unexpected results discussed above suggest that MIX and Ro 20-1724 may not be useful as tools to examine the hypothesis.

Support for this conclusion concerning the usefulness of phospho-

diesterase inhibitors as tools in smooth muscle can be found in the literature. Phosphodiesterase is a term for a family of isoenzymes and multiple forms of the enzyme have been found in every mammalian tissue examined (Robison et al., 1971; Appelman et al., 1973; Wells and Hardman, 1977). The enzyme has a non-uniform distribution in smooth muscle (Triner et al., 1971; Volicer et al., 1973; Hidaka et al., 1975; Wells et al., 1975; Kuo et al., 1977). Although in some cases relaxation of smooth muscle has been correlated with inhibition of phosphodiesterase (Kukovetz and Poch, 1970; Poch and Kukovetz, 1972; Kukovetz et al., 1976; Kramer and Wells, 1978), other investigators found no such correlation between the two effects (Daniel and Crankshaw, 1974; Collins and Sutter, 1975). Furthermore, in some instances the contracting action of agents has been enhanced by phosphodiesterase inhibitors (Bartelstone et al., 1967; Kalsner, 1971; McNeill et al., 1973). Finally, both MIX and Ro 20-1724 have been reported to have effects on smooth muscle contractility which appeared to be unrelated to their action as inhibitors of phosphodiesterase (Sawynok and Jhamandas, 1979).

In summary, concentration-dependent relaxation of vascular and non-vascular smooth muscle was observed with forskolin. Forskolin caused a concentration-dependent stimulation of adenylate cyclase activity in broken cell preparations of guinea pig taenia caeci and the longitudinal muscle of the rabbit small intestine. Holzmann (1982) [illegible] direct evidence of forskolin effects on cyclic AMP in beef [illegible]teries. Therefore, forskolin-mediated relaxation of these [illegible]s may occur via a mechanism involving cyclic AMP.

In addition, phosphodiesterase inhibitors, MIX and Ro 20-1724, were used as tools to test the hypothesis that forskolin-mediated relaxation of smooth muscle occurs via cyclic AMP. The results obtained with these agents were inconsistent and unexpected. These results suggest that interpretation of data obtained with phosphodiesterase inhibitors in smooth muscle is difficult and may lead to erroneous conclusions. Therefore, MIX and Ro 20-1724 have limited value as tools for the investigation of cyclic AMP mediated events in the smooth muscles used in this study.

4.4 CONCLUSION

The suggestion of Sutherland and Rall (1960) concerning the role of cyclic AMP in hormone-mediated relaxation of smooth muscle forms the basis of this thesis. This hypothesis implies that cyclic AMP *per se* can cause relaxation of smooth muscle. Two hormone receptors, adenosine-receptors and beta-receptors, were investigated in three smooth muscle preparations; beef coronary artery, guinea pig taenia caeci and the longitudinal muscle of the rabbit small intestine. Specifically, the following questions were asked:

1) Does adenosine increase levels of cyclic AMP in strips of beef coronary artery?
2) Is adenylate cyclase activity in broken cell preparations of guinea pig taenia caeci and the longitudinal muscle of the rabbit small intestine increased by isoproterenol, adenosine or analogs of adenosine?
3) Can forskolin be used as a tool to investigate the effect of cyclic

AMP on smooth muscle contractility?

4) Can phosphodiesterase inhibitors, MIX and Ro 20-1724, be used as tools to investigate the role of cyclic AMP in forskolin-mediated relaxation of smooth muscle?

