MYOCARDIAL ADENYLATE CYCLASE: INTERACTIONS WITH GUANYL NUCLEOTIDES, HORMONES AND MAGNESIUM

by

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INTRODUCTION

The marked effects of catecholamines on cardiac function point to an important role of sympathetic innervation in regulating the heart beat. The first report of the cardiovascular effects of adrenal extracts was made by Oliver and Schäfer (1) in 1895. The similarity of the effects of exogenous epinephrine and those of sympathetic nerve stimulation was noted somewhat later by Elliot (2). Experimental proof of chemical neurotransmission, however, was not provided until publication of the experiments of Loewi (3) and by Cannon and Uridil (4) in 1921. Despite recognition at that time of the ability of catecholamines to increase heart rate (3) and to modify systolic ejection patterns (5) the major efforts of the succeeding decades in research on the control of cardiac performance lay in other directions (6). In the last twenty years, however, recognition of the role of changes in myocardial contractility in the regulation of cardiac function has led to intense study of the effects of catecholamines on the heart. Table 1 presents a recent listing of the effects of catecholamines on isolated cardiac muscle and the intact heart.

TABLE 1 Parallel Actions of Catecholamines on Cardiac Muscle and Intact Heart

	Cardiac Muscle	Intact Heart
Inotropy	Isometric contractions	
	↑Rate of tension development (+dP/dt)	↑Rate of isovolumic pressure rise (+dP/dt)
	↑Rate of Tension decay (-dP/dt)	↑Rate of isovolumic pressure fall (-dP/dt)
	↓Twitch duration	↓Duration of systole
	↓Time to peak tension (TPT)	↓Isovolumic contraction time
	↓Relaxation time (RT)	↓Isovolumic relaxation time
	↑Peak contractile tension	↑Peak pressure
	Isotonic contractions	
	↑Magnitude of shortening	↑Stroke volume, end-systolic volume, ↑ ejection fraction
	↑Rate of shortening	↑Rate of volume ejection (dV/dt)
	↓Twitch duration	↓Ejection time
Chronotropy	↑Rate of diastolic depolarization	↑Heart rate
	Activation of latent pacemaker cells	Ectopic beats
Conduction	↓Refractory period of atrioventricular node	Facilitated atrioventricular conduction
	↑Conduction velocity in His-Purkinje fibers	Greater synchrony of contraction
Energetics	↑Activation heat	↑Oxygen consumption

Reproduced from reference 74

The discovery that a hormone produces an effect raises the question of how such a response is produced. It is a premise of molecular endocrinology that the initial event leading to the response of the target tissue involves the binding of the hormone to a specific receptor. This receptor concept was originally suggested by studies of drug actions on the neuromuscular junction which prompted Langley's suggestion (7) that a specific "receptor substance" mediates the action of neurohumors. Concurrently, the concept of specific receptors was invoked by Ehrlich in describing the interaction of toxins with cells (8), and antigens with antibodies (9).

The first comprehensive review of receptors was that of A.J. Clark (10) who concluded that "the more accurate quantitative data can be interpreted as the expression of a chemical reaction between the drug and specific receptors, which later in a large number of cases can be interpreted as being situated on the cell surface". The receptor concept does much to explain the specificity of hormonal effects, the relation of hormone concentration to the magnitude of the observed response and the ability of drugs to block selectively the effects of hormone. No insight, however, is provided as to how the response of the target tissue is related to events taking place when the hormone binds to the receptor.

The second messenger hypothesis, formulated by Sutherland co-workers (11), provided a model explaining how the interaction of a hormone with a receptor on the outer surface of the cell can affect metabolic events within the cell. The concept is based on stimulation by the hormone of the enzyme adenylate cyclase. This enzyme catalyzes the conversion of ATP to 3'-5' cyclic AMP, which has the ability to stimulate the activity of a class of cyclic AMP-dependent protein kinases within the cell. The process is illustrated schematically in Figure 1.

Simply stated the hypothesis postulated that the hormone receptor is part of, or closely associated with, the adenylate cyclase system. The hormone-receptor interaction modifies adenylate cyclase activity, thereby leading to a change in the cyclic AMP content of the cell. This model regards the hormone as the first messenger and cyclic AMP as the second messenger which transfers the information carried by the hormone to the internal mechanism of the cell.

In order to establish that the response to a hormone in a given tissue is indeed mediated by cyclic AMP acting as a second messenger, the following criteria must be satisfied (12).

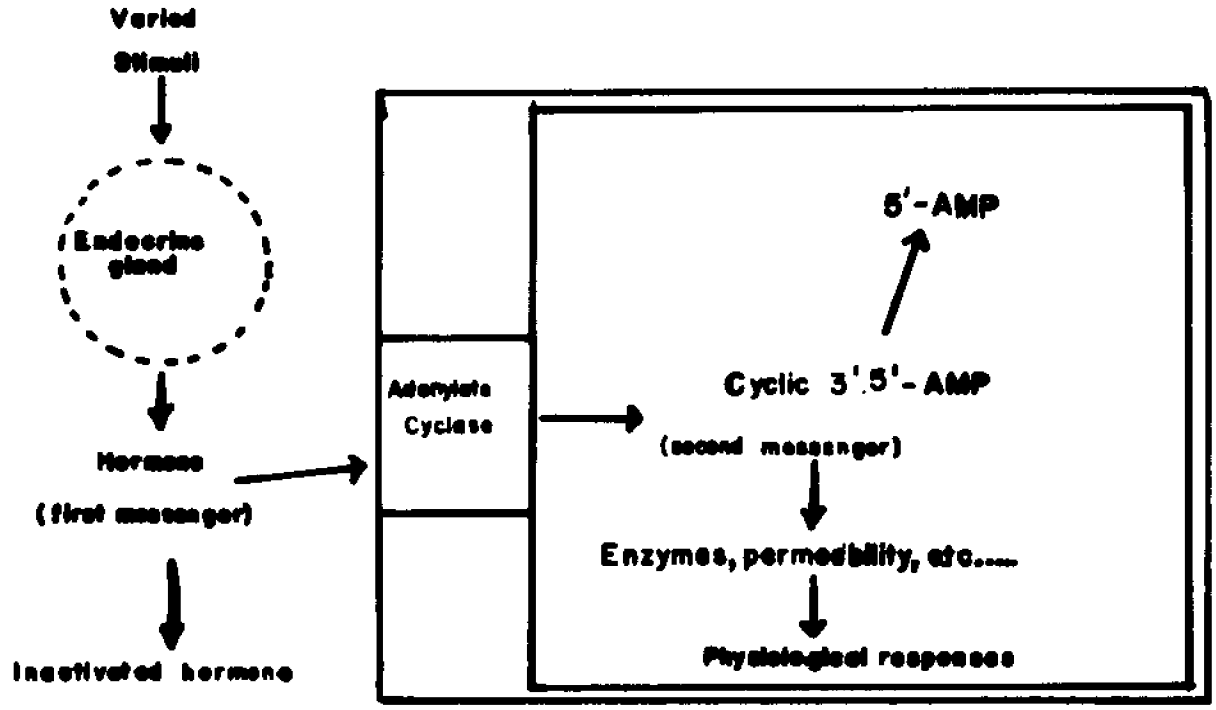

Figure 1. Schematic representation of the second messenger concept. Reproduced from Robison et al, (151).

Firstly: adenylate cyclase in broken cell preparations should respond to the same hormones which produce biological responses in intact tissue. In cases where a number of hormone analogs are available the order of potency should be the same *in vitro* as *in vivo*. Similarly, if competitive antagonists are available they should be effective *in vitro* as well as *in vivo*.

Secondly: since the concentration of cyclic AMP is also modified by changes in phosphodiesterase activity that converts cyclic AMP to 5'-AMP, the actions of hormones which stimulate adenylate cyclase should be potentiated by drugs that inhibit phosphodiesterase activity. This criterion is subject to several qualifications. In order for a phosphodiesterase inhibitor to potentiate the response to a hormone, phosphodiesterase activity must be present in the broken cell preparation. It is also necessary that the concentration of hormone is low enough so that the maximal response is not elicited in the absence of the phosphodiesterase inhibitor, and that the effects of the phosphodiesterase inhibition is not obscured by other effects of the drug.

Thirdly: the level of cyclic AMP in intact tissue should change in response to hormonal stimulation. Optimally these studies should be done under conditions in which a physiological response can be monitored simultaneously. The increase in cyclic AMP should precede or at least appear concomitant to the physiological response.

Finally: it should be possible to demonstrate that application of exogenous cyclic AMP or a suitable analog can mimic the effects of the hormone.

Catecholamine-sensitive adenylate cyclase activity in cardiac muscle was first reported in 1958 by Sutherland and Rall (13). These investigators were able to detect measurable amounts of cyclic AMP in the incubation medium of a cardiac particulate preparation only in the presence of the hormone epinephrine. Later reports by Murad et al, (14, 15) presented a more complete study of the effects of catecholamines on cyclic AMP formation. Using a particulate preparation of canine ventricular muscle, these investigators tested a series of catecholamines for relative potency in stimulating cyclic AMP production as well as the ability of the competitive beta blocking agent dichloroisoproterenol (DCI) to inhibit this effect. The results obtained were very similar to those previously reported by Mayer and Moran (16) when measuring contractile force and phosphorylase a content in vivo. In both studies the increase in the measured response was dependent on the concentration of the beta adrenergic agonist used in the experiment. Furthermore, the increase in cyclic AMP production and the rises in contractile force and phosphorylase a activity produced by the addition of catecholamine could be blocked by DCI. Other studies of the effect of beta adrenergic blocking agents by Robison et al, (17) lend further support to the

hypothesis that interaction of adrenergic agonists with their receptor leads to an increase in the cyclic AMP level in the heart.

Fulfillment of Sutherland's second criterion for the establishment of cyclic AMP mediation of the effects of epinephrine on the heart; potentiation of the myocardial response to epinephrine by phosphodiesterase inhibitors, is complicated by the nonspecific effects of the phosphodiesterase inhibitors. Theophylline was found to potentiate the inotropic response to norepinephrine (18) as well as to increase the force of contraction when administered alone (19). The response to theophylline, however, is associated with an increase in the time to peak tension, a decrease in the rate of relaxation and an overall prolongation of systole (20) which is quite different from the response to catecholamines (21). These differences may be explained by the finding that methylxanthines interfere with calcium retention by the sarcoplasmic reticulum (22, 23). The effects of the phosphodiesterase inhibitors would therefore represent a balance of different effects. Blinks et al, (24) have suggested that methylxanthines produce at least two different responses: 1) an increase in sarcolemmal calcium flux (which is perhaps cyclic AMP mediated) and 2) a direct inhibition of calcium accumulation by the sarcoplasmic reticulum.

Development of a potent phosphodiesterase inhibitor, Ro 7-2956 (4-(3,4-dimethoxybenzyl-2-imidazolidinone) which is chemically unrelated to the methylxanthines, provided a new tool which is apparently free of side effects (25). Tsien et al, (26) showed that low concentrations of this agent were able to mimic the effects of catecholamines on the level and duration of the plateau phase of the action potential of Purkinje fibers as well as to accelerate the pacemaker potential which follows repolarization.

An alternative approach was utilized by Kukovetz and Pöch (27) who found that imidazole, which has the ability to stimulate phosphodiesterase activity in vitro, was able to inhibit the positive inotropic effect of methylxanthines as well as the response to low concentrations of catecholamine. In general, therefore, it can be concluded that the effects of phosphodiesterase inhibitors on the heart are compatible with the second messenger hypothesis.

Sutherland's third criterion for the establishment of cyclic AMP as a second messenger; a correlation between tissue cyclic AMP and the physiologic response to hormone, led to considerable controversy. The effect of epinephrine to increase the cyclic AMP content of perfused hearts was first demonstrated in the rat by Øye et al, in 1964 (28) and in the rabbit by Hammermeister et al, in 1965 (29). In the preliminary study of Øye et al, no dissociation in the increases in cyclic AMP, phosphorylase a activity or

contractility was apparent. In later work from the same laboratory, however, (30) the rise in cyclic AMP clearly preceded the increase in contractile force or phosphorylase a activity. A similarly rapid rise in cyclic AMP content after exposure to epinephrine was also noted by Cheung and Williamson (31) who reported the increase in cyclic AMP not only preceded the rise in contractile force but that changes in phosphorylase followed both of these parameters. Further evidence of this sequence of events, presented by Williamson (32) and Mayer et al, (33), suggested that the effects on contractility and glycogenolysis either followed a different time course or differed greatly in sensitivity to the effector. Experiments by Drummond et al, (34) that compared the time course of cyclic AMP accumulation with phosphorylase b kinase activation showed that these events occured almost simultaneously, the inotropic response to epinephrine occuring several seconds later. A similar relationship between cyclic AMP content and phosphorylase b kinase activity was demonstrated by Namm and Mayer (35). These results imply that the conversion of phosphorylase b to phosphorylase a is the rate limiting step in glycogenolysis, and therefore, the lag of phosphorylase activation relative to the inotropic response cannot be taken as evidence for separate mediatory mechanisms for the two effects.

Several studies, however, fail to support the concept that cyclic AMP mediates the inotropic response to the catecholamines. Mayer et al, (36) reported that glucagon, which produces both positive inotropic and chronotropic effects (37-39), caused increases in both phosphorylase a and contractile amplitude before an increase in cyclic AMP could be detected. Shanfeld et al, (40) reported a dissociation between increases in cyclic AMP and the positive inotropic effect of norepinephrine by the drug N-isopropyl methoxamine (IMA). When IMA was present, norepinephrine did not increase either cardiac cyclic AMP or phosphorylase activity although the increase in contractile force was not impaired. Similarly, Benfey and Carolin reported that although phenylephrine increased the force of contraction in rabbit and chicken hearts, it did not increase adenylate cyclase activity in particulate preparations of these tissues (41). Dopamine was also found to be ineffective in increasing cyclic AMP content of heart slices or adenylate cyclase activity in rabbit heart homogenates (42). The inotropic effects of phenylephrine and dopamine have been well characterized (43-45).

The concensus of opinion has returned to favor cyclic AMP as an important mediator of the inotropic effects of the catecholamines. In the discussion of their results, Mayer et al, (36) pointed out that their data were not conclusive inasmuch as their assay for cyclic AMP content was not as

sensitive as their contractile force measurements. The lag in cyclic AMP changes, therefore, could result from failure to detect an increase in the nucleotide that was still large enough to produce a physiologic response.

McNeill and Verma (46) pointed out that the failure of Benfey to detect an effect of phenylephrine on adenylate cyclase activity might be due to tissue disruption. When measurements were made of cyclic AMP content of whole hearts, phenylephrine was found to be effective, albeit at much higher concentrations than required to elicit responses with norepinephrine. Similar increases in cyclic nucleotide content by phenylephrine or dopamine were reported by Osnes et al, (47, 48). The latter group found, however, that when the tissue was treated with the beta blocking agent propranolol, phenylephrine still produced a positive inotropic effect even though the increase in cyclic AMP was fully inhibited. This latter inotropic effect is attributable to an alpha adrenergic component to the effects of phenylephrine as Schuman et al, (49) reported that the alpha agonist methoxamine alone increased the contractile force of rabbit papillary muscle without significant increase in cyclic AMP content. In the presence of the phosphodiesterase inhibitor papaverine, methoxamine increased contractile force while cyclic AMP concentration decreased. The inotropic response to alpha adrenergic agonists therefore appears to be mediated by mechanisms independent of cyclic AMP. The

difference in mechanism is also reflected in qualitative differences in effects on the shape of the isometric tension development curves, as phenylephrine in the presence of propranolol leads to an increase in the duration of systole (50), which is in contrast to the shortening of systole associated with the effects of beta agonists (see below). The failure of IMA to inhibit the inotropic response to norepinephrine may perhaps also be explained by the well known alpha adrenergic activity of norepinephrine.

It can be concluded that most evidence favors the hypothesis that cyclic AMP mediates the effects of beta adrenergic stimulation in the heart and that the interaction of adrenergic amines with cardiac alpha receptors produce a positive inotropic effect by a different mechanism that does not require cyclic AMP.

Attempts to fulfill the fourth requirement of Sutherland et al, duplication of the response to adrenergic stimulation by treatment with cyclic AMP or its analogs, began in 1965. At that time Robison et al, (51) reported the failure of cyclic AMP and several of its derivitives to elicit a positive inotropic response in perfused rat hearts. Later attempts by that group were also unsuccessful (17), perhaps because of the low concentrations of nucleotide that were used for those studies.

The first report of cardiovascular effects of cyclic AMP appeared in 1966 when Levine and Vogel (52) found an increase in heart rate, cardiac output and blood glucose after injection of large doses of cyclic AMP into unanesthetized dogs, whereas no significant changes followed administration of 2'3'-AMP, 5'-AMP or ATP. Similar responses were elicited by cyclic AMP in human patients (53, 54). The authors of the clinical studies concluded that these were direct effects on the heart and not due to reflex action. This hypothesis was substantiated by studies of the effects of dibutyryl cyclic AMP (db-cyclic AMP) on isolated perfused hearts from the guinea pig (55) and rat (56-58). Skelton et al, (59) noted that the increments in tension and rate of tension development in isolated cat papillary muscle caused by a maximally effective dose of db-cyclic AMP were similar to those produced by optimal concentrations of norepinephrine. Ahren et al, (58) showed that db-cyclic AMP could mimic the metabolic effects of epinephrine as well.

In all of these studies there was a delay of several minutes before any change in the measured parameter was observed. In contrast, the responses to norepinephrine were much more rapid, maximal effects being seen after less than one minute. Measurements of the entry of labeled db-cyclic AMP into perfused rat hearts indicated that cell membrane permeability to this nucleotide was very low so

that some time would be required to achieve a physiologically active level (60). In contrast, cultured isolated rat heart cells showed an immediate positive chronotropic response to db-cyclic AMP (61). This rapid effect was attributed to the trypsin treatment of the heart cells prior to culture.

Although studies of the effects of catecholamines or phosphodiesterase inhibitors on the pacemaker current of cardiac Purkinje fibers are consistent with the hypothesis that cyclic AMP mediates the effects of these agents, attempts to demonstrate the ability of dibutyryl (62) or monobutyryl (63, 64) cyclic AMP to mimic the action of epinephrine on the pacemaker current have not succeeded. This is especially puzzling as monobutyryl cyclic AMP has profound effects on the plateau (calcium) currents of the same preparation (26). The fact that it is possible to duplicate the pacemaker effects of epinephrine by intracellular iontophoresis of cyclic AMP into Purkinje fibers (65) or sino-atrial node (66) suggests that it is perhaps some difference in the structure of dibutyryl or monobutyryl cyclic AMP that limits their access to or effects on the relevant site of action within the cell.

Studies on the inotropic effects of epinephrine and cyclic AMP have proved more amenable to analysis. Most investigators now believe that myocardial contractility is determined, at least in part, by the amount of calcium delivered to the contractile proteins during systole (67).

It is well established that catecholamines increase the amplitude of a slow inward current carried largely by calcium ions during the plateau phase of the cardiac action potential (68-70). This increase in the slow inward current appears to be mediated by an increase in the specific conductance of the membrane for calcium with no change in the associated membrane parameters (69). The effect of catecholamines on this ionic channel has been duplicated by superfusion with mono (60,71) and dibutyryl cyclic AMP (69) as well as intracelluarly applied cyclic AMP (65).

The independent effect of epinephrine to increase the rate of relaxation of cardiac muscle has also been studied (72-74). A direct relaxant effect of db-cyclic AMP on cat papillary muscle in potassium-induced contracture has been reported by Heinertz et al, (75). The addition of cyclic AMP itself was also found to increase the rate of relaxation in "skinned" cardiac fibers (76). The mechanism by which cyclic AMP increases the rate of relaxation has been identified. Entman et al, (77) reported an effect of cyclic AMP to increase the rate of calcium sequestration by sarcoplasmic reticulum vesicles (microsomes) isolated from canine cardiac muscle. This was partly confirmed by Shineborne and White (78) who found that only very high (1 mM) concentrations of cyclic AMP produced this effect. Kirchberger et al, (79) found that in the presence of partially purified protein kinase much lower concentrations of the nucleotide were required to stimulate calcium transport, half maximal activation being seen at 2×10^{-7} M cyclic AMP.

This value was similar to the apparent Km for cyclic AMP stimulation of phosphorylation of cardiac microsomes by protein kinase. An intrinsic protein kinase of cardiac microsomes was also identified by LaRaia and Morkin (80) and Wray et al, (81). This intrinsic protein kinase is able to phosphorylate a substrate in the sarcoplasmic reticulum (81). It now appears that a the phosphorylated substrate that mediates the relaxant effect of catecholamines is a 22,000 dalton protein, phosphorylation of which is strongly correlated with increased calcium transport activity by cardiac sarcoplasmic reticulum vesicles (82-84). Conversely, when the 22,000 dalton phosphoprotein in these membranes is dephosphorylated, the rate of calcium transport falls to control values (85).

Another mechanism by which cyclic AMP may influence contractility is by the phosphorylation of contractile proteins. Troponin has been phosphorylated by partially purified cyclic AMP-dependent protein kinase (86, 87) and by a kinase endogenous to the native troponin-tropomyosin complex (88). Phosphorylation of cardiac troponin appears to decrease the calcium sensitivity of actomyosin ATPase (89, 90). Although these data are consistent with the increased rate of tension development seen in the presence of catecholamines, there is as yet no proof of their physiological releveance.

The wide range of processes sensitive to change in myocardial cyclic AMP makes studies of the properties of cardiac adenylate cyclic and factors that regulate its interaction with the catecholamines of central importance to an understanding of cardiac function. In view of these considerations, the work presented here was aimed toward the characterization of the control of a hormone sensitive cardiac adenylate cyclase.

The first objective of these studies is the localization of adenylate cyclase within the cardic cell. Amine and polypeptide hormones that stimulate adenylate cyclase are generally agreed to interact with receptors on the plasma membrane, and cardiac membrane preparations enriched in sarcolemma (plasma membrane) markers show a highly active, hormone sensitive, adenylate cyclase (91-94). Other studies, however, have shown that an epinephrine-sensitive adenylate cyclase is copurified with membranes enriched in ATP-dependent calcium transport activity; the latter being a marker for the sarcoplasmic reticulum (77, 95, 96). This adenylate cyclase activity is unlikely to be due to contamination by sarcolemma as preparations showing both the highest calcium transport activity and the highest adenylate cyclase activity were virually free of (Na + K)•ATPase and ouabain-binding sites, which are markers for the sarcolemma.

Evidence from cytochemic studies performed by Shulze et al, suggest that there are two classes of adenylate cyclase in cardiac muscle (97). These studies were based on the property of lead ions to precipitate with pyrophosphate which is one of the products of the adenylate cyclase reaction. When ventricular slices were incubated with lead and then examined under the electron microscope precipitate was found along the plasma membrane, T tubules, subsarcolemmal cisternae, and the places of contact between the T tubules and the sarcoplasmic reticulum. Incubation of the tissue with either epinephrine, glucagon, or NaF, agents which stimulate cardiac adenylate cyclase, increated the density of all lead deposits. On this basis Sulze et al, concluded that there are two sites of catecholamine-sensitive adenylate cyclase in the cardiac ventricular cell; one on the plasma membrane and the other on the subsarcolemmal cisternae and the sarcoplasmic reticulum.

Other approaches to the localization of the catecholamine receptor in cardiac muscle measured the effects of catecholamines on responses other than cyclic AMP production, i.e. membrane potential changes and force of contraction. Since an intact cell was necessary for these studies, special techniques were utilized to differentially apply catecholamines to either the outside or inside of the cell. Ventner and coworkers (98, 99) found that isoproterenol immobilized

on glass beads, and presumably only able to interact with extracellular binding sites, produced an inotropic response. These investigators concluded that the isoproterenol receptors were on the cell surface as immobilization did not reduce the effect of isoproterenol, but the stability of the glass bead-isoproterenol complex has been questioned by Yong (100) who calculated that enough free drug could have leached off the glass to account for these effects.

Reuter (69) utilized intracellular iontophoresis of norepinephrine and isoproterenol and found no effect on either the pacemaker activity or the plateau level of the action potential of cardiac Purkinje fibers. Similar stimulation after the electrode was withdrawn from the cell produced marked acceleration of pacemaker activity and increased the plateau level of the action potential. Fabiato and Fabiato (76, 101) prepared mechanically skinned ventricular cells and found that epinephrine had no effect on tension development unless at least a small area of the sarcolemma was left intact. These results imply that cardiac beta adrenergic receptors and therefore catecholamine-sensitive adenylate cyclase are localized on the sarcolemma.

The discrepancies between the two conflicting groups of experiments, one of which supports the idea of two locations for catecholamine-sensitive adenylate cyclase, the other limiting the location of the beta receptor to

the sarcolemma, may be explained if latent enzyme activity in nonsarcolemmal membranes becomes unmasked during isolation (91).

The second objective of these studies is to characterize the effects of GTP on the adenylate cyclase of the sarcolemma. The first report of the interaction of this nucleotide with adenylate cyclase appeared in 1969 in which Cryer et al, (102) noted that GTP inhibited basal adenylate cyclase activity in fat cells at concentrations ranging from 5×10^{-7} to 5×10^{-4} M. Later work by Rodbell et al, showed that GTP stimulated basal activity of the hepatic adenylate cyclase and greatly increased the response of that enzyme to glucagon (103). This latter effect of GTP, to enhance the hormone sensitivity of adenylate cyclase, appears to be a general mechanism as similar phenomena were observed in the adrenal gland (104-106), bovine (107) and human thyroid (108), frog bladder epithelia (109), pancreatic islets (110-112), platelets (113), fat cells (114, 115), the anterior pituitary (116), canine heart (117), and avian (118, 119) and mammalian erythrocytes (120), as well as being amply confirmed in the liver (121-124). One notable exception to this rule is the vasopressin-sensitive adenylate cyclase of the bovine renal medulla in which the action of the hormone is inhibited by GTP while basal activity is increased (125).

In most systems studied GTP also increases basal activity, but this effect is usually slight. Much larger increases in adenylate cyclase activity, both in the absence and presence of hormones, have been elicited by the synthetic analogs 5' guanylyl-imido-diphosphate (GMP-PNP) and 5' guanylyl (β-γ) methylene diphosphonate (GPP-CH_2-P) (104, 106, 119, 120, 124, 126-132). These synthetic analogs apparently bind at the same site as GTP, but differ from the naturally occuring nucleotide in that the activation by GMP-PNP and GPP-CH_2-P is irreversible (133-135).

Both the localization of cardiac adenylate cyclase and the interaction of this enzyme with the guanyl nucleotides have been examined in this study. The effects of catecholamines and other agents known to modify adenylate cyclase activity were tested with sarcolemmal and sarcoplasmic reticulum preparations derived from the ventricular muscle of the guinea pig heart.

The present studies on the effects of GTP on the sarcolemmal adenylate cyclase had already been begun when the effects of GMP-PNP on canine cardiac adenylate cyclase were reported by Lefkowitz (127). Differences in the effects of nucleotides on the two enzymes prompted a more complete investigation of the guinea pig enzyme. These studies which were carried out in light of newer work by Rodbell and co-workers (136-139) stressed the importance of time as a variable affecting the observed response to added

nucleotides. In addition to the testing of various guanyl and other nucleotides for effect on basal activity of the adenylate cyclase of the guinea pig sarcolemma, special attention was devoted to the interaction of the guanyl nucleotides with other enzyme ligands, i.e., catecholamines, NaF, substrate, and Mg^{2+}. Careful time course experiments were routinely employed in an effort to develop a more complete kinetic model that could account for the observed effects.

METHODS

Preparation of Guinea Pig Ventricular Sarcolemma

Sarcolemma was prepared from guinea pig ventricles by a modified method of Tada et al, (91). Adult female guinea pigs (Cavia cobava) weighing 300-400 gm were sacrificed by a sharp blow to the head and their hearts excised and trimmed of the atria and large vessels. Ventricular tissue was washed in ice-cold 2.5 mM imidazole - 0.2 mM EDTA (pH 7.4), suspended in five volumes of the same buffer and homogenized in a Virtis Omnimixer at full speed for 1-2 sec. The homogenate was allowed to settle and the supernatant was filtered through four layers of gauze. The remaining slices were homogenized again in the same buffer and the process repeated until all the tissue was broken down to single cell fragments as determined by phase contrast microscopy. The pooled supernatants were centrifuged at 1600 x g for 30 min and the pellets resuspended in 10% sucrose - 0.2 mM EGTA - 10 mM Tris-HCl (pH 7.5) and dispersed gently by hand in all glass Dounce homogenizer with a loose fitting pestle. The suspension was centrifuged at 1600 x g for 30 min and resulting pellets were resuspended in the same buffer. The cells were again collected by centrifugation at 1600 x g for 30 min and suspended in an equal volume plus 2 ml of 40% sucrose - 0.2 mM EDTA -

10 mM Tris-HCl (pH 7.5) with the Dounce homogenizer. Three volumes of 40% sucrose - 1.3 mM KCl - 0.26 mM EDTA - 13 mM Tris-HCl (pH 7.0) were then added and the suspension stirred overnight at 4°C to extract contaminating contractile proteins. After extraction the solution was diluted with three volumes of 5 mM Tris-HCl (pH 7.5) and centrifuged at 1600 x g for 30 min. The pellets were resuspended in half the initial volume of the same buffer and centrifuged again at 1600 x g for 30 min. The final pellets were dispersed in the appropriate buffer (as dictated by assay conditions) with the Dounce homogenizer.

Protein concentration of the resuspended membranes was determined by a biuret procedure with bovine serum albumin as standard.

Preparation of Guinea Pig Cardiac Microsomes

Guinea pig cardiac microsomes were prepared by a modified method of Harigaya and Schwartz (140). Ventricular tissue was minced with scissors and homogenized in a Waring blender in 30 ml of ice-cold 10 mM sodium bicarbonate for 30 sec at maximum speed. The homogenate was centrifuged at 9,000 x g for 10 min. The supernatant was filtered through four layers of gauze and centrifuged again at 9,000 x g for 20 min. The supernatants were removed and centrifuged at 37,000 x g for 30 min. The pellets were resuspended in 0.6 M KCl - 20 mM Tris-HCl (pH 7.0) and

centrifuged at 37,000 x g for an additional 30 min. The final pellets were resuspended in a buffer appropriate for subsequent assays by gentle homogenization with a Potter-Elvehjem homogenizer fitted with a Teflon pestle.

Protein concentration was assayed by a biuret method with bovine serum albumin as standard.

Preparation of Microsomal Fractions of Mucosal Epithelia of the Urinary Bladder of the Turtle (Pseudymis scripta)

Microsomal fractions of mucosal epithelia were prepared by the method of Solinger et al, (141). All steps in the procedure prior to centrifugation were carried out at room temperature; centrifugation and later procedures were performed at 4°C. Three bladders in the form of closed sacs were immersed in Ca-free, choline Ringer solution containing 2 mM EDTA. The mucosal surface was washed 3-4 times in the same solution. The sacs were then filled with 20 ml of the modified EDTA-containing Ringer solution and immersed in the same solution for 30 min. During the incubation the serosal fluid was gassed with 99% O_2-1% CO_2. The sacs were removed and their walls gently rubbed together between thumb and forefinger to release the epithelial cells into the mucosal fluid. The cell-containing fluid was removed and centrifuged at 10,000 x g for 15 min. The pellets were homogenized in an all-glass Dounce homogenizer and resuspended in 100 ml of a solution

containing 0.25 M sucrose, 1 mM Tris-EDTA and 0.1% Na deoxycholate (pH 7.4). The cell suspension was centrifuged at 10,000 x g for 15 min and the resulting supernatant re-centrifuged at 65,000 x g for 1 hr. The pellet so obtained was resuspended, homogenized in the Dounce homogenizer and diluted to 50 ml with a solution containing 0.25 M sucrose, 1 mM Tris-EDTA and 0.05% Na deoxycholate (pH 7.4). This suspension was centrifuged at 20,000 x g for 20 min and the resulting supernatant was centrifuged at 65,000 x g for 1 hr. The final pellet was homogenized and suspended in 10 ml of 0.25 M sucrose - 1 mM EDTA solution (pH 7.4).

Protein concentration of these preparations was determined by the method of Lowry et al, (142) with bovine serum albumin as standard.

Purification of Cyclic AMP-Dependent Protein Kinase

Cyclic AMP-dependent protein kinase was partially purified from bovine hearts following the method of Miyamoto et al, (143) through the DEAE-cellulose chromatography step. Frozen bovine hearts were thawed and trimmed of atria, large vessels and fat. The ventricular muscle was sliced into small pieces and homogenized for 2 min in a Waring blender in 3 volumes of 4 mM EDTA (pH 7.0). The homogenate was centrifuged at 27,000 x g for 30 min and the supernatant filtered through four layers of gauze. Acetic acid (1 M) was added dropwise to the filtered supernatant until a pH

of 4.8 was reached. After 10 min the precipitated protein was removed by centrifugation at 27,000 x g for 30 min. The supernatant was again filtered through four layers of gauze and the pH adjusted to 6.5 by the addition of 1 M potassium phosphate buffer (pH 7.0). The volume of the solution was measured and 37.5 g of solid ammonium sulfate was added per 100 ml of solution. Thirty minutes after the salt was fully dissolved the solution was centrifuged at 16,000 x g for 20 min. The pellets were dissolved in a volume of 5 mM potassium phosphate, 2 mM EDTA, pH 7.0 buffer equal to 6% of the volume of the initial muscle homogenate and dialyzed overnight against 20 volumes of the same solution. During the dialysis period the external buffer was changed twice. After dialysis the solution was centrifuged at 27,000 x g for 30 min and the precipitate discarded.

The supernatant was applied to a DEAE-cellulose column that had been equilibrated with 5 mM potassium phosphate buffer (pH 7.0). The column was then washed with 2 volumes of 0.1 M potassium phosphate buffer, pH 7.0, and the enzyme eluted with 0.3 M potassium phosphate, pH 7.0. All potassium phosphate buffers contained 2 mM EDTA. Active fractions were pooled and dialized for 14 hours against 20 volumes of 5 mM histidine-HCl, (pH 6.8) with two changes of the external buffer.

After dialysis the concentration of protein was determined by the method of Lowry et al, (142) and 0.5 ml aliquots were frozen in a dry ice-acetone mixture and stored at -20°C.

Preparation of Mg-ATP

Disodium ATP was freed of metal ions by cation exchange chromatography on Dowex 50 (H^+ form). An equimolar amount of $MgCl_2$ was added and the solution adjusted to pH 6.8 with the addition of Tris.

Preparation of Calcium EGTA Buffers

In some experiments the calcium ion concentration was maintained with a calcium-EGTA buffer system. The concentration of EGTA required to maintain a given ionic calcium concentration under assay conditions was calculated according to the equations of Katz et al, (144) with a calcium-EGTA binding constant of 4.4×10^5 M^{-1} (pH 6.8) as determined by Ogawa (145).

Adenylate Cyclase Assay

The adenylate cyclase activity of the isolated membrane preparations was assayed according to the method of Bär and Hechter (146) as modified by Bär (147). Under standard assay conditions 30-55 µg of membrane protein were incubated at 37°C for 5 min in a total volume of 50 µl containing 50 mM Tris HCl (pH 7.5 at 37°C),

0.2 mM ATP, 1-2 μCi [α^{32}P] ATP, 10 mM $MgCl_2$, 1 mg/ml creatine phosphokinase, 25 mM creatine phosphate, 0.5 mM cyclic AMP, 1 mM EGTA and 1 mg/ml bovine serum albumin.

The reaction was started by addition of protein and terminated by addition of 5 μl of a solution containing 20 mM each of ATP, AMP and cyclic AMP in 0.2 M EDTA (pH 7.0) and the tubes placed on ice. When the time course of cyclic AMP production was determined, the total reaction volume was increased to 100 μl. After the start of the reaction, 10 μl aliquots were removed from the reaction mixtures and transfered to tubes containing an equal volume of an ice-cold solution containing 4 mM each of ATP, AMP and cyclic AMP in 40 mM EDTA (pH 7.0). The contents of the tubes were mixed thoroughly and the tubes placed on ice.

Labeled nucleotides were separated by application of 5 μl aliquots of the reaction mixture to polyethyleneimine (PEI) impregnated cellulose TLC plates, which were then developed in 0.25 M LiCl. After the plates were dried, cyclic AMP, ATP and AMP spots were located under UV illumination. These were then cut out and counted in toluene scintillation fluid [4.0 g PPO (2,5-diphenyloxazole) and 0.2 g POPOP (1,4-bis (2- (5-phenyloxazole))-benzene /1] with a liquid scintillation spectrometer. The ratio of cyclic AMP associated radioactivity to total radioactivity was used as an estimated of the conversion of substrate to cyclic AMP and the specific activity of

the enzyme was expressed as pmol cyclic AMP produced per mg protein per min.

To test the adequacy of the regenerating system, aliquots of the reaction mixtures were applied to PEI-impregnated cellulose TLC plates and chromatographed in 0.5 M LiCl - 2 N formic acid. The plates were dried and the nucleotides visualized as described above. Estimates of the ratio between ATP and total radioactive nucleotide demonstrated that nearly 90% of the substrate was intact at the end of the incubation period.

$(Na^+ + K^+)$-ATPase Assay

The assay of $(Na^+ + K^+)$-ATPase activity was carried out according to the method of Siegel and Albers (148). Cardiac sarcolemmal membranes (25-40 µg) were incubated for 5 min in a volume of 100 µl containing 40 mM Tris-HCl (pH 7.4, 37°C), 0.1 mM Tris-EDTA, 3 mM ATP, 0.4 µCi [γ^{32}P] ATP, 3 mM $MgCl_2$, and either 60 mM NaCl alone or 60 mM NaCl + 25 mM KCl. Reactions were started by the addition of substrate and terminated by the addition of 25 µl of 25% perchloric acid. The reaction tubes were centrifuged at 20,000 x g for 20 min and 50 µl aliquots of the resulting supernatants were added to clean tubes containing 35 µl of 5% ammonium molybdate in 1 N H_2SO_4. The contents of the tubes were mixed and 200 µl of isobutanol added. The contents of the tubes were mixed again and then centrifuged for 5 min at 10,000 x g to

separate organic and aqueous phases. Aliquots (100 µl) of the organic layer in each tube were removed for counting in 10 ml of Bray's solution (60 gm naphthalene, 4 g PPO, 0.2 g POPOP, 100 ml methanol and 20 ml of ethylene glycol/l). $(Na^+ + K^+)$-ATPase activity was determined by the difference in activity in the presence and absence of KCl. Activity was expressed in terms of µmoles of Pi liberated per h per mg sarcolemmal protein.

Assay of Calcium Transport Activity

Guinea pig cardiac sarcoplasmic reticulum (1 mg) was incubated in 4 ml of a solution containing 120 mM KCl, 40 mM histidine (pH 6.8), 5 mM potassium oxalate, calcium-EGTA buffer (0.125 mM $CaCl_2$, 7.5×10^{-7} M Ca^{2+}), and 0.3 µCi ^{45}Ca. Concentrations of bovine cardiac protein kinase and cyclic AMP, when present, were 0.2 mg/ml and 1 µM, respectively.

The membranes were added to the reaction mixtures which were then preincubated for 5 min at 25°C. Calcium transport was started by the addition of the calcium buffer. Aliquots of the reaction mixture were removed in disposable syringes and the reaction stopped by filtration through a 0.45 µm Millipore filter held in a Swinny adapter. Aliquots (50 µl) of the filtrates were removed for counting in 5 ml of Bray's solution. Calcium transport was calculated from the decrease in

radioactivity of the filtrates as compared to unfiltered portions of the same reaction mixture. Results are expressed in terms of μmoles of calcium transported per mg membrane protein.

RESULTS

Characterization of Sarcolemmal Beta Receptors

The beta adrenergic receptor associated with the adenylate cyclase of the guinea pig ventricular myocardium was characterized in several series of experiments. In the first, the ability of the two stereo-isomers of epinephrine to stimulate adenylate cyclase activity of the sarcolemmal membrane preparation was compared (Table 2). Although basal activity under the conditions used was low, 10^{-4} M l-epinephrine produced a 320% increase in the rate of cyclic AMP production whereas the same concentration of d-epinephrine did not stimulate adenylate cyclase activity. For this reason only the l-stereoisomers of catecholamines were tested in later experiments. The extent of stimulation by l-epinephrine was greatly reduced by a five-fold excess of the beta adrenergic blocking agent, l-propranolol. The ability of propranolol to inhibit the effect of l-epinephrine suggests that the receptor involved in the stimulation of adenylate cyclase is a beta adrenergic receptor.

The effects of a series of adrenergic agonists were compared to evaluate the beta receptor properties of the sarcolemmal preparations. Equimolar concentrations (10^{-4} M) of isoproterenol, epinephrine, norepinephrine, dopamine and phenylephrine were added to assay tubes and the ability of these agents to stimulate adenylate cyclase

TABLE 2

Effects of l- and d-Epinephrine and of d, l-propranolol on Cardiac Sarcolemmal Adenylate Cyclase Activity

Addition	Adenylate Cyclase Activity (pmol cAMP mg^{-1} min^{-1})
None	2.27 ± 1.83
10^{-4} M l-epinephrine	9.54 ± 1.81*
10^{-4} M d-epinephrine	2.27 ± 3.14
10^{-4} M l-epinephrine + 5×10^{-4} M d, l-propranolol	3.20 ± 0.91†

Assays were performed at 25°C for 10 min in a medium containing 2 mM [α-^{32}P]ATP, 5 mM $MgCl_2$, 0.5 mM unlabeled cyclic AMP, 2.5 mg/ml sarcolemmal protein, a calcium-EGTA buffer (125 µM $CaCl_2$, 138 µM EGTA, 10 µM Ca^{2+}) and 100 mM imidazole buffer at pH 6.8. The values of adenylate cyclase activity are means ± SE of six determinations.

* $t = 2.815$, $P<0.01$ compared with control.

† $t = 2.782$, $P<0.01$ compared with 10^{-4} M l-epinephrine.

activity was determined. Representative results are shown in Figure 2. In general the order of effectiveness of these agents was: isoproterenol > epinephrine > norepinephrine > dopamine > phenylephrine. In some experiments (Table 3) epinephrine was somewhat more effective than isoproterenol. The order of potency of the other agonists was the same in all experiments.