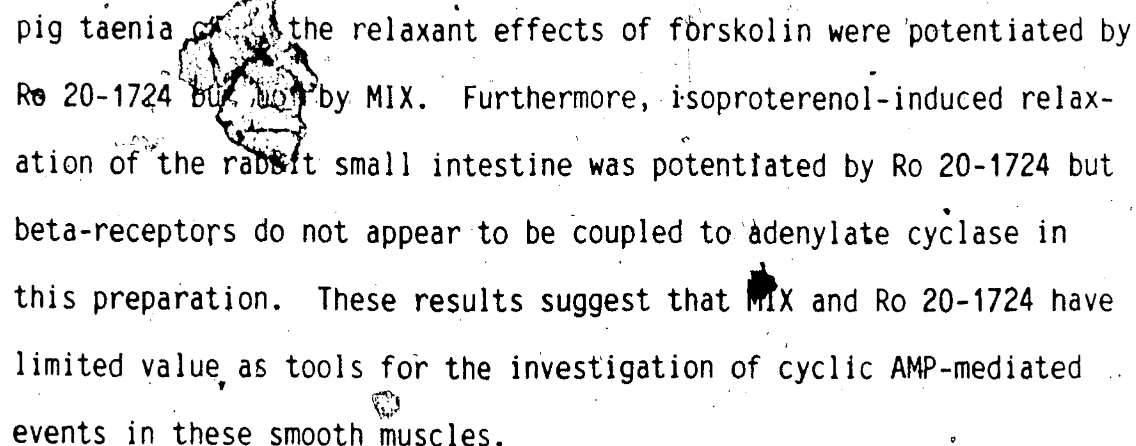
We found that a high concentration (1 mM) of adenosine increased cyclic AMP levels in strips of beef coronary artery. However, 0.1 mM adenosine did not significantly alter cyclic AMP levels in this tissue. The variability of basal levels of cyclic AMP was large. Our results are discussed in view of the observations of others. It was concluded that this approach proved to be unfruitful and neither supports nor refutes the findings of others.

In broken cell preparations from the guinea pig taenia caeci and the longitudinal muscle of the rabbit small intestine, the guanine nucleotide binding site and the catalytic unit of the adenylate cyclase complex were present and functional. Neither adenosine-receptor nor beta-receptor-mediated stimulation of adenylate cyclase activity was observed in these smooth muscle preparations despite the following measures which were taken to optimize the experimental conditions: Deoxy-alpha-[^{32}P]-ATP was used as substrate in the assay system, preparations were treated with adenosine deaminase, GTP or Gpp(NH)p were used in combination with adenosine or analogs of the nucleoside and the longitudinal muscle of the rabbit small intestine was fractionated. These results suggest that adenosine-receptors and beta-receptors in these smooth muscles differ from those known to be coupled to adenylate cyclase in other cell types. This conclusion is supported for adenosine-receptors by tissue-bath studies with

analogs of adenosine. The reproducibility of the results obtained in broken cell preparations is discussed with regard to receptor-mediated stimulation of adenylate cyclase activity. Thus, the question cannot be answered finally until ligand-binding studies are performed to investigate the state of the receptors present in the broken cell preparations used in this study. Furthermore, a method to ensure that only smooth muscle cells are present in the preparation must be found and specific inhibitors of proteolytic enzymes and/or endogenous inhibitors of adenylate cyclase must be found before definite conclusions can be drawn.

Forskolin caused a concentration-dependent relaxation of a variety of vascular and non-vascular smooth muscle preparations *in vitro*. Also, forskolin caused a concentration-dependent stimulation of adenylate cyclase activity in broken cell preparations of guinea pig taenia caeci and the longitudinal muscle of the rabbit small intestine. Holzmann (1982) has found that forskolin increased cyclic AMP levels in beef coronary artery. Therefore, forskolin-mediated relaxation of these three smooth muscles appears to be mediated by cyclic AMP. It follows that cyclic AMP *per se* can cause relaxation of these smooth muscles. Furthermore, forskolin can be used as a tool to investigate the effect of cyclic AMP on smooth muscle contractility.

The phosphodiesterase inhibitors, MIX and Ro 20-1724, were used as tools to investigate the role of cyclic AMP in forskolin-mediated relaxation of smooth muscle. The results obtained with these agents were inconsistent and unexpected in some cases. Thus, in the guinea



pig taenia coli the relaxant effects of forskolin were potentiated by Ro 20-1724 but not by MIX. Furthermore, isoproterenol-induced relaxation of the rabbit small intestine was potentiated by Ro 20-1724 but beta-receptors do not appear to be coupled to adenylate cyclase in this preparation. These results suggest that MIX and Ro 20-1724 have limited value as tools for the investigation of cyclic AMP-mediated events in these smooth muscles.

In conclusion, this thesis has raised more questions than it has answered. The relevance of measurements of levels of cyclic AMP in intact cells has been questioned. Our ability to evaluate the effect of receptor-mediated alteration of adenylate cyclase activity in broken cell preparations from smooth muscle at this time has been questioned. The use of phosphodiesterase inhibitors as tools to investigate the role of cyclic AMP in smooth muscle relaxation induced by forskolin has been questioned. The answers to many of these questions must await further developments in our understanding of the molecular events comprising the adenylate cyclase complex in the smooth muscles in question.

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