A similar pattern of effectiveness was seen when the concentration dependency of the different agonists was studied in the range of 10^{-7} to 10^{-4} M (Figure 3). The concentration of drug required to produce half of the maximal response to that drug was used as a measure of the receptor affinity for that drug. Isoproterenol showed the highest apparent affinity for the receptor as well as the greatest maximal effect, which was seen at a concentration of 10^{-5} M. Epinephrine was almost as effective as isoproterenol at a concentration of 10^{-5} M but showed a lower affinity for the receptor. Concentrations of isoproterenol or epinephrine above 10^{-5} M were inhibitory. As in the experiments shown in Table 3, 10^{-4} M epinephrine was slightly more effective in increasing adenylate cyclase activity than was 10^{-4} M isoproterenol. Norepinephrine elicited a much smaller maximal response; its affinity for the receptor, however, was somewhat higher than that of epinephrine. Dopamine produced significant stimulation of cyclase activity only at concentration above 10^{-5} M. The response to phenyl-

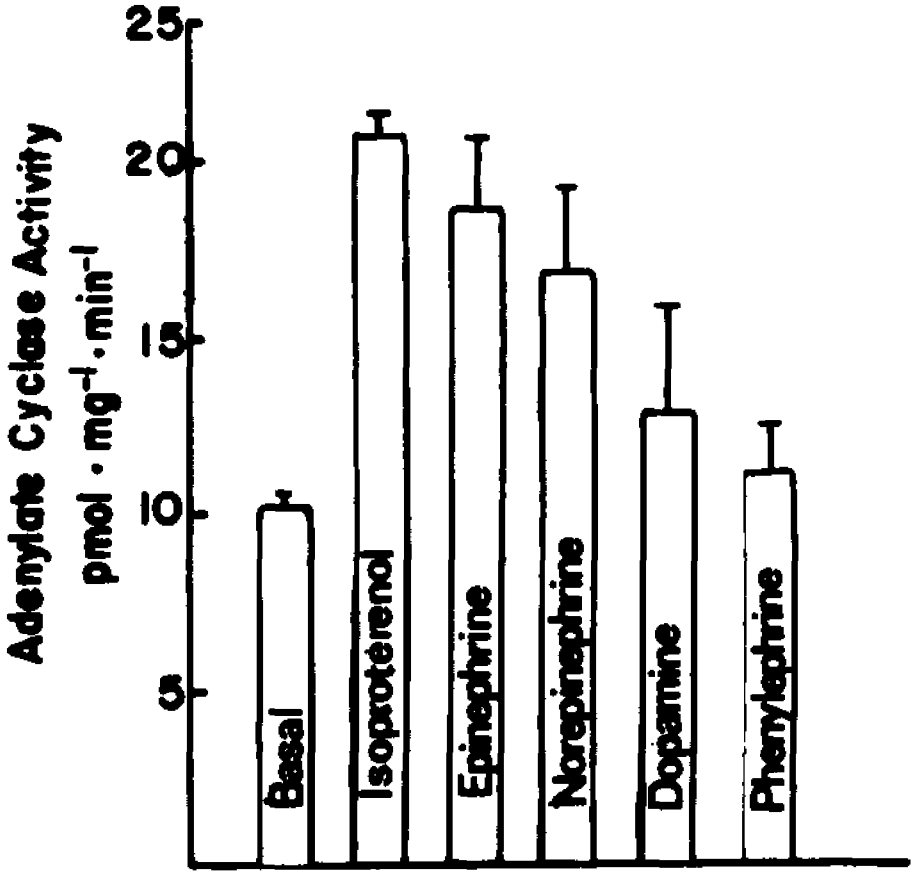

Figure 2. Comparison of effects of adrenergic agonists on sarcolemmal adenylate cyclase activity. Adenylate cyclase activity of guinea pig sarcolemmal membranes was determined in the presence and absence of 10^{-4} M concentrations of indicated agonists. Bars represent the mean ± SE of three replicates. Reactions were carried out at 25°C for 10 min in a medium containing 2 mM [α^{32}P] ATP 5 mM $MgCl_2$, 0.5 mM $CaCl_2$ and 50 mM HEPES buffer at pH 7.5.

TABLE 3

Comparison of the Effects of Adrenergic Agonists on Sarcolemmal Adenylate Cyclase Activity

	Expt. 1 n = 6	Expt. 2 n = 5	Expt. 3 n = 6	F
BASAL	10.35 ± 0.35	17.89 ± 0.93	21.85 ± 1.27	
ISO	20.68 ± 0.99	36.32 ± 3.37	51.68 ± 1.68	16.7, P <0.001
EPI	18.78 ± 2.15	24.37 ± 1.84	34.3 ± 1.35	44.86, P <0.001
NE	17.89 ± 2.54	28.62 ± 1.17	31.55 ± 1.74	36.49, P <0.001
DOPA	12.48 ± 0.24	20.14 ± 1.80	27.47 ± 2.48	5.68, P <0.05
PHENYL	11.36 ± 1.39	13.91 ± 0.96	21.82 ± 1.49	1.19 ns

The results are the means ± SE of the indicated number of determinations. All values are expressed in terms of pmol cyclic AMP formed/min/mg sarcolemmal protein. The F values for the effect of each drug when compared to basal enzyme activity were derived from analysis of variance for the combined data of the three experiments. Assays were performed under the conditions listed in Fig. 2. All drugs, when present, were at 10^{-4} M final concentration. ISO = isoproterenol, EPI = epinephrine, NE = norepinephrine, DOPA = dopamine, PHENYL = phenylephrine, ns = not significant.

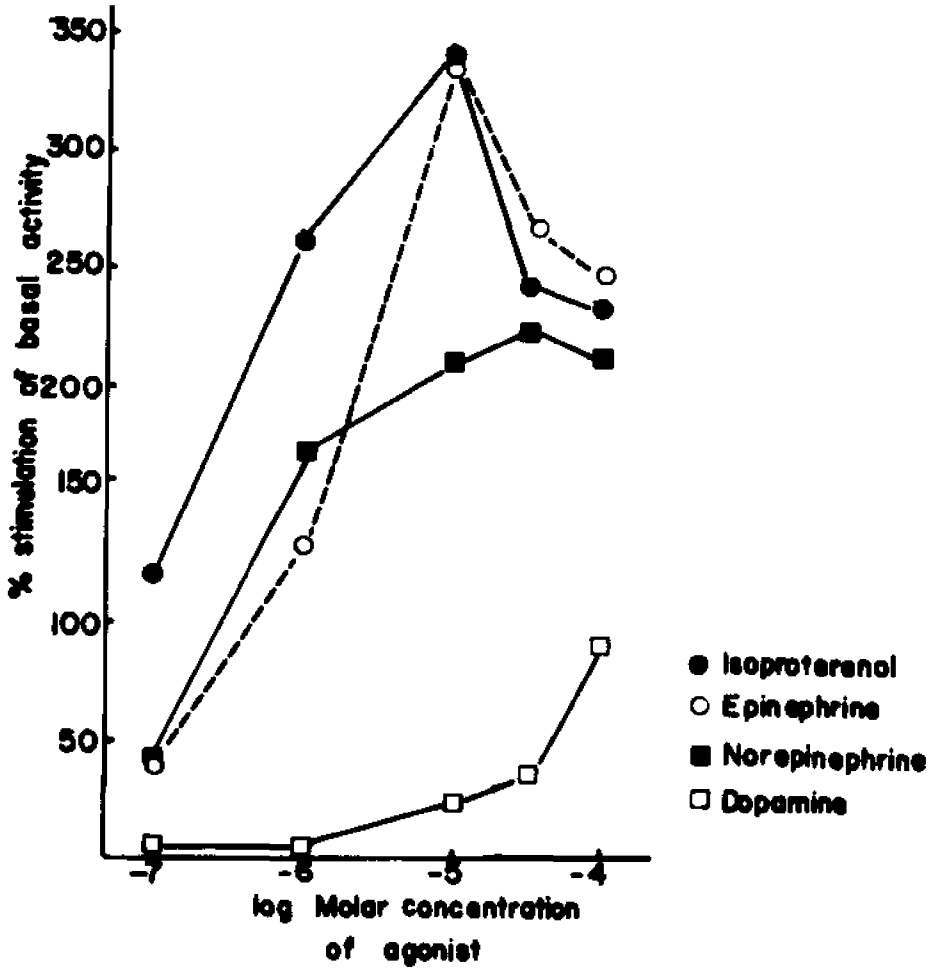

Figure 3. Dose response relationship of adrenergic agonists and sarcolemmal adenylate cyclase activity. Each point represents the mean of two replicate determinations. Assays were performed under the conditions listed in the legend to Figure 2 except that the pH was 6.8.

ephrine was minimal and so is not included in Figure 3. The order of affinity determined for the adrenergic receptors in this tissue (Table 4) is consistent with that of a typical beta receptor.

Partial Characterization of Sarcoplasmic Reticulum Adenylate Cyclase

The affects of beta agonists on sarcoplasmic reticulum adenylate cyclase were studied by the use of a microsomal fraction of the guinea pig ventricles as a source of enzyme. Microsomes were prepared as described under "Methods" and were assayed for calcium transport activity as a marker for the presence of sarcoplasmic reticulum. Significant amounts of calcium were accumulated in the presence of 5 mM potassium oxalate and the rate of calcium accumulation was greatly enhanced by preincubation of the microsomal vesicles with protein kinase and cyclic AMP (Figure 4). The high calcium transport activity indicates that these membrane preparations contain vesicles derived from the sarcoplasmic reticulum.

Comparison of the ability of beta agonists at levels of 10^{-4} M to stimulate the microsomal adenylate cyclase is shown in Figure 5. As was seen previously with the sarcolemmal enzyme, isoproterenol was most effective in stimulating microsomal adenylate cyclase activity followed by epinephrine and norepinephrine. Neither phenylephrine nor

TABLE 4

Apparent Dissociation Constants of Adrenergic Agonists for Sarcolemmal Beta Receptors

Isoproterenol	2×10^{-7} M
Norepinephrine	3×10^{-7} M
Epinephrine	6×10^{-7} M
Dopamine	$> 3 \times 10^{-5}$ M

Values shown are means derived from three dose response experiments of the type represented by Figure 3.

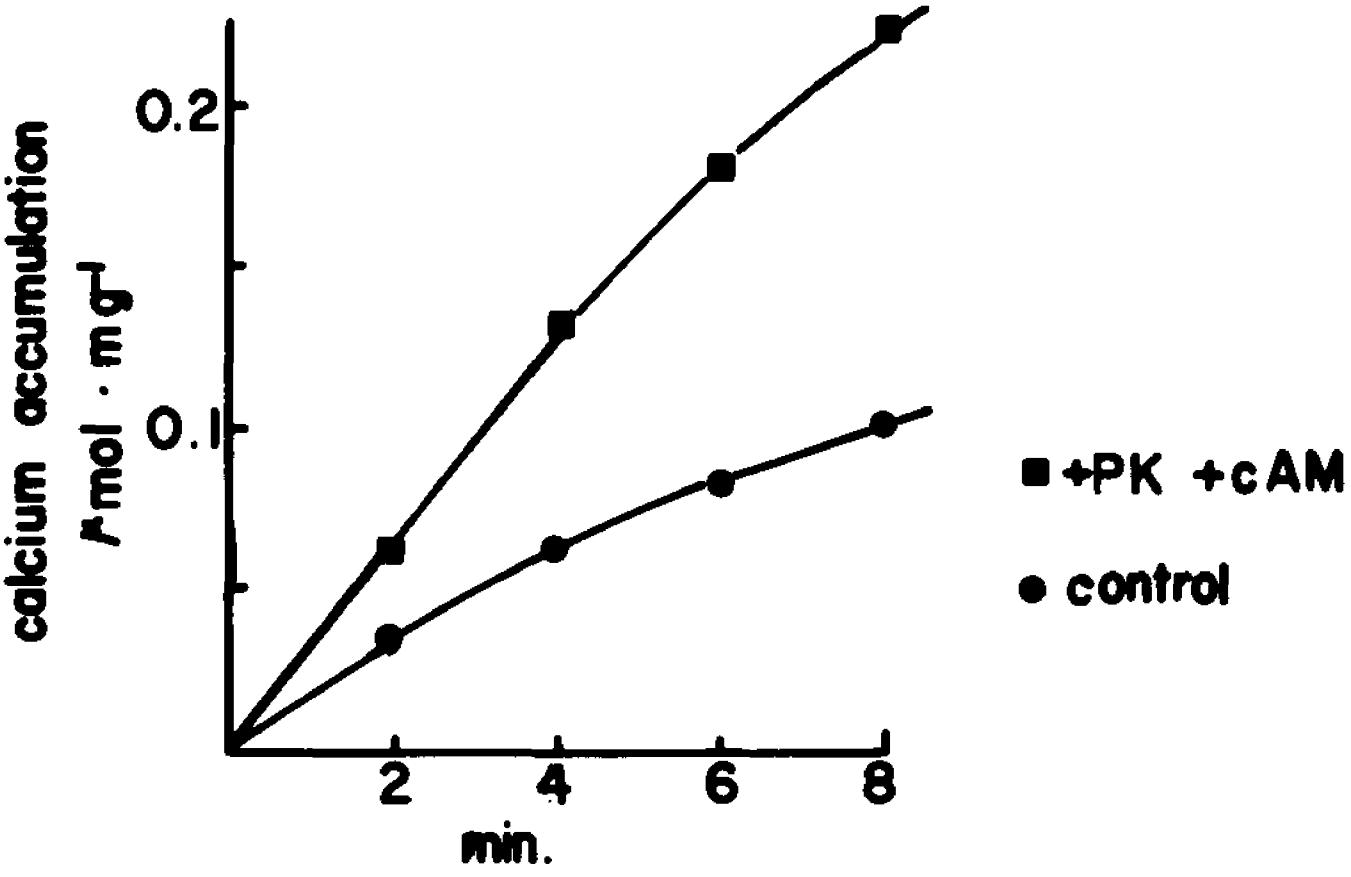

Figure 4. Calcium transport activity of guinea pig cardiac sarcoplasmic reticulum. Calcium transport was assayed as decribed in "Methods" in the presence and absence of 0.2 mg/ml protein kinase and 1 μM cyclic AMP.

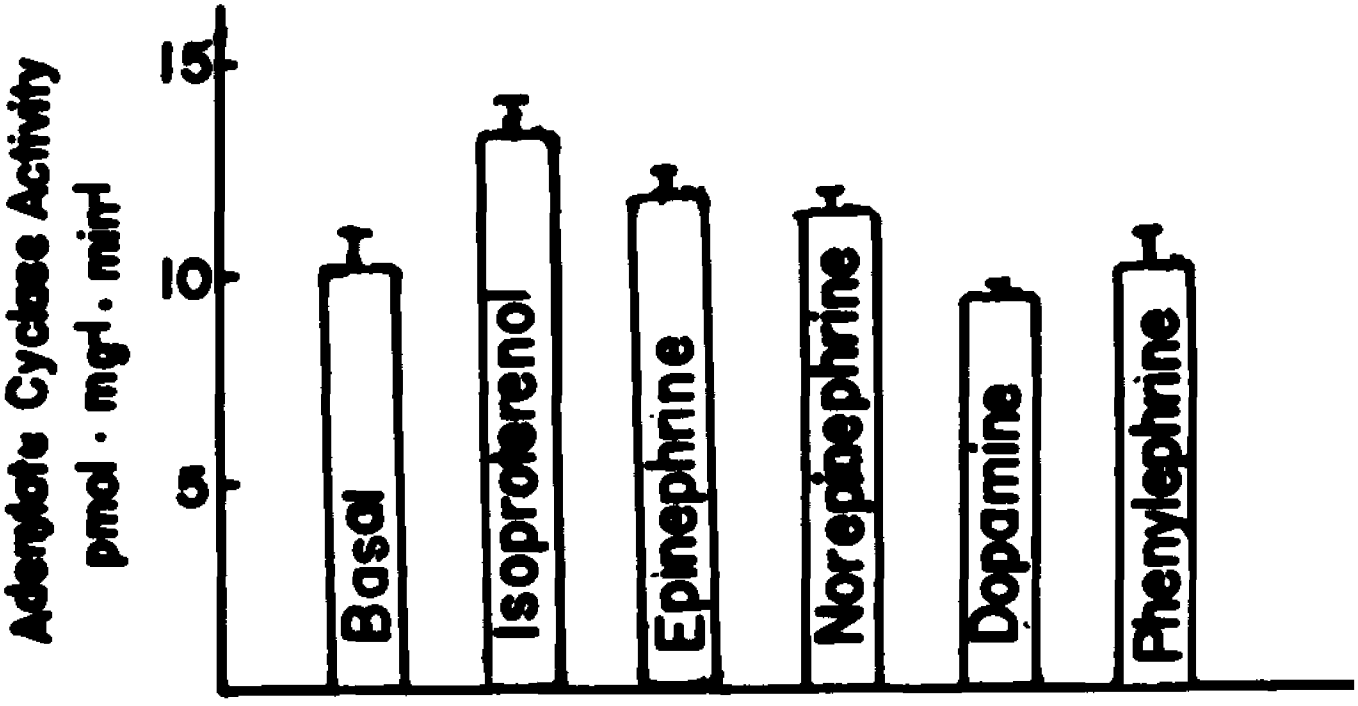

Figure 5. Comparison of effects of adrenergic agonists on sarcoplasmic reticulum adenylate cyclase activity. Adenylate cyclase was measured in the presence and absence of 10^{-4} M concentrations of the indicated adrenergic agonists. Bars represent the mean ± SE of four replicates. The assays were performed under the conditions described in the legend to Figure 2.

dopamine significantly affected microsomal adenylate cyclase activity. Attempts to find conditions that would increase the extent of stimulation by these beta adrenergic agonists and allow more accurate dose-response studies to be performed were not successful.

The pH dependency of the microsomal adenylate cyclase, shown in Figure 6, was determined in the presence of Tris-HCl. Microsomal adenylate cyclase activity was maximal at pH 7.5 in the presence or absence of 10^{-5} M isoproterenol or 5 mM NaF contrasting with the pH dependency of the sarcolemmal adenylate cyclase (Figure 11). The adenylate cyclase contained in the sarcoplasmic reticulum preparations differed from the sarcolemmal enzyme in other ways as well. Increasing the ionic strength of the reaction medium with either KCl or NaCl was found to increase sarcolemmal adenylate cyclase activity (see Figure 12). Similar concentrations of these ions slightly inhibited microsomal adenylate cyclase activity (Table 5).

GTP at a concentration of 10^{-5} M did not significantly affect adenylate cyclase activity in sarcoplasmic reticulum preparations (Figure 7). In other experiments the concentration of GTP was varied from 10^{-5} to 10^{-4} M (Figure 8). The effect of GTP on both basal and NaF stimulated activity was not significant at any concentration of the nucleotide.

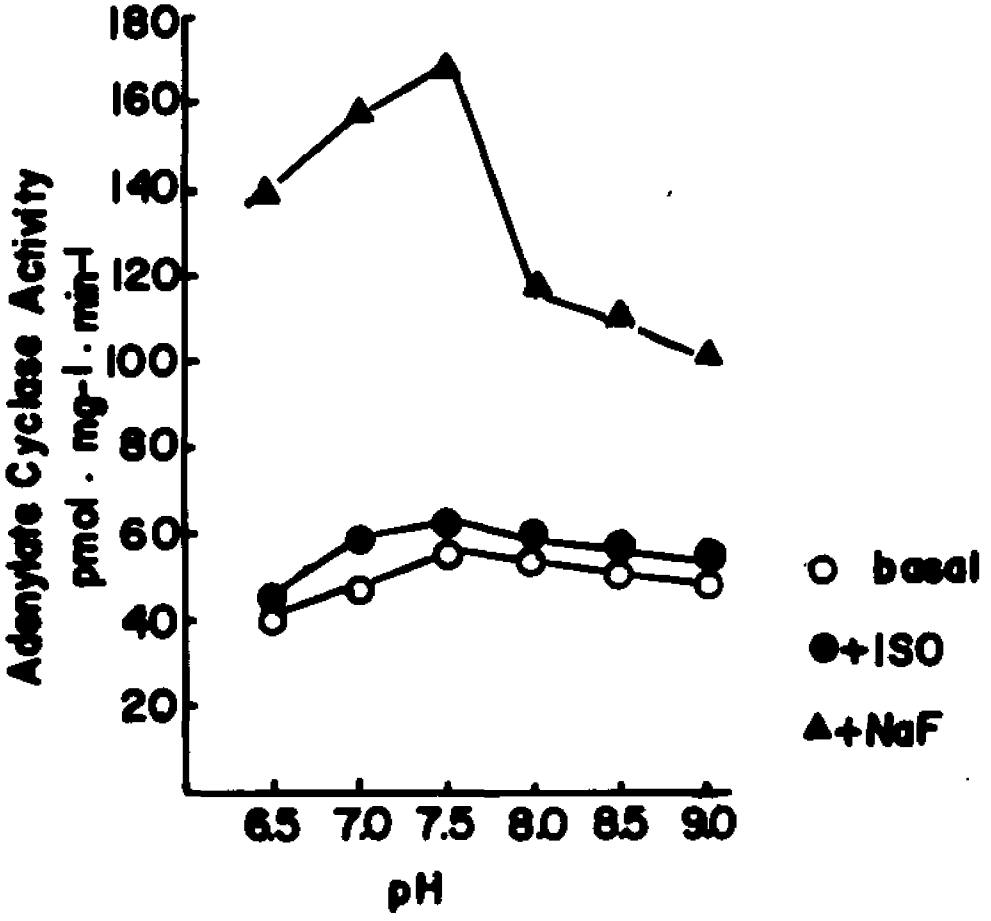

Figure 6. pH dependence of sarcoplasmic reticulum adenylate cyclase activity. Each point represents the mean of three replicate determinations. Reactions were carried out in 50 mM Tris buffer under the standard conditions listed in the "Methods".

TABLE 5

Effects of Increasing Ionic Strength on Sarcoplasmic Reticulum Adenylate Cyclase Activity.

	Basal	+ 10^{-5} M Isoproterenol	+ 8 mM NaF
control	97.96 ± 0.56	97.26 ± 0.97	258.30 ± 33.00
+ 50 mM KCl	92.20 ± 7.15	110.85 ± 9.60	260.18 ± 8.43
+ 100 mM KCl	93.63 ± 7.72	101.75 ± 2.71	226.00 ± 23.58
+ 50 mM NaCl	80.45 ± 1.80*	78.00 ± 7.03	258.08 ± 7.86
+ 100 mM NaCl	88.15 ± 0.88*	88.60 ± 6.13	227.84 ± 14.70

Values are expressed as pmol cyclic AMP formed mg^{-1} min^{-1} and are the mean ± SE of three assays of a single preparation. Assays were performed under standard conditions in the presence or absence of added salts.

* $p < 0.05$ when compared to controls.

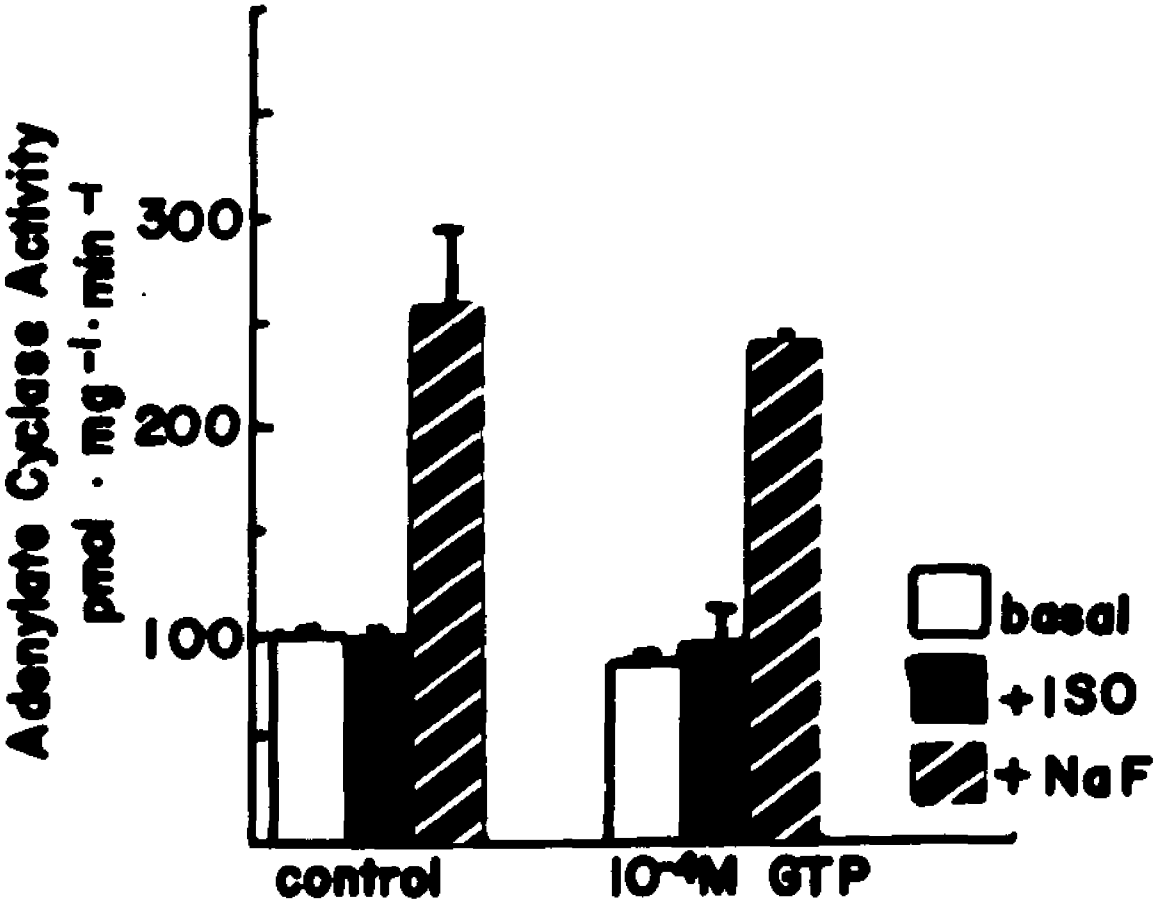

Figure 7. Effect of GTP on sarcoplasmic reticulum adenylate cyclase activity. Adenylate cyclase activity was assayed under standard conditions in the presence and absence of 10^{-5} M isoproterenol of 8 mM NaF. Bars indicate the mean ± SE of three replicates.

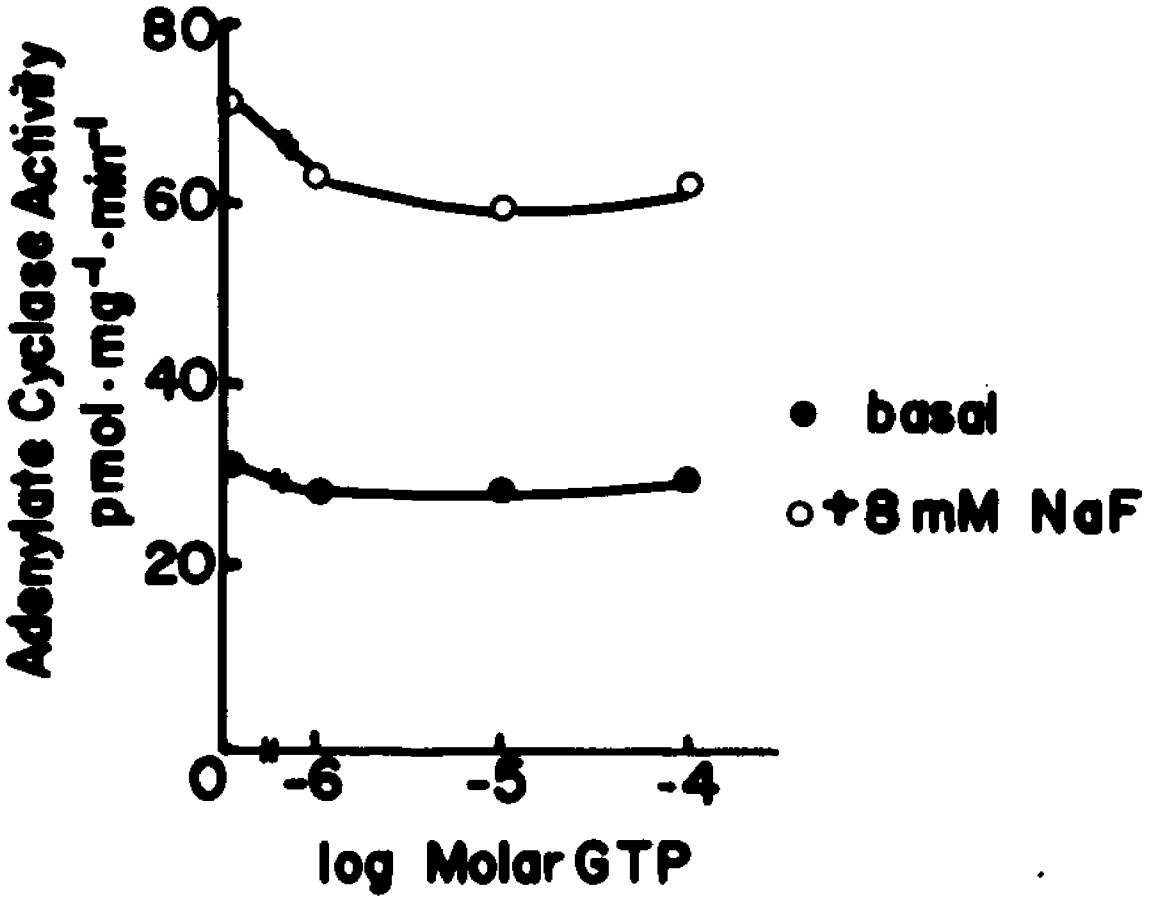

Figure 8. Concentration dependence of the GTP effect on sarcoplasmic reticulum adenylate cyclase activity. Assays were performed under standard conditions in the presence and absence of 8 mM NaF and the indicated concentrations of GTP. Each point represents the mean of three replicate determinations.

Characterization of Sarcolemmal Adenylate Cyclase

The standard conditions listed under "Methods" (pH 7.5, 37°C) were adopted for this series of experiments. The concentration of ATP in the reaction medium was reduced to 0.2 mM with a ten-fold increase in the specific activity of the substrate. An ATP regenerating system was included to offset the increased ATPase activity observed at 37°C. Figure 9 shows the time course of the adenylate cyclase reaction under these standard conditions. The rates of basal and NaF-stimulated enzyme activity remained linear for at least 8 minutes. A reaction time of 5 minutes was used in all subsequent experiments.

A more direct test of the adequacy of the regenerating system was provided by concomitant measurements of the amount of ATP remaining in the assay mixture as the reaction progressed. As shown in Table 6, where the amount of ATP remaining is expressed in terms of percentage of ATP at the start of the assay, nearly 90% of the ATP remained after 5 minutes. For this reason the regenerating

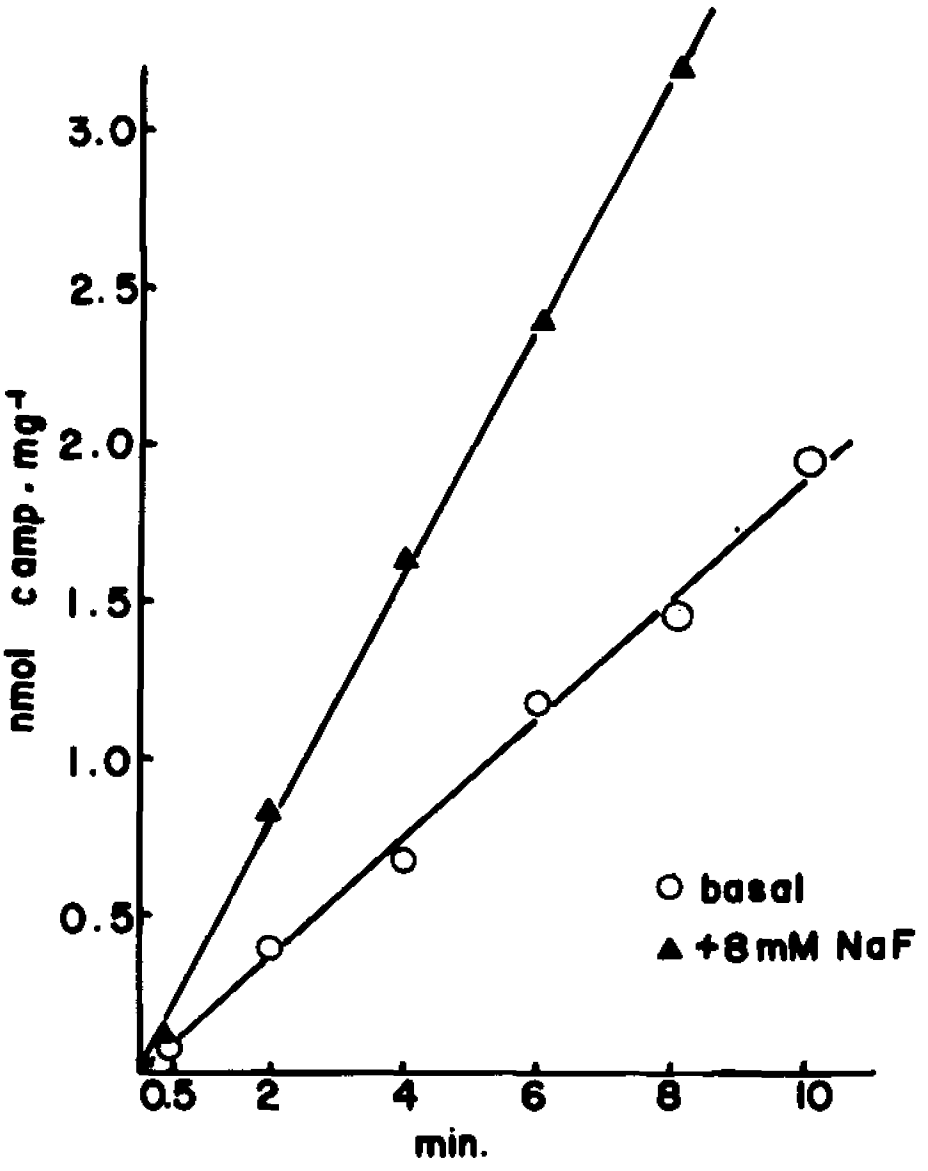

Figure 9. Linearity of the adenylate cyclase reaction with time. Assays were performed under standard conditions in the presence and absence of 8 mM NaF. Sarcolemmal protein concentration was 1 mg/ml. Each point represents the mean of three replicate determinations.

TABLE 6

Change in Substrate Level During the Adenylate Cyclase Reaction: Effect of [illegible].

Time (min.)	[illegible]	+ [illegible]
0.5	97.08	97.45
2.0	94.67	95.10
4.0	89.11	91.39
7.0	87.09	90.61
8.0	84.64	88.99

The results are expressed as percentage of radioactivity remaining as ATP as compared to control tubes not containing pericolonial membranes. Control values remained constant throughout the 8 minute reaction.

system was considered adequate and was not modified further. The level of ATP remaining in the tubes containing NaF was higher than that of controls. This effect of NaF was seen consistently and can be accounted for by the inhibitory action of F^- on the membrane-bound ATPases (Table 7).

The effect of increasing concentrations of NaF on the adenylate cyclase activity of the sarcolemma is shown in Figure 10. The response to NaF was maximal at 5 mM while higher concentrations were inhibitory.

NaF was found to modify the pH dependency of the adenylate cyclase in addition to stimulating the rate of cyclic AMP formation by this enzyme. The effects of changing pH on adenylate cyclase activity in the presence or absence of 5 mM NaF or 10^{-5} isoproterenol are shown in Figure 11. Both basal and isoproterenol-stimulated activities were maximal at pH 8.0. NaF (5 mM) shifted the pH optimum to 7.5. Stimulation of cyclase activity by isoproterenol was minimal at both extremes of the pH range with a maximal effect seen at pH 7.0-8.0 .

Increasing ionic strength produced small but significant increases in basal and isoproterenol-stimulated adenylate cyclase activity (Figure 12). NaCl and KCl were equally effective in stimulating adenylate cyclase activity. The percent stimulation of cyclase activity by isoproterenol was independent of ionic strength.

TABLE 7

Effect of NaF on Sarcolemmal ATPase Activity.

Preparation	Addition	ATPase activity: nmol P_i released/mg hr^{-1}
1, n = 3	none	10.76 ± .75
	+ 50 mM NaF	6.02 ± .49
2, n = 3	none	11.31 ± .95
	+ 50 mM NaF	5.68 ± .73

Assays were performed as described in the Methods. The data shown are the means ± SE of the indicated number of replicates for each preparation.

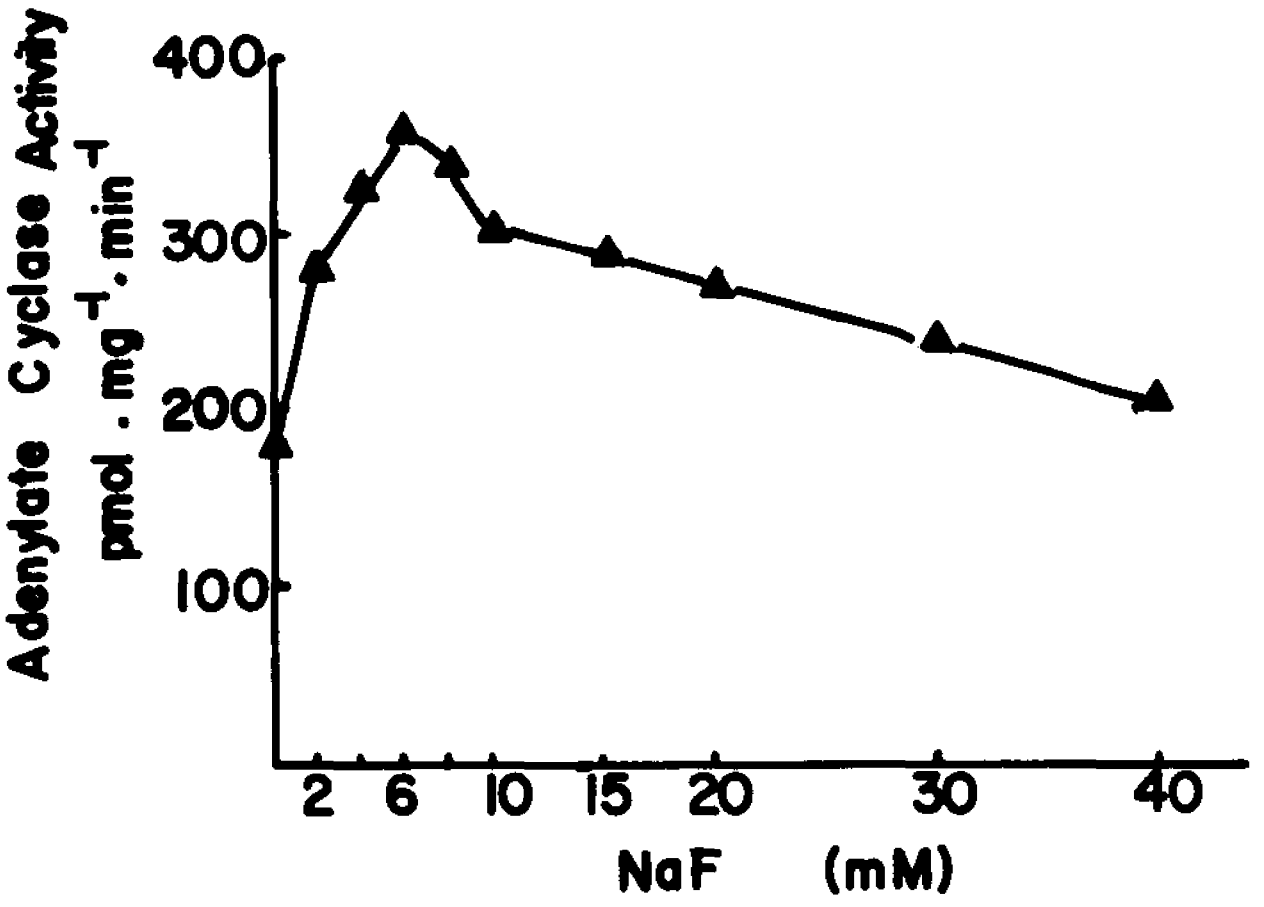

Figure 10. NaF dependency of sarcolemmal adenylate cyclase. Assays were performed under standard conditions in the presence of indicated concentrations of NaF. Each point represents the mean of three replicates.

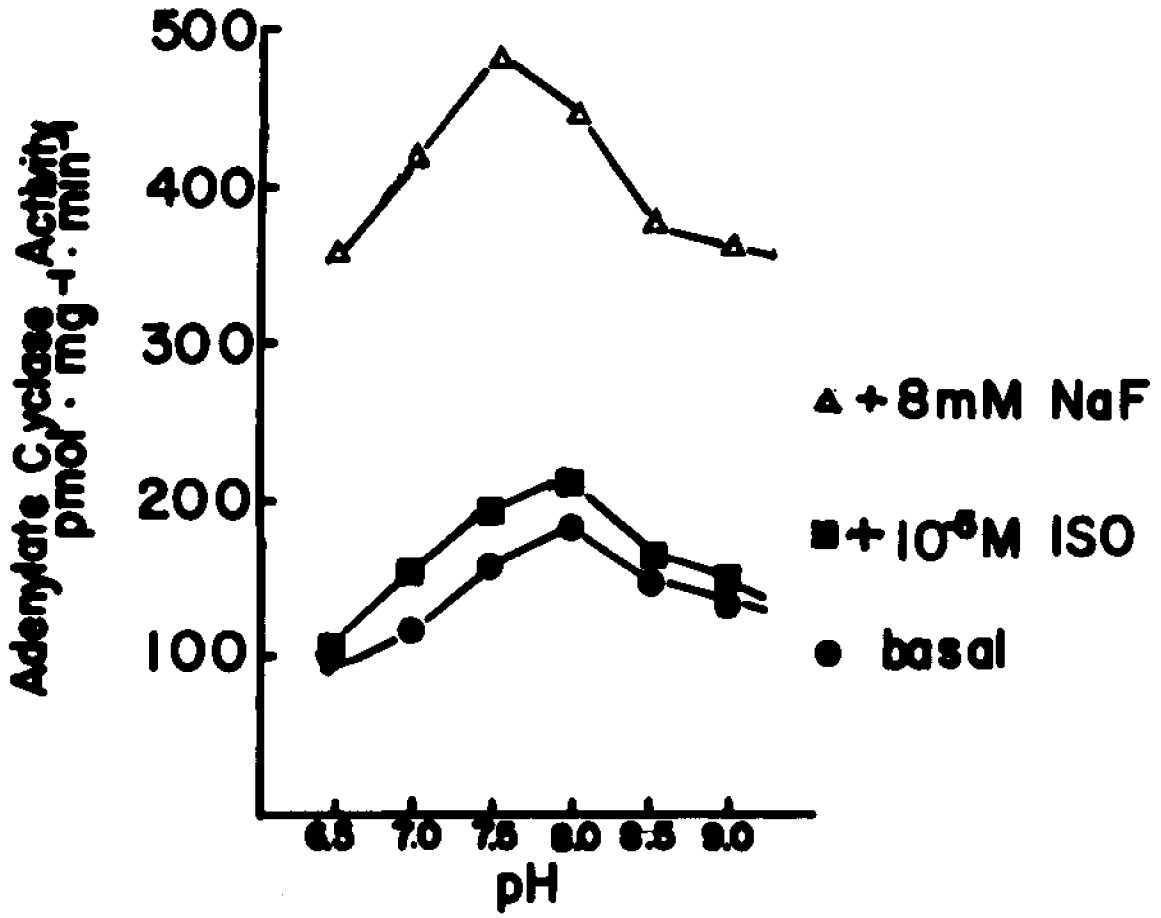

Figure 11. pH dependence of sarcolemmal adenylate cyclase. Reactions were carried out in 50 mM Tris buffer under standard conditions. Each point represents the mean of three replicate determinations.

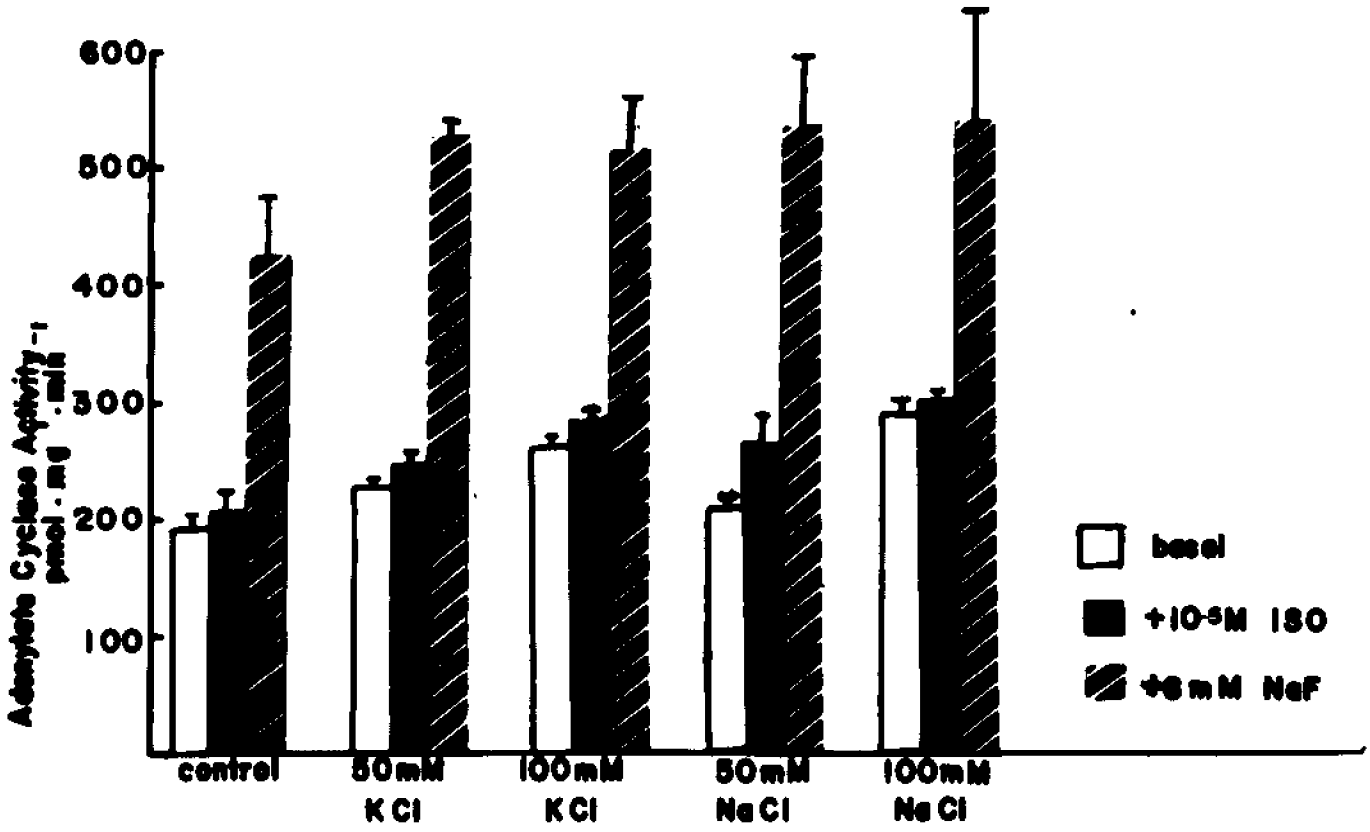

Figure 12. Effect of increased ionic strength on sarcolemmal adenylate cyclase activity. Adenylate cyclase activity was assayed under standard conditions in the presence and absence of 10^{-5} M isoproterenol or 8 mM NaF. Bars represent the mean ± SE of three replicate determinations.

Sarcolemmal adenylate cyclase was inhibited by GTP under all conditions tested (Figure 13). Basal activity appeared to be somewhat more sensitive to GTP than isoproterenol-stimulated activity, resulting in an increase in the response of the enzyme to catecholamines.

Because the more usual response to GTP is an elevation of hormone-stimulated adenylate cyclase activity (see introduction), the possibility of trace contaminants in the assay reagents being the cause of an anomalous response to GTP was examined by testing the effect of the nucleotide on a different enzyme preparation. Plasma membranes of the mucosal epithelia of the urinary bladder of the turtle Pseudemis scripta were prepared as described in the Methods and assayed for adenylate cyclase activity using aliquots of the same reagents used for the previous experiments. In the absence of GTP, 10^{-4} M norepinephrine only slightly increased adenylate cyclase activity. The increment due to isoproterenol was greatly increased in the presence of 10^{-5} M GTP (Table 8). It was therefore considered unlikely that the inhibition of sarcolemmal adenylate cyclase activity was due to the presence of contaminants in the assay system and the reaction conditions were left unmodified.

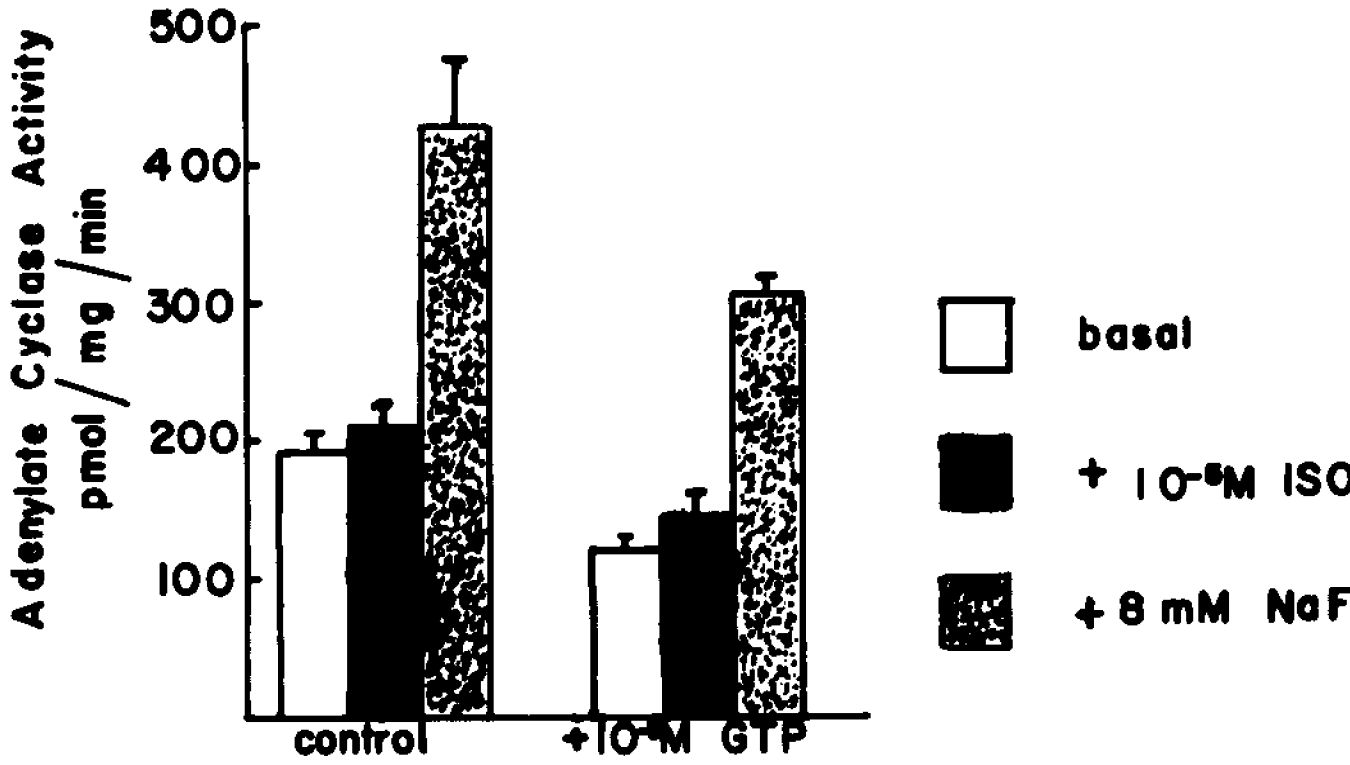

Figure 13. Effect of GTP on sarcolemmal adenylate cyclase activity. Adenylate cyclase activity was assayed under standard conditions in the presence or absence of 10^{-5} M isoproterenol or 8 mM NaF. Bars represent the mean ± SE of three replicate determinations.

TABLE 8

Effects of GTP on the Stimulation of Turtle Bladder Adenylate Cyclase by Norepinephrine (NE)

Expt.	Addition	Adenylate Cyclase Activity pmol cyclic AMP mg^{-1} min^{-1}
1.	none	30.25 ± .40
	10^{-4} M NE	38.93 ± .73*
	10^{-4} M NE + 10^{-5} M GTP	46.05 ± 1.05†
	10 mM NaF	95.49 ± 9.96
2.	none	29.77 ± 4.17
	10^{-4} M NE	37.57 ± 4.69§
	10^{-4} M NE + 10^{-5} M GTP	70.69 ± 2.83††
	10 mM NaF	104.84 ± 6.87

The results are expressed as the mean ± SE of three determinations.

*t = 14.91, P <.001 when compared to control.
†t = 5.18, P <.02 when compared to 10^{-4} M NE.
§t = 1.24, ns when compared to control.
††t = 6.04, P <.01 when compared to 10^{-4} M NE.

An attempt was made to characterize the nature of the inhibition of adenylate cyclase activity produced by GTP. Since the structure of GTP and ATP are similar, it was initially assumed that GTP was acting as a competitive inhibitor of the cyclase. In order to test this hypothesis the following experiment was performed. Adenylate cyclase activity was measured at either 0.2 or 2.0 mM ATP in the presence of varying concentrations of GTP. When the data were plotted on a Dixon plot ((I) vs. 1/v), which is designed to yield K_i for a pure competitive inhibitor, the resulting nonlinearity of the curves indicated partial or mixed inhibition (Figure 14). These results suggest, therefore, that the GTP binding site is independent of the catalytic site of the adenylate cyclase molecule.

Before the GTP inhibition was more closely examined, a clearer understanding of the substrate dependency of the adenylate cyclase was sought. The velocities of basal, isoproterenol-stimulated and NaF-stimulated activity were determined at ATP concentrations ranging from 0.05 to 2.0 mM. The concentration of $MgCl_2$ was held constant at 10 mM throughout the entire ATP concentration range. The results are shown in Figure 15 where the velocity of the reaction is plotted as a function of ATP concentration. With the exception of slight inhibition seen at 2 mM ATP, the enzyme activity increased with increasing substrate concentration.

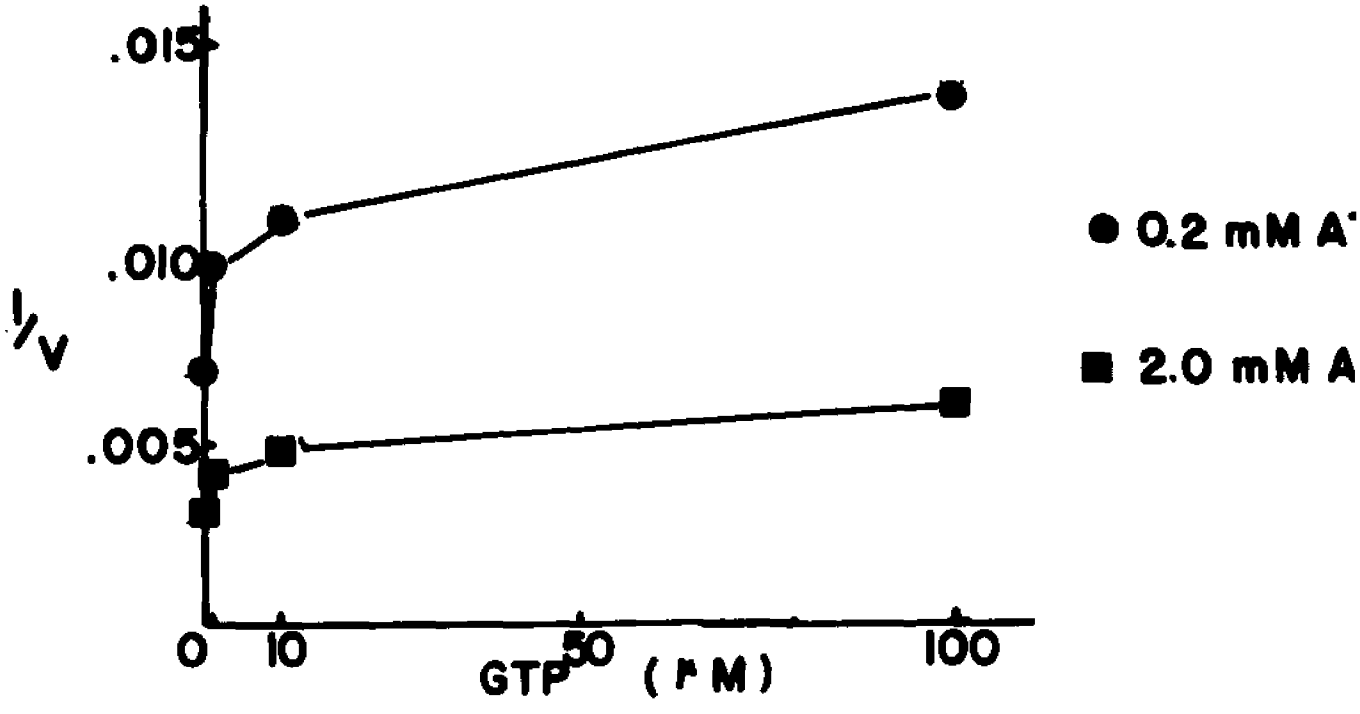

Figure 14. Dixon plot of the effect of GTP on sarcolemmal adenylate cyclase activity. Adenylate cyclase activity was assayed under standard conditions in the presence of either 0.2 or 2.0 mM ATP and the indicated concentrations of GTP. Each point represents the mean of three replicate determinations.

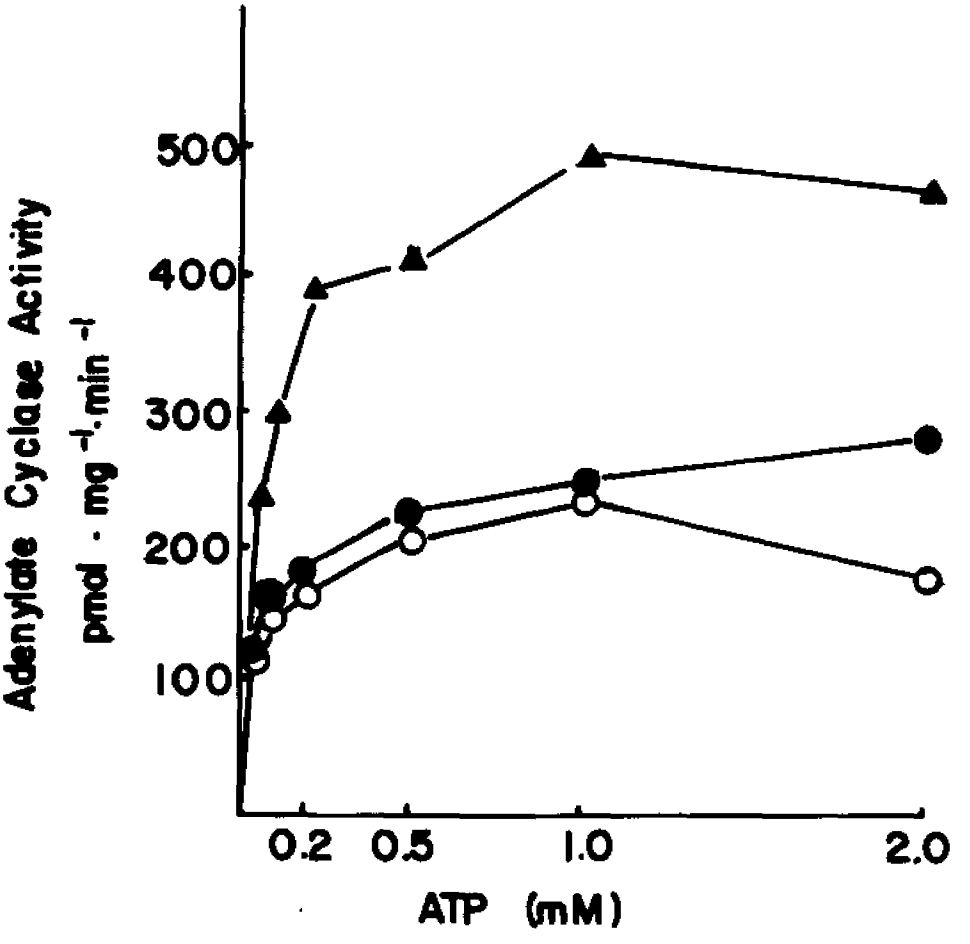

Figure 15. ATP dependency of sarcolemmal adenylate cyclase. Adenylate cyclase activity was assayed under standard conditions in the absence (O) and presence of 10^{-5} M isoproterenol (●) or 8 mM NaF (▲) and the indicated concentration of ATP.

When subjected to analysis by Lineweaver-Burk plots (Figure 16), however, the data deviated from classical Michaelis-Menten kinetics. Instead of a linear relationship between 1/v and 1/S, a nonlinear (concave downward) pattern was seen. The nonlinearity was not abolished by the addition of isoproterenol or NaF (Figure 16). Regression analysis of the data indicated that the deviations from linearity were statistically significant ($P < 0.01$). The inhibition seen at 2 mM ATP (Figure 15) is reflected in the Lineweaver-Burk plots as a small concave upward segment adjacent to the ordinate.

The nonlinearity of the Lineweaver-Burk plots can be explained by the presence of an allosteric binding site for ATP in addition to the catalytic site of the enzyme. Because Figure 14 suggests that GTP interacts at such a site, it was of interest to test the effect of that nucleotide on the ATP dependency of the cardiac adenylate cyclase. As shown in Figure 16, GTP linearized the double reciprocal plots, indicating that this nucleotide can occupy one of the ATP binding sites. GTP was inhibitory throughout the range of ATP concentrations used in this study, but as the concentration of ATP was raised the extent of inhibition by GTP was greatly reduced. This finding provides evidence that GTP and ATP compete for a regulatory site.

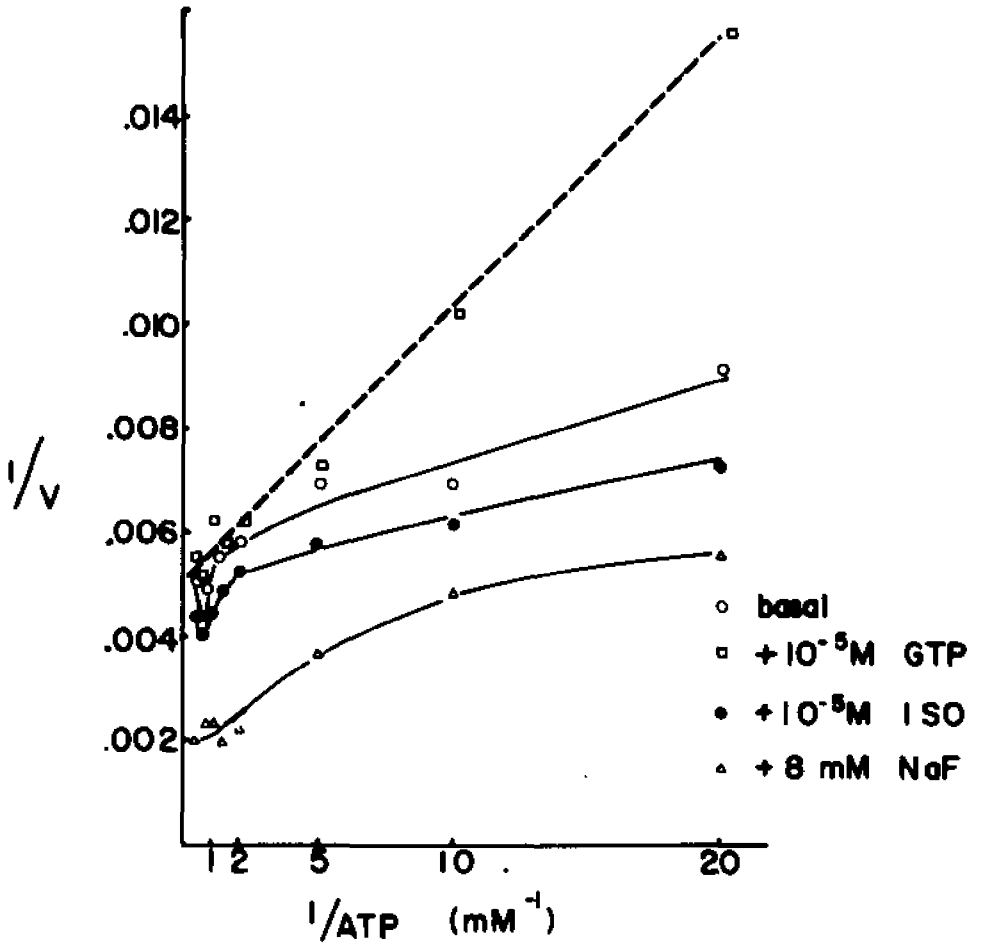

Figure 16. Effect of GTP on the ATP dependence of sarcolemmal adenylate cyclase. Lineweaver-Burk replot of adenylate cyclase activity measured under standard conditions in the presence and absence of 10^{-5} M isoproterenol, 8 mM NaF or 10^{-5} M GTP. Each point represents the mean of three replicate determinations.

The linear double reciprocal plots seen in the presence of 10^{-5} M GTP allowed a K_m value for ATP of 0.1 mM and a value of 200 nmol·mg^{-1}·min^{-1} for V_{max} to be calculated.

Characterization of the GTP Effect on Sarcolemmal Adenylate Cyclase

If GTP exerts its effect at an allosteric regulatory site, then a change in the conformation of the enzyme system should occur subsequent to nucleotide binding at the regulatory site. If the rate of this change is slow, the extent of inhibition measured by a single 5 minute time point would underestimate the effect of the nucleotide at the time that the new steady state is achieved. Alternatively, it is possible that after an initial inhibition the final steady state velocity achieved is the same or even higher than the basal reaction rate. To examine these possibilities the effect of GTP on the time course of cyclic AMP production was therefore determined.

The extent of inhibition of cyclase activity by 10^{-5} M GTP remained constant throughout the course of the reaction both in the presence and absence of isoproterenol (10^{-5} M). Reaction rates under all conditions remained linear for at least 8 minutes. The percent inhibition produced by GTP in the presence of isoproterenol (9%) was much less than the inhibition of basal activity (26%), so that the effect of isoproterenol was considerably increased when GTP was

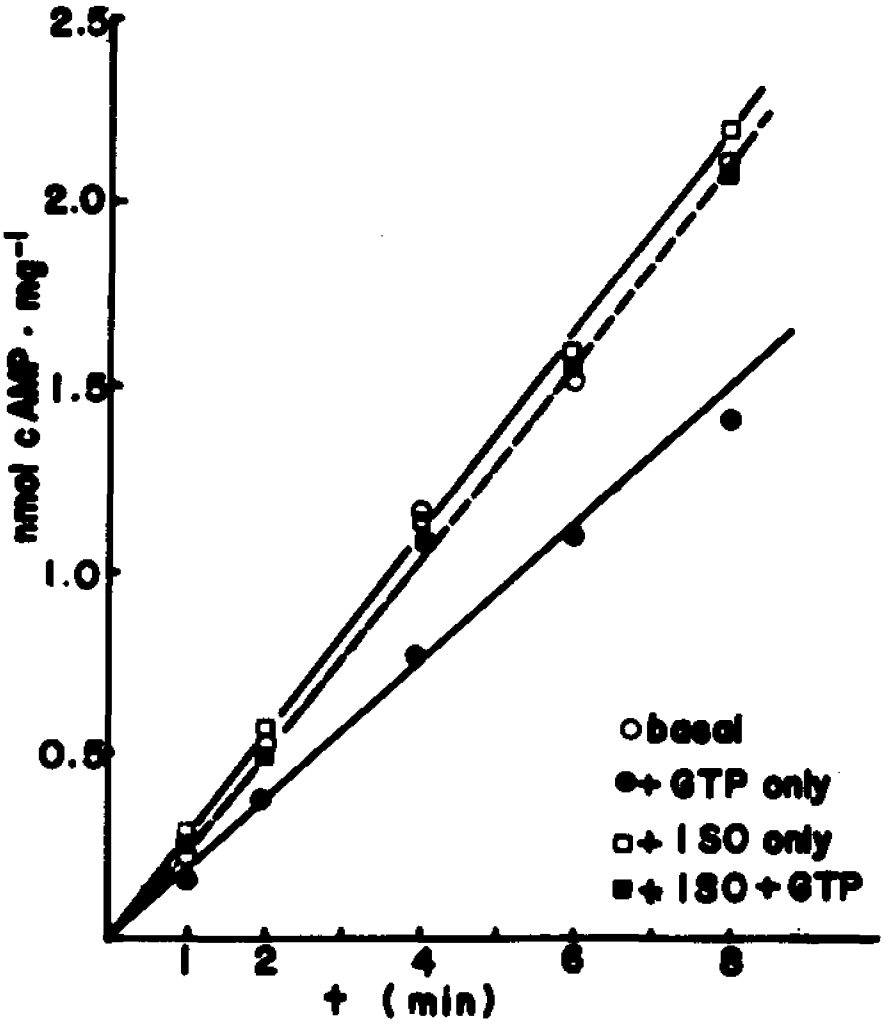

Figure 17. Time course of the effect of GTP on sarcolemmal adenylate cyclase activity. Assays were performed under standard conditions in the presence and absence of 10^{-5} M isoproterenol, 10^{-5} M GTP or both 10^{-5} M isoproterenol and 10^{-5} M GTP. Each point represents the mean of three determinations. The dashed line represents cyclic AMP production under both basal and "+ISO + GTP" conditions.

present. Because the reaction rates of the basal and isoproterenol + GTP treated enzymes were nearly identical only a single dashed line is drawn in Figure 17 to represents cyclic AMP production under both conditions.

The absence of a lag in cyclic AMP production in the presence of GTP suggested that either GTP itself is inhibitory and that hydrolysis to GDP is not necessary for the inhibition to be observed or that FTP is hydrolyzed very rapidly. Support for the former explanation was derived from a survey of several nucleoside triphosphates for their effects on myocardial adenylate cyclase activity (Figure 18). Of special interest, in this respect, is the effect of the FTP analog 5' guanylyl imidodiphosphate (GMP-PNP). Although GMP-PNP is resistant to membrane bound phosphohydrolases (149), it is a potent inhibitor of both basal and fluoride stimulated adenylate cyclase activity. Of the other nucleotides tested only the purines ITP and XTP were inhibitory, pyrimidine based compounds, i.e., CTP and UTP were without effect.

The large increase in isoproterenol-stimulated adenylate cyclase activity in the presence of GMP-PNP prompted testing of a series of guanyl nucleotides for their effects on cyclic AMP production (Figure 19). Uniform concentrations (10^{-5} M) of GDP, GTP, GMP-PNP and GPP-CH_2-P were added to reaction tubes in the presence or absence of isoproterenol (10^{-5} M) or NaF (8 mM). GDP was added only 5 sec. prior to

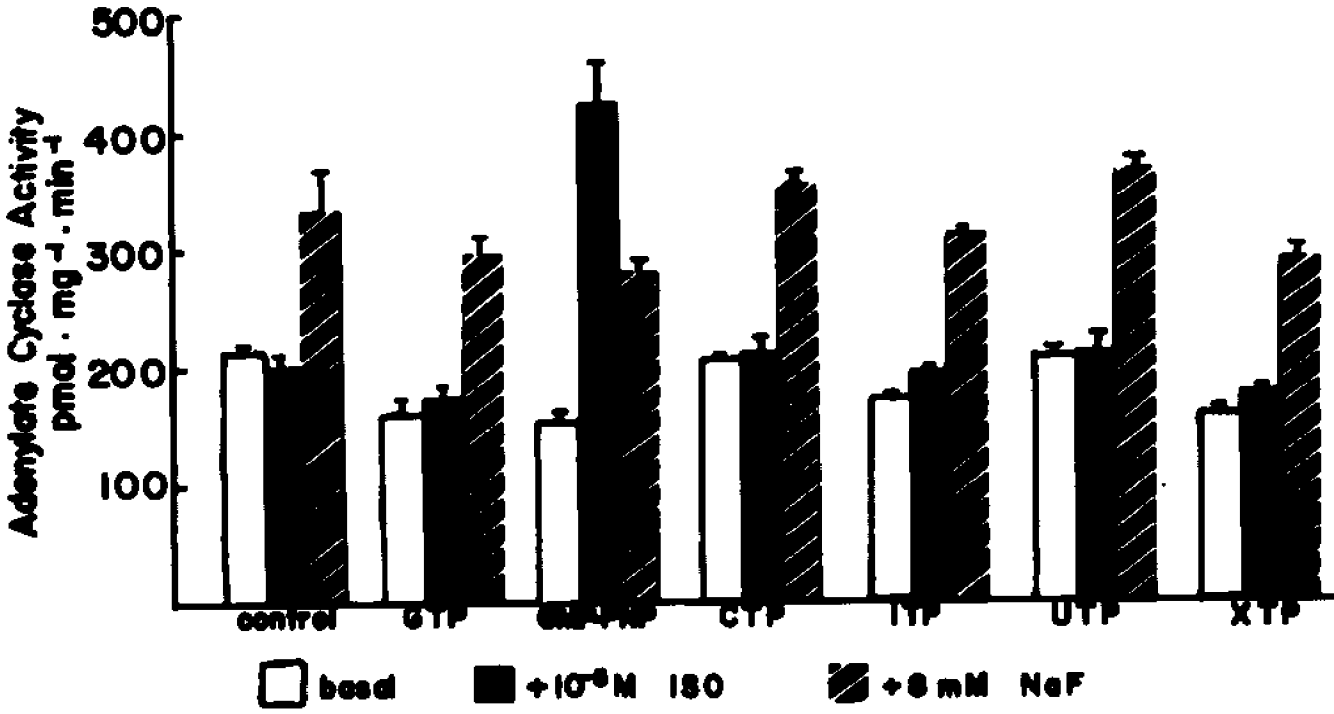

Figure 18. Survey of nucleoside triphophates for effect on sarcolemmal adenylate cyclase activity. Assays were performed under standard conditions in the presence and absence of 10^{-5} M isoproterenol or 8 mM NaF. The concentration of all nucleoside triphosphates was 10^{-4} M. Bars represent the mean $\pm$ SE of three replicates.

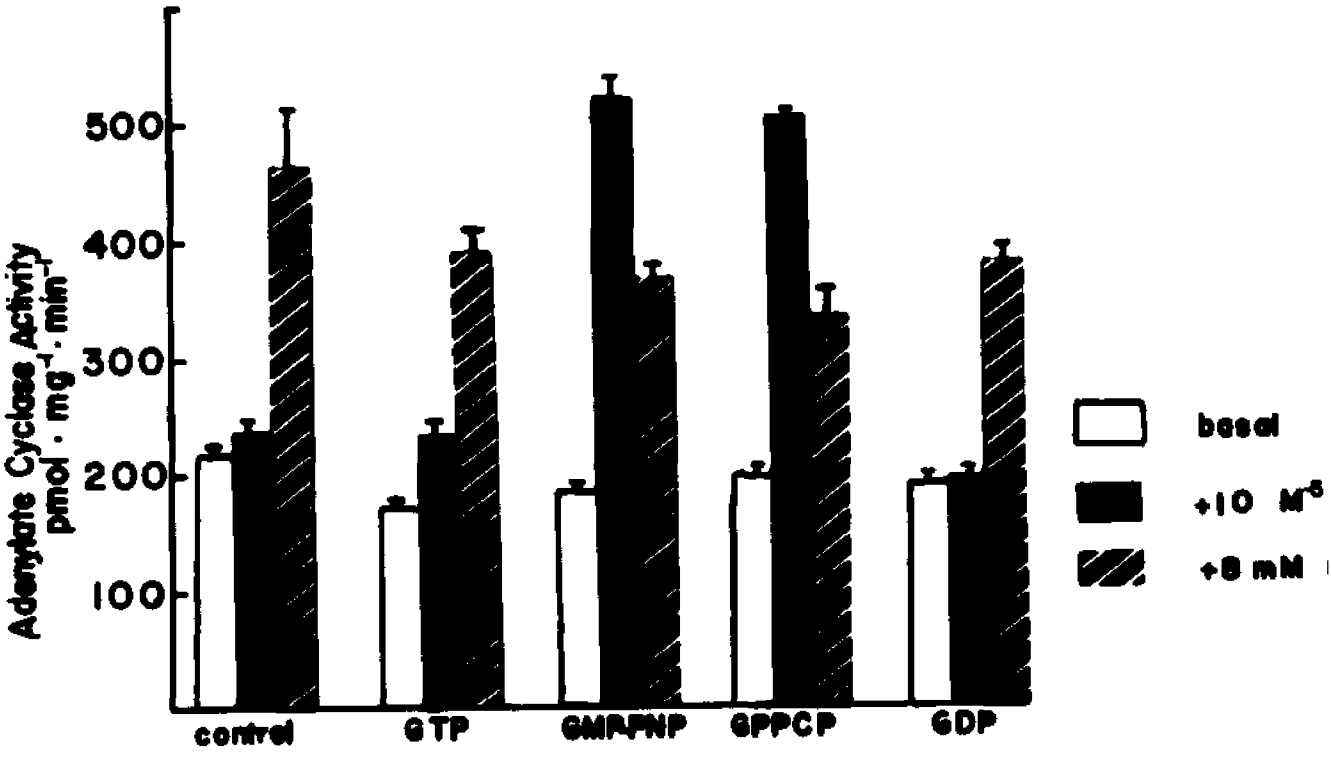

Figure 19. Survey of guanyl nucleotides for effect on sarcolemmal adenylate cyclase activity. Assays were performed under standard conditions in the presence and absence of 10^{-5} M isoproterenol or 8 mM NaF. The concentration of all guanyl nucleotides was 10^{-5} M. GDP was added 5 sec prior to the initiation of the reaction by the addition of sarcolemmal membranes. Bars represent the mean $\pm$ SE of three replicate determinations.

the start of the reaction with the addition of sarcolemmal membranes. Control activity and the activity in the presence of GTP corresponded to results obtained in previous experiments. GPP-CH_2-P, which like GMP-PNP is not susceptible to hydrolysis (150), inhibited both basal and NaF stimulated adenylate cyclase activity. The effect of isoproterenol, however, was greatly increased. The effect of GDP on basal and NaF stimulated activity was similar to that of GTP. The response to GDP did differ in that no enhancement of the effect of isoproterenol could be observed.

Figure 20 shows the time course of the effect of GDP on basal adenylate cyclase activity. Basal adenylate cyclase activity was linear throughout the 6 min. reaction period. Adenylate cyclase activity in the presence of GDP was essentially linear with the reaction rate being approximately 70% of control values.

The effects of guanyl nucleotides at a concentration of 10^{-5} M indicate that GTP can play a regulatory role at concentrations normally found <u>in vivo</u>. It was therefore of interest to examine the dose response relationship for the GTP effect on sarcolemmal adenylate cyclase. GTP was inhibitory of basal, isoproterenol-stimulated and NaF stimulated cyclase activity in doses above 3×10^{-7} M (Figure 21), half-maximal activity being obtained at ap-

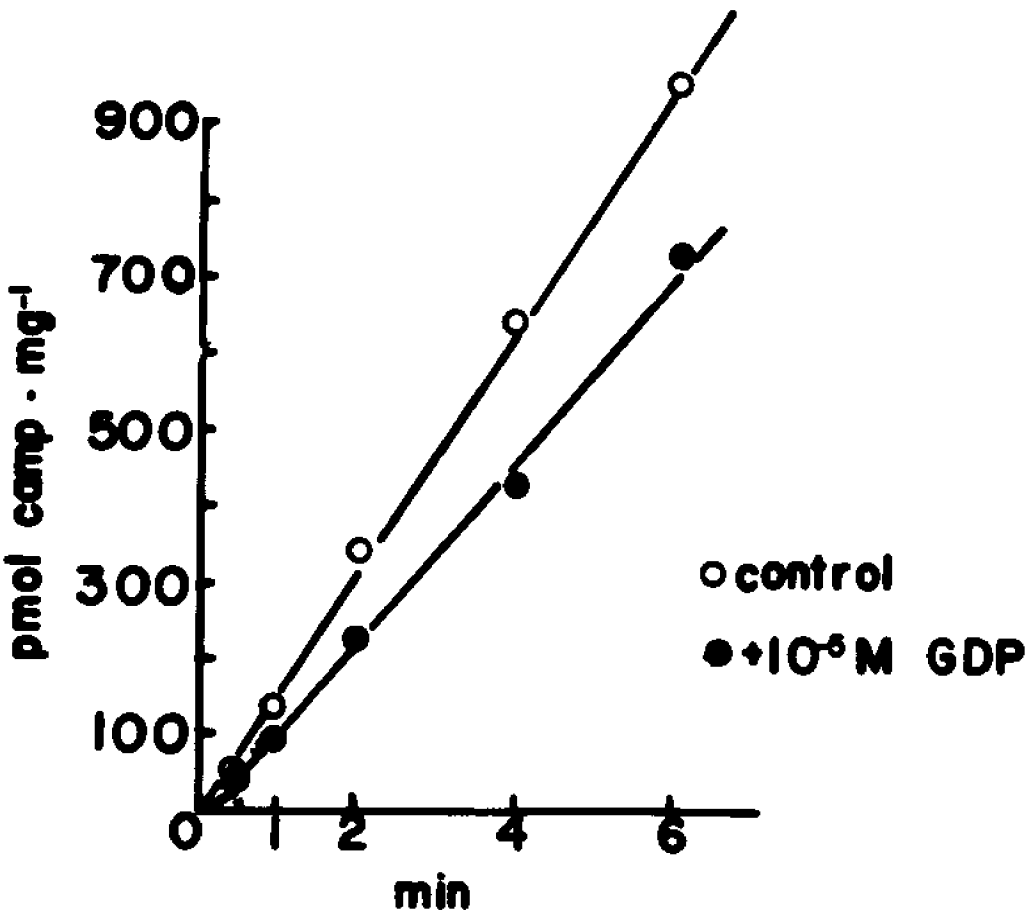

Figure 20. Time course of the effect of GDP on sacrolemmal adenylate cyclase activity. Assays were performed under standard conditions in the presence and absence of 10^{-5} M GDP. GDP was added 5 sec prior to the initiation of the reaction by the addition of sarcolemmal membranes. Each point represents the mean of three replicates.

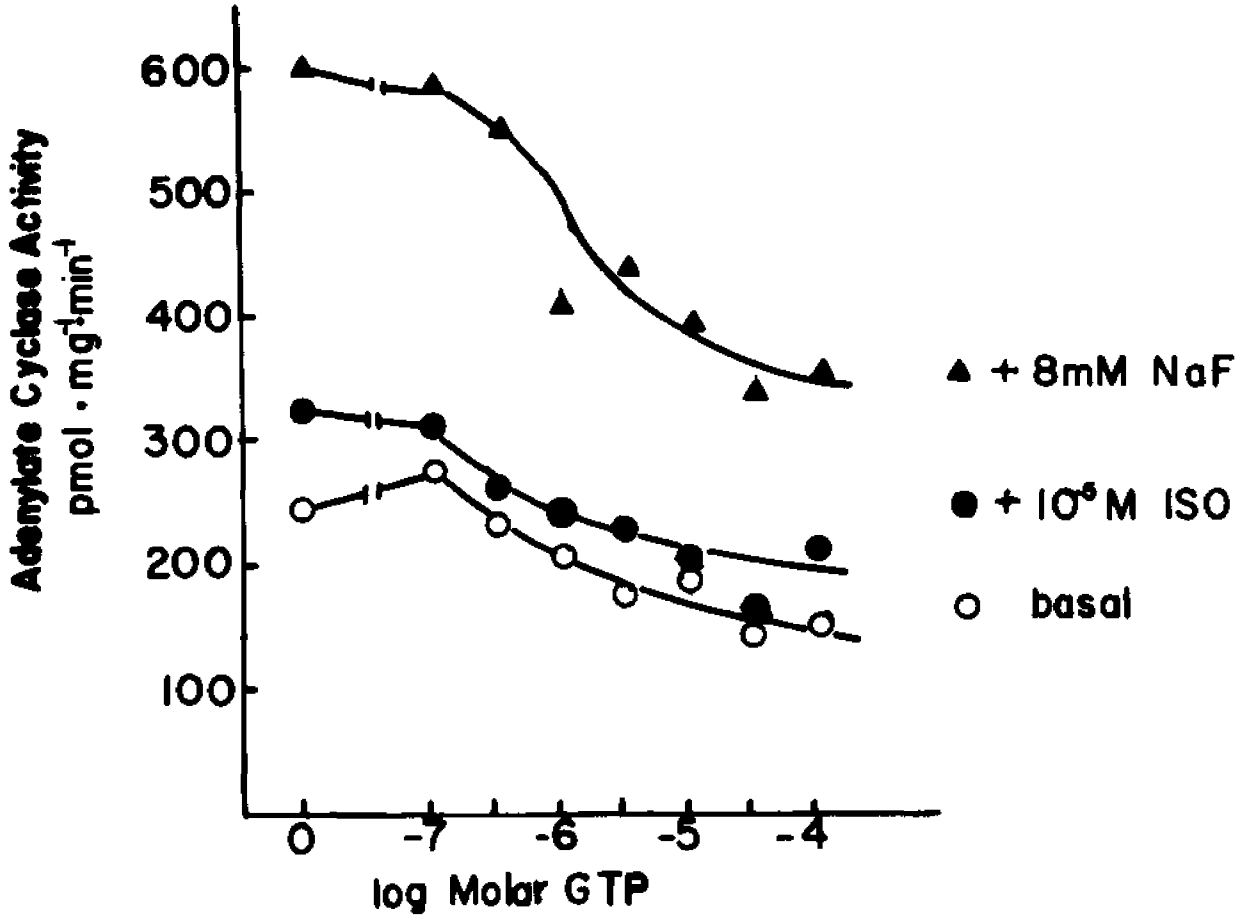

Figure 21. Dose response relationship for the effect of GTP on sarcolemmal adenylate cyclase. Assays were performed under standard conditions in the presence and absence of 10^{-5} M isoproterenol or 8 mM NaF. Each point represents the mean of three replicate determinations.

proximately 2 x 10^{-6} M. Raising the concentration of GTP also increased the increment due to isoproterenol so that the effect of a given dose of isoproterenol is determined by the concentration of GTP in the assay medium.

Because of the large increase in isoproterenol-stimulated adenylate cyclase activity seen in the presence of [illegible], dose response studies were also performed for this nucleotide analog. Figure 22 shows the effects of varying concentrations of [illegible] on sarcolemmal adenylate cyclase activity in the presence and absence of NaF (5 mM) or isoproterenol (10^{-5} M). The stimulatory effect of [illegible] in the presence of isoproterenol was evident at all concentrations above 10^{-7} M, with half-maximal stimulation at 10^{-6} M [illegible]. At the highest concentration tested (10^{-4} M) the activity in the presence of isoproterenol was 16% above that seen in the absence of [illegible], representing a 14-fold increase in the effect of the catecholamine. NaF-stimulated activity was somewhat more sensitive to [illegible] with inhibition detected at concentrations as low as 10^{-7} M. Inhibition was half maximal at approximately 2 x 10^{-7} M [illegible] and maximal at 10^{-6} M [illegible] (Figure 22). Absence of further effects on NaF-stimulated adenylate cyclase activity at higher concentrations was similar to the findings with GTP.

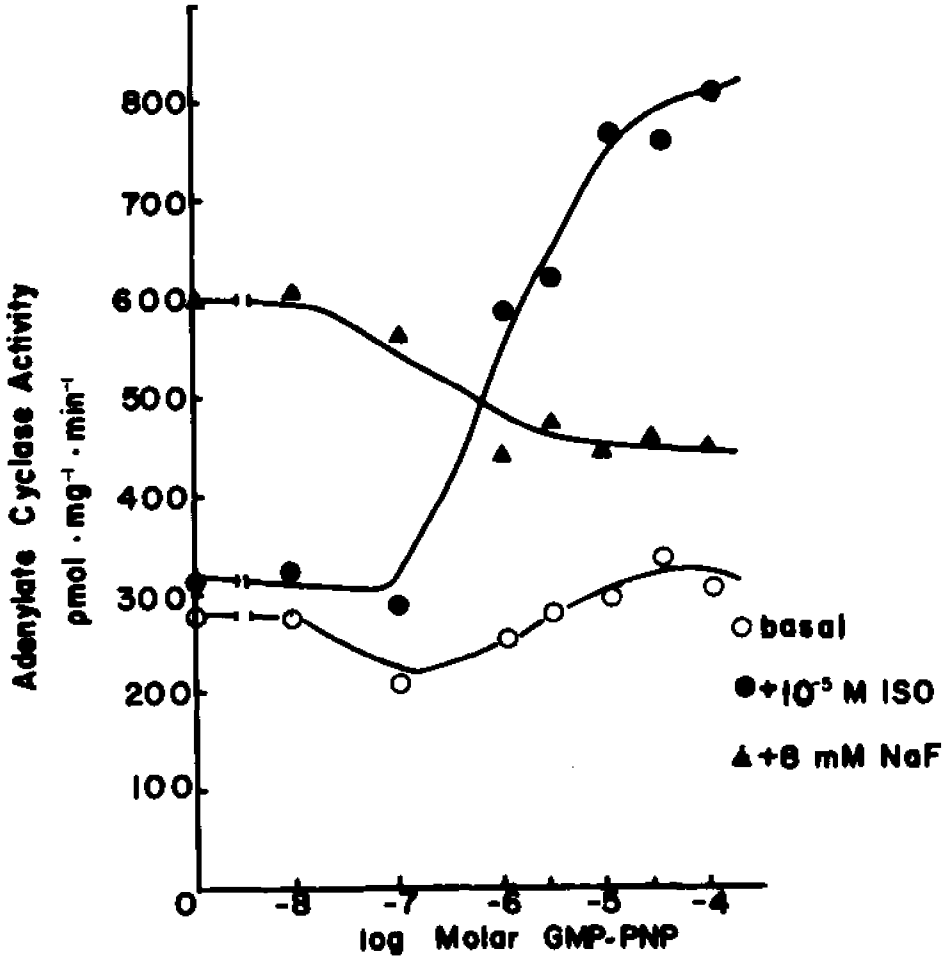

Figure 22. Dose response relationship for the effect of GMP-PNP on sarcolemmal adenylate cyclase activity. Assays were performed under standard conditions in the presence and absence of 10^{-5} M isoproterenol or 8 mM NaF. Each point represents the mean of three replicate determinations.

The effects of GTP-Mg on basal adenylate cyclase activity were complex. At low concentrations (10^{-7} M) GTP-Mg was inhibitory. The extent of inhibition, however, decreased as the nucleotide concentration was raised. At concentrations above 3×10^{-6} M a small but consistent increase above control activity was observed, the maximal stimulatory effect being apparent at 3×10^{-5} M. This biphasic dose-response relationship suggested that the nucleotide analog might have more than one effect on the sarcolemmal adenylate cyclase. Studies of the time course of the adenylate cyclase reaction in the presence of GTP-Mg also support this hypothesis. GTP-Mg (10^{-5} M) in the absence of isoproterenol was inhibitory throughout the entire reaction period (Figure 23), but the inhibition was much more severe during the initial part of the reaction. In some experiments (Figure 24-A) the reaction rate in the presence of GTP-Mg eventually surpassed that of the basal enzyme. In the insert to Figure 23, where the data are plotted on a much expanded scale, it is apparent that even in the presence of isoproterenol GTP-Mg inhibits the initial rate of cyclic AMP production. The dominant effect of GTP-Mg, the enhancement of the response to isoproterenol, was not apparent until 2 minutes after the start of the reaction. Varying the level of GTP-Mg, whether in the presence or absence of isoproterenol, changed the final rate of cyclic AMP production in a manner consistent with the concentration

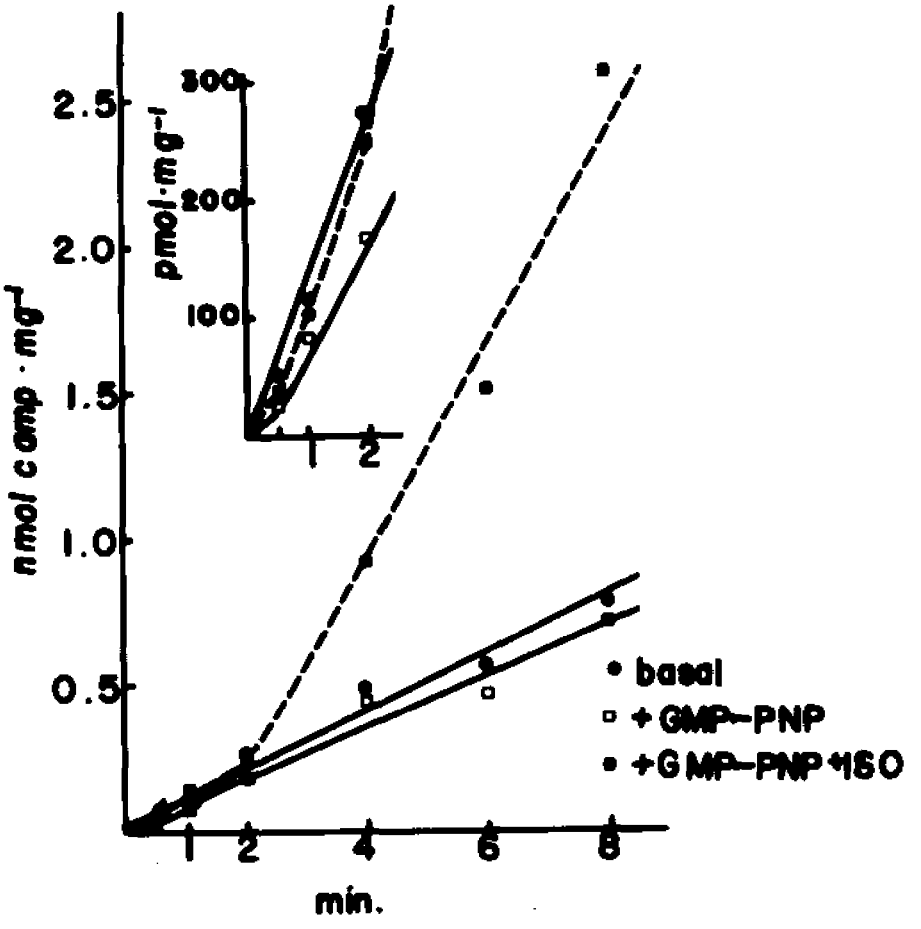

Figure 23. Time course of the effect of GMP-PNP on sarcolemmal adenylate cyclase activity. Assays were performed under standard conditions. The concentration of both GMP-PNP and isoproterenol, when present, was 10^{-5} M. The insert reproduces the first three points of each curve plotted on an expanded scale. Each point represents the mean of three replicate determinations.

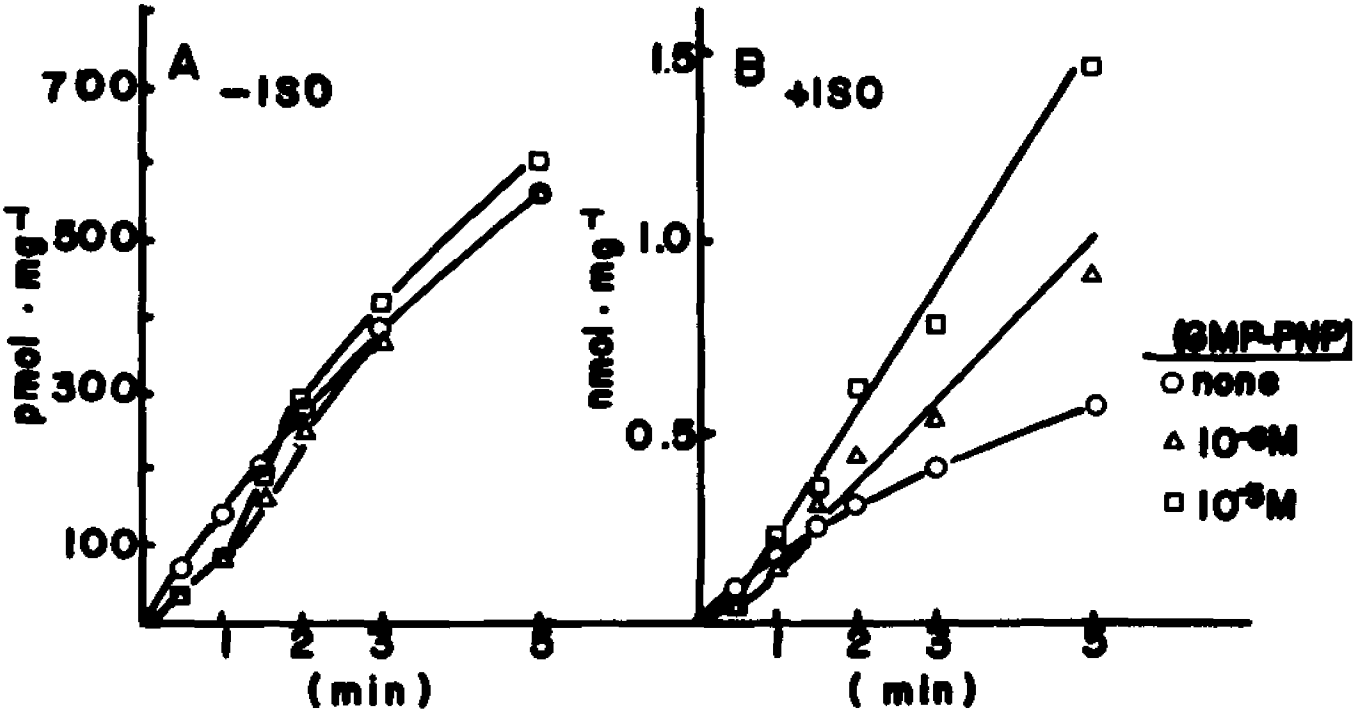

Figure 24. Assays were performed under standard conditions in the absence (A) and presence (B) of 10^{-5} M isoproterenol and the indicated concentrations of GMP-PNP. Each point represents the mean of three replicate determinations.

dependence studies (Figure 24). The duration of the inhibitory "lag" period was also concentration dependent with the lag decreasing with increasing nucleotide concentration.

Ca^{2+} Dependency of Sarcolemmal Adenylate Cyclase

The adenylate cyclases of all tissues studied show a Ca^{2+} requirement for enzyme activity (151). Inasmuch as GTP, as well as ATP, binds magnesium with high affinity, the effects of varying the Ca^{2+} ion concentration on adenylate cyclase activity in the presence and absence of that nucleotide were studied. At low free Ca^{2+} levels, 10^{-5} M GTP greatly inhibited basal activity (Figure 25), as the Ca^{2+} concentration was raised, however, the percent inhibition of cyclase activity by GTP gradually decreased until adenylate cyclase activity was identical in the presence and absence of the nucleotide. A similar relationship was observed in the presence of 10^{-5} M isoproterenol. The effect of Ca^{2+} on the NaF-stimulated enzyme differs in that no further increases were observed when the Ca^{2+} level was raised above 20 mM. Similar Ca^{2+} dependencies were observed when the ATP concentration was raised to 2 mM.

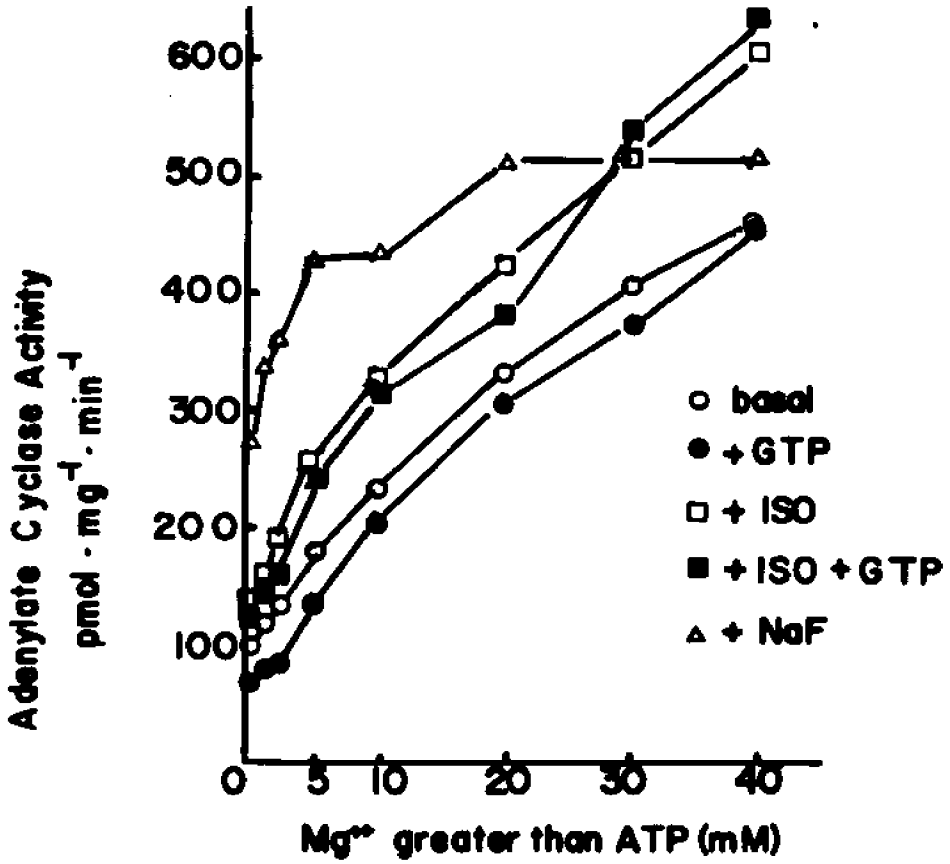

Figure 25. Mg^{2+} dependency of sarcolemmal adenylate cyclase. Assays were performed under standard conditions. The concentrations of GTP, NaF and isoproterenol, when present, were 10^{-5} M, 8 mM and 10^{-5} M respectively. Each point represents the mean of three replicate determinations.

The presence of several points of inflection in the curves in Figure 25 suggested that Mg^{2+} has multiple effects on the adenylate cyclase reaction. This is shown more clearly in Figure 26 where the data are presented in double reciprocal ($1/v$ vs. $1/Mg^{2+}$) plots. One effect of Mg^{2+}, independent of its general stimulatory effect on enzyme activity, is to modify the enhancement of the response of isoproterenol by GTP. This phenomenon is illustrated in Figure 27 where the percent increase in cyclase activity produced by isoproterenol is plotted as a function of the Mg^{2+} concentration. At low free Mg^{2+} and in the presence of GTP, isoproterenol (10^{-5} M) produced a 100% increase in cyclase activity when compared to controls with added GTP only. As the Mg^{2+} concentration was raised, the stimulatory effect of isoproterenol fell rapidly, approaching the value observed when isoproterenol alone was present. In the absence of GTP, elevation of Mg^{2+} concentration did not greatly affect the percent response to isoproterenol which remained approximately 40% throughout the entire range of magnesium concentrations tested (Figure 27).

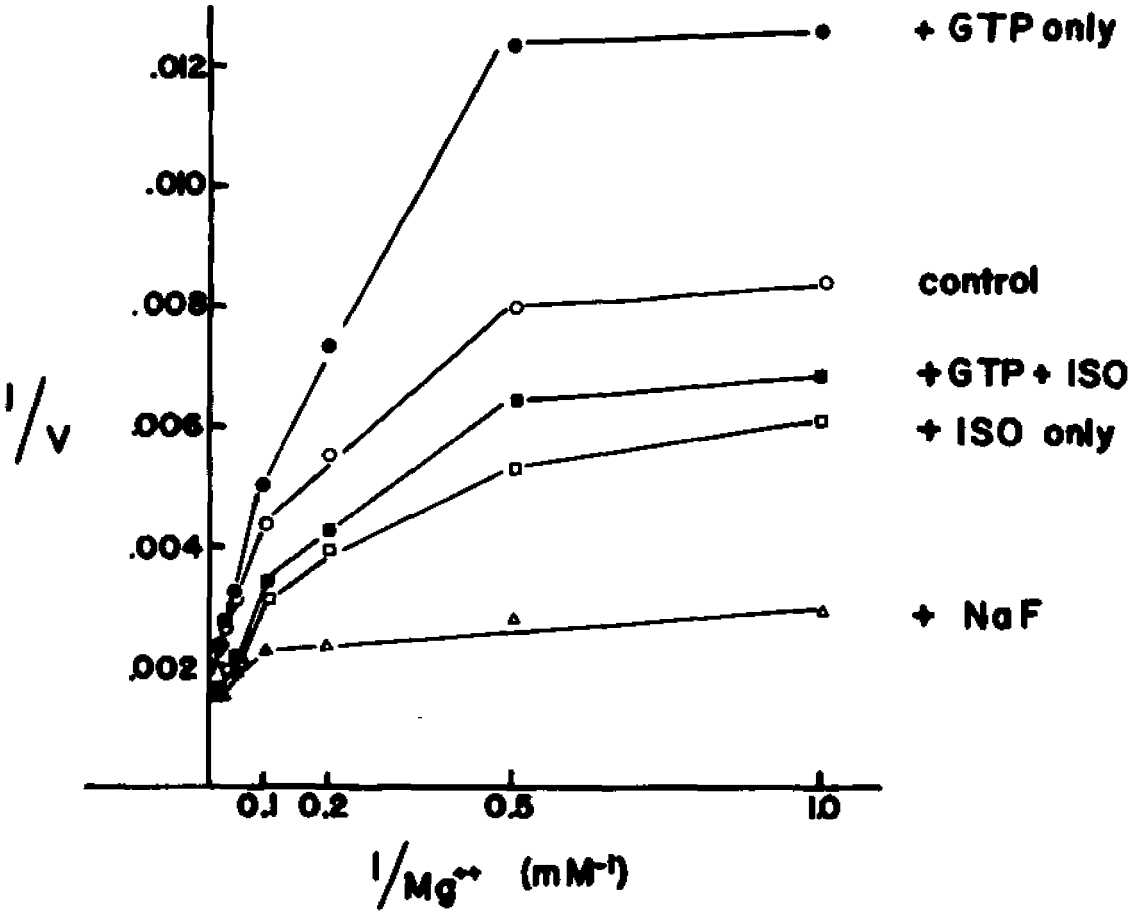

Figure 26. Double reciprocal plots of the Mg^{2+} dependence of sarcolemmal adenylate cyclase. Data derived from Figure 25.

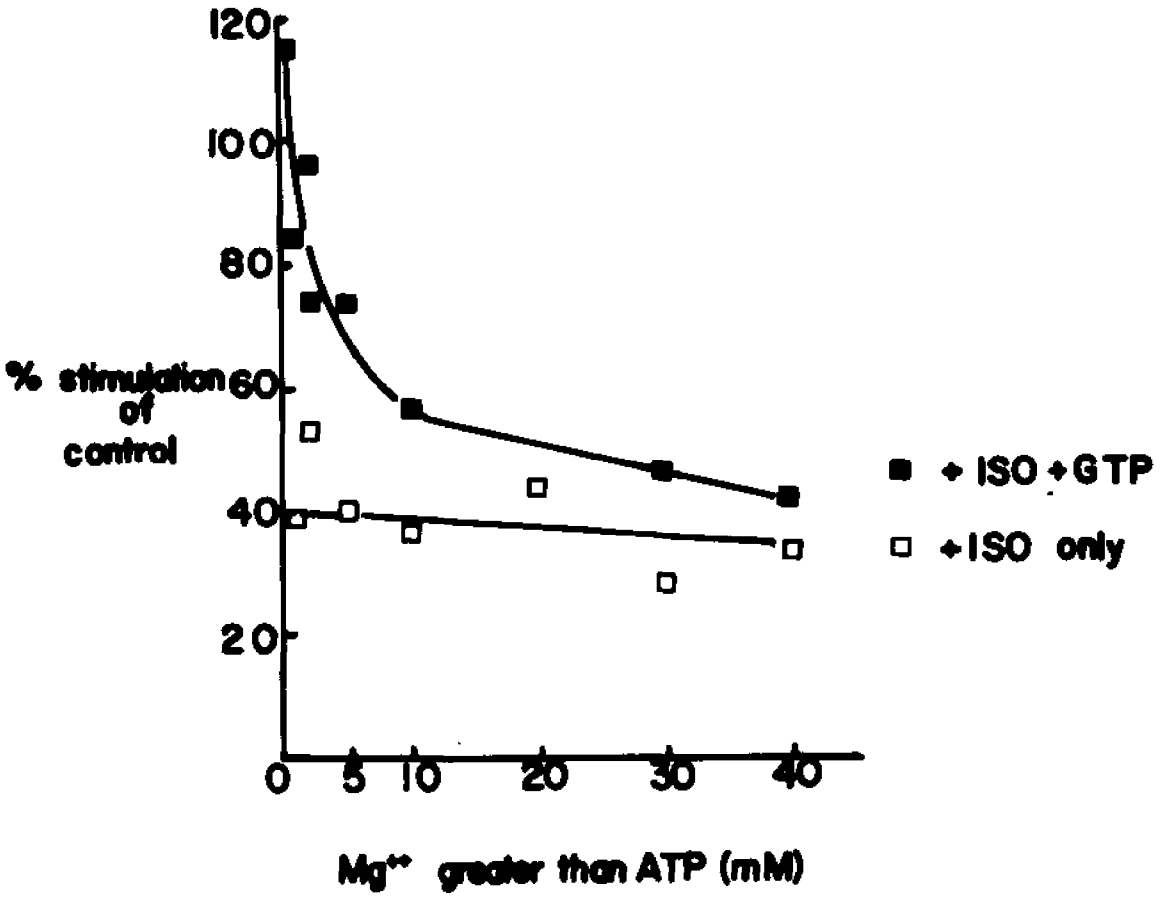

Figure 27. Mg^{2+} dependence of the enhancement of the response to isoproterenol by GTP. Data derived from Figure 25. See text for details.

DISCUSSION

In view of the large number of cardiac functions affected by cyclic AMP (see Introduction) it is not surprising that cyclic AMP production by the myocardial adenylate cyclase is itself a highly regulated process. The experiments described in the previous section examined the interactions of three classes of endogenous agents with the adenylate cyclase of the ventricular sarcolemma of the guinea pig. These agents, catecholamines, guanyl nucleotides and free magnesium ion, appear to act at different sites of the adenylate cyclase system. In the following paragraphs the results of these experiments will be related to previous studies of myocardial and other adenylate cyclases, after which an attempt will be made to construct a model to account for the experimental findings.

It has long been known that the stimulation of cardiac adenylate by catecholamines is mediated by a <u>beta</u> adrenergic receptor (15) as originally defined by Ahlquist (152). This assignment has been confirmed by the present study in two ways: by the relative potency of the adrenergic agonists tested for ability to stimulate adenylate cyclase and by the ability of the <u>beta</u> blocking agent propranolol to inhibit the response of the enzyme to epinephrine. Stimulation of cyclic AMP production by l-epinephrine cannot be attributed

to an antioxidant effect as the chemically identical d-stereoisomer had no effect on adenylate cyclase activity.

The affinities of the various agonists for the receptor, as estimated from the dose response curves, were approximately one order of magnitude greater than those reported by previous investigators of cardiac adenylate cyclase (15, 153-5). This discrepancy may be due to the use of HEPES buffers for this series of experiments. Previous experiments with guinea pig cardiac sarcolemmal adenylate cyclase, performed in this laboratory under the same conditions as the present study except for the use of imidazole buffers, showed a half maximal response at a somewhat higher epinephrine concentration (156), in accord with estimates from other laboratories. The maximal stimulation of adenylate cyclase activity was the same in the present and previous studies, so that it appears that the hydrogen ion buffers may account for the different apparent affinities of beta adrenergic agonists for the receptor.

The concentration of adrenergic agonists required for half-maximal stimulation of adenylate cyclase activity in the presence of HEPES is still much higher than that required to elicit half-maximal inotropic responses in papillary muscle (157). It is possible that this finding reflects disruption of receptor-catalytic unit coupling during the homogenization and centrifugation required for the isola-

tion of pure membrane fractions. Alternatively, the difference in the sensitivity of the adenylate cyclase and physiologic responses of the heart could be due to 'receptor reserve' in which the hearts capacity for cyclic AMP production greatly exceeds the amount required for maximal elaboration of physiologic responses. The latter hypothesis is most likely, in view of the common finding of redundancy in biological control systems.

Comparison of Sarcoplasmic Reticulum and Sarcolemmal Adenylate Cyclase

The characteristics of the beta adrenergic receptor activity associated with the adenylate cyclase found in sarcoplasmic reticulum vesicles prepared from guinea pig ventricle differed significantly from those of the corresponding sarcolemmal preparations. At pH 7.5 and 25°C, the adenylate cyclase activity of the sarcoplasmic reticulum preparations was significantly increased by several adrenergic agonists. The order of potency of these agonists was: isoproterenol > epinephrine > norepinephrine >> dopamine = phenylephrine, indicates that these effects are mediated by a beta adrenergic receptor. Catecholamine-sensitive adenylate cyclase activity has also been demonstrated in sarcoplasmic reticulum prepared from canine ventricles (95, 96) with half-maximal activation of cyclic AMP production elicited by 5×10^{-6} M epinephrine. The low levels of this

beta receptor activity in the guinea pig sarcoplasmic reticulum preparations, in contrast to the more active sarcolemmal preparations, precluded a complete characterization of the beta adrenergic receptors in the sarcoplasmic reticulum.

The sarcoplasmic reticulum preparations tested for adenylate cyclase activity did, however, exhibit significant calcium transport activity. The rate of calcium transport at 0.75 M Ca^{2+} in the presence of potassium oxalate (0.015 μmol/mg/min), was about one half that exhibited by canine cardiac sarcoplasmic reticulum under the same conditions (83), and compares favorably with values for the guinea pig reported by other laboratories (158).

The pH dependency of the sarcoplasmic reticulum adenylate cyclase differed from that of the sarcolemmal enzyme in that maximal activity for the former was seen at pH 7.5, even in the presence of NaF. Furthermore, several modulators of the sarcolemmal adenylate cyclase did not change the rate of cyclic AMP production by the enzyme found in the sarcoplasmic reticulum. Thus, no significant increase in sarcoplasmic reticulum cyclase activity was observed when the ionic strength of the medium was increased with KCl or NaCl, and the addition of 10^{-5} M GTP had almost no effect on either basal or isoproterenol stimulated adenylate cyclase activity. The slight decrease in NaF-stimulated adenylate cyclase activity of the sarcoplasmic reticulum preparations was

not significant even when GTP was present in high concentration.

Inasmuch as only the sarcolemma but not the sarcoplasmic reticulum were exposed to isotonic sucrose buffers during isolation, the differences between the two enzymes might have arisen from the different methods used to purify these membrane fractions. Sucrose, however, has no striking ability to activate sarcoplasmic reticulum (96) or sarcolemmal (156) adenylate cyclase, nor do the procedures used to purify the latter increase hormone-sensitivity (Katz et al., unpublished observation). Thus, it is most likely that these two enzymes differ chemically, although the functional significance of the lesser regulation of the sarcoplasmic reticulum enzyme cannot be evaluated by these studies.

Effects of Fluoride on the Sarcolemmal Adenylate Cyclase

Stimulation of cardiac adenylate cyclase by catecholamines can be mimicked by millimolar concentrations of fluoride ion. Because the effect of fluoride is independent of cell surface receptors and is apparent in all eukaryotic systems studied, the percent stimulation by fluoride can be used to compare adenylate cyclases from disparate sources.

Sarcolemmal adenylate cyclase was maximallay stimulated by 6 mM NaF, which differs only slightly from the 8 mM optimum previously reported by Drummond and Duncan (159) and Tada et al, (156). Decreased activity seen at slightly higher fluoride concentrations (8-10 mM) suggests that the anion may bind at more than one site on the adenylate cyclase system, while the more gradual fall in activity at still higher levels (Figure 12), is likely to reflect, at least in part, the complexation of ionized Mg^{2+} by F^-, the MgF_2 solubility product being $10^{-8.2}$. The resulting reduction in Mg^{2+} concentration can account for the decrease in the activity of this Mg^{2+}-dependent enzyme.

pH Dependence of the Sarcolemmal Adenylate Cyclase

The shift in the pH optimum for the sarcolemmal adenylate cyclase in the presence of NaF (Figure 11), which is not apparent in sarcolemmal prepared from canine hearts (92), cannot be explained. The pH dependency reported by Drummond and Duncan (159) for the adenylate cyclase activity of a particulate preparation of guinea pig ventricle is strikingly different from present findings. Inasmuch as the nature of the pH buffer used often affects enzyme activity (160) the difference in pH profile may be due to the difference in the buffer systems used by Drummond and Duncan to maintain hydrogen ion concentrations. The difference in the preparations used adds further difficulties to the comparison

of the data of Drummond and Duncan with the results of the present study. Wantanabe and Besch (155), who prepared guinea pig membranes by both the Tada technique and the method of Drummond and Duncan, found that purification of the sarcolemma increased the percent response to epinephrine and NaF in addition to raising the basal cyclase activity of the preparation. Thus, qualitative as well as quantitative properties of adenylate cyclase depend on the preparatory method employed. Such changes may be due to the removal of loosely bound proteins that are in association with the enzyme system in intact tissue.

Effects of Alkali Metal Salts on the Sarcolemmal Adenylate Cyclase

The effects of increasing ionic strength on cardiac sarcolemmal adenylate cyclase have been previously been described by Tada et al, (156) who reported small but consistent increases in basal activity with increasing concentrations of either NaCl or KCl. Non-electrolytes such as sucrose or urea did not change adenylate cyclase activity in this tissue. Dousa (161) has demonstrated that similar concentrations of Na^+ or K^+ salts increased the response of canine renal adenylate cyclase to vasopressin or NaF in addition to stimulating basal activity. The results of the present study (Figure 12) show that increased ionic strength does not modify the response of sarcolemmal adenylate cyclase

to isoproterenol. The difference in the effect of electrolytes on the renal and cardiac adenylate cyclase may reflect the specialized functions of the organs from which they were isolated. In the presence of vasopressin the kidney reabsorbs large amounts of water at the distal convoluted tubule and collecting duct. This effect of vasopressin is believed to be mediated by cyclic AMP (162, 163). The action of salts to increase the efficacy of a given concentration of vasopressin provides a short loop negative feedback pathway by which salt levels may be regulated by vasopressin in addition to the independent effect of increased tonicity on the release of that hormone from the posterior pituitary.

In the heart, however, physiological regulation by changing extracellular Na^+ or K^+ is less important. Cardiac function, instead, is exquisitely sensitive to cytoplasmic levels of Ca^{2+}, which are regulated, in part, by cyclic AMP (see Introduction). The work of Tada et al, (156) suggests a role for Ca^{2+} in the feedback regulation of cardiac cyclic AMP levels. A similar relation between physiological regulation and the control of adenylate cyclase is seen in the case of free fatty acids. These end products of cyclic AMP-stimulated lipolysis inhibit adenylate cyclase activity of rat adipose tissue (164) [but not of avian adipocytes (165)].

Although there are impressive similarities in the properties of adenylate cyclases from different tissues, these studies emphasize that there are differences in the regulation of adenylate cyclase in different tissues or species. The use of information about one adenylate cyclase in the evaluation of new data about a second must, therefore, be exercised with care.

Effects of Guanyl and Other Nucleotides on Sarcolemmal Adenylate Cyclase

Tissue specificity in the response of adenylate cyclase to physiological modulators must also be considered in evaluating reports of the effects of GTP on cardiac adenylate cyclase. Lefkowitz (117) reported that GTP stimulates basal activity of canine cardiac adenylate cyclase and greatly increases activity in the presence of isoproterenol. Other guanyl nucleotides were found to be similar to GTP in their effects on cyclase activity, but were less potent. All nucleoside triphosphates tested inhibited NaF-stimulated adenylate cyclase activity. Only guanyl nucleotides stimulated basal activity; ITP, XTP and GTP were inhibitory. Several nucleotides increased the absolute value of isoproterenol-stimulated activity. ITP and XTP slightly reduced the absolute value of isoproterenol stimulated activity, but, since basal activity was inhibited to a greater extent, percent stimulation of cyclase activity by iso-

proterenol was increased in the presence of the nucleotide. The GTP-induced stimulation of canine cardiac adenylate cyclase was reported to be time dependent with a lag of several minutes preceding any increase in the rate of cyclic AMP production.

These previously reported effects of GTP on the dog heart differ greatly from the present study of the guinea pig sarcolemma, where GTP was found to inhibit basal, isoproterenol-stimulated and NaF-stimulated adenylate cyclase activity. The nucleotide-binding site on the guinea pig enzyme was more selective than that of the dog as only the purine nucleotides ITP and XTP reproduced the effects of GTP, whereas the pyrimidine nucleotides UTP and CTP had no effect on cyclase activity. Thus, the myocardial adenylate cyclases of the guinea pig and dog differed not only in their response to GTP but in their interactions with other nucleotides as well. The different responses of these enzymes in vitro, which may reflect inherent differences in the regulation of myocardial adenylate cyclase in these species, could be shown not to reflect an artefact due to reagent contaminants by the demonstration of the stimulatory effect of GTP on turtle bladder epithelial adenylate cyclase (Table 8).

Since guanyl nucleotides generally stimulate adenylate cyclases, especially in the presence of hormone (See Introduction), the mechanism of the GTP-induced inhibition of cyclase activity in the sarcolemma was of great interest. The non-linear Dixon plots of the concentration dependence of the GTP effect (Figure 14) indicate that GTP was acting as either a partial or mixed-type inhibitor. Either type of inhibition requires a binding site for the inhibitor which is distinct from the catalytic substrate-binding site. Since the second, regulatory, site is selective for purine nucleotides it seemed likely that the substrate ATP might also interact with that site. Such an interaction could be reflected in deviations of the substrate dependency from Michaelis-Menten kinetics. The results of the substrate-dependency experiments (Figure 16), which exhibited non-linear reciprocal plots, could indeed be explained by a non-cooperative allosteric model in which ATP binds to a regulatory site similar to that postulated to account for the effects of GTP on this enzyme.

The non-linear, concave downward, double reciprocal plots, shown in Figure 16, can also be accounted for by at least two other models. Similar kinetic patterns would be obtained if there were two forms of adenylate cyclase present on the sarcolemma which differ in their kinetic constants such that $Km_1 < Km_2$ and $Vmax_1 < Vmax_2$. This interpretation is consistent with the finding that two

forms of adenylate cyclase can be isolated from solubilized membranes of the canine renal medulla which differ in apparent molecular weight and in their response to GMP-PNP (166). The kinetic properties of the two enzymes have not yet been fully described.

Alternatively, cardiac adenylate cyclase could be a multimeric enzyme with a catalytic site on each protomer. If these subunits interacted in a negatively cooperative fashion such that substrate binding to one subunit lowered the affinity of associated subunits for substrate, the same non-linearities would appear in the double reciprocal plots.

With the information currently available, the non-cooperative allosteric model remains the most likely explanation for the sarcolemmal adenylate cyclase substrate-dependency. Although the data presented to support the existance of two forms of adenylate cyclase in the kidney are convincing, these findings may reflect heterogeneity of cell types in the renal medulla or the use of a crude homogenate and not a purified membrane fraction as the source of soluble enzyme. The cardiac adenylate cyclase preparations used for the present study were derived from a more homogeneous tissue, but the possibility that there are multiple membrane components that introduced additional forms of adenylate cyclase cannot be excluded. The activity of the sarcoplasmic reticulum adenylate cyclase is, however, too low for a sarcoplasmic reticulum contaminant to

modify the kinetics of the sarcolemmal adenylate cyclase. The negative-cooperativity model of the adenylate cyclase-substrate interaction is tenuous as there is no direct evidence for an oligomeric structure for adenylate cyclase in any tissue.

Linear double reciprocal plots of the substrate dependency of sarcolemmal adenylate cyclase of both guinea pig (156, 159) and canine (92) hearts have been reported earlier, contrasting sharply with the results of the present study. The nature of the observed kinetics has been shown by Garbers and Johnson (107) to depend on the type of ATP regenerating system used. Linear double reciprocal plots were observed in the presence of a pyruvate kinase-PEP regenerating system, while non-linear plots were seen when creatine kinase-phosphocreatine regenerating systems were employed. Although the observation made by Garbers and Johnson accounts for the difference between previous and present ATP dependency studies, no insight is provided as to which regenerating system is producing artefactual results. Such information could be provided only by cross-over experiments in which individual components of each regenerating system is examined for effect on the ATP dependency in the presence of the other regenerating system. Alternatively, regenerating systems could be avoided entirely by the use of AMP-PNP as substrate, as this synthetic analog of ATP is resistant to hydrolysis by membrane-bound ATPases (149).

It may be argued that until definitive studies are made, the data obtained in the presence of pyruvate kinase-PEP regenerating systems should be accepted on the basis of simplicity as they conform to classical Michaelis-Menten kinetics. The attractiveness of such a simple model is illusory, however, as a second nucleotide-binding site provides the simplest explanation of the effects of GTP on enzyme activity. Furthermore, investigations of the adenylate cyclases of both the adrenal gland (104) and the liver (138) have shown non-linear reciprocal plots of the substrate-velocity curves even when AMP-PNP was used as substrate. Reexamination of the original data from this laboratory regarding the guinea pig cardiac adenylate cyclase demonstrates that the fit of a curved line to the data in the double reciprocal plot is at least as close as the straight line that has been published (156). Subsequent analysis of the mechanism of the GTP-produced inhibition of sarcolemmal adenylate cyclase will therefore be based on the non-cooperative allosteric model of the enzyme.

According to this model, GTP exerts its inhibitory effect on basal adenylate cyclase activity by binding at an allosteric regulatory site on the enzyme. As a result of this interaction, the affinity of the catalytic site for substrate is reduced, leading to a decreased reaction velocity. Since even 10^{-7} M GTP was somewhat inhibitory in the presence of a much higher con-

centration of ATP, it is apparent that the regulatory site has a much higher affinity for GTP than for ATP (Figures 14, 16). The identity of allosteric binding sites for ATP and GTP is strongly suggested by the ability of GTP to linearize the double reciprocal plot of the ATP dependency of basal activity. A similar effect of GTP on the double reciprocal plots for adrenal adenylate cyclase has been reported by Londos and Rodbell (104).

The observation that the time course of the GTP-inhibited adenylate cyclase reaction was linear had at first suggested that GTP itself was the inhibitory species, for if GDP, its hydrolytic product, was the inhibitor, the extent of inhibition might be expected to increase with time. This hypothesis was supported by the demonstration that the nucleotide analogs GMP-PNP and GPP-CH_2-P, which are not hydrolyzed to GDP, also inhibited basal activity. The findings that the inhibitory effects of GDP were similar to those of GTP, and that the extent of the GDP-induced inhibition also remained essentially constant with time, suggest that the presence of the γ-phosphate moiety neither enhances nor decreases the extent of inhibition of basal activity. Thus, the initial rate of cyclase activity in the presence of guanyl nucleotides a function of total guanyl nucleotide concentration but is independent of the GTP/GDP ratio. These experiments, therefore, could not be used to estimate the steady state level of GTP in the reaction medium.

Although the γ-phosphate of GTP is not required for inhibition of basal adenylate cyclase activity, it is required for the enhancement of the response to isoproterenol as GDP failed to modify the response to the catecholamine. Furthermore, the greater enhancement of isoproterenol-stimulated activity in the presence of GTP-PNP and GPP-CH_2-P, compared to GTP, suggested that this response is a function of the γ-phosphate. The much smaller effect of GTP, therefore, implies that a substantial amount of GTP hydrolysis occured before GTP and GDP levels reached steady state. The linearity of the adenylate cyclase reaction in the presence of both GTP and isoproterenol (Figure 17) indicated that under the conditions used the steady state GTP level is reached within the first 30 sec of the reaction. For this reason most of the later experiments were performed using the more stable GTP-PNP.

Basal adenylate cyclase activity changed in a biphasic manner as the concentration of GTP-PNP was raised (Figure 22), with low concentrations of the nucleotide being inhibitory and high concentrations stimulatory. The biphasic concentration dependence can be accounted for by either a "two site" model or by one which proposes multiple changes in the conformation of the enzyme following the binding of guanyl nucleotides (multiple transition state model).

The two site model postulates the existance of two GTP-PTP-binding sites in association with the catalytic subunit of the sarcolemmal adenylate cyclase that differ in their affinity for GTP-PTP. At low nucleotide concentrations, when only the high affinity site is occupied, basal activity is inhibited. The second, low affinity site is saturated only at much higher concentrations of GTP-PTP. Occupation of the second site, however, induces an enzyme conformation with higher catalytic activity; thus, high concentrations of GTP-PTP are stimulatory.

The multiple transition state model, which was first proposed by Rodbell and co-workers for the hepatic adenylate cyclase (135), can account for the biphasic GTP-PTP dependency in a different way. Only a single GTP-PTP-binding site is contained within the adenylate cyclase system. The initial effect of nucleotide binding is to induce a conformation of the enzyme with lowered activity (State I). Subsequently, the enzyme undergoes a second, much slower, conformational change that when complete results in a more active enzyme system (State II). The observed effect of a given concentration of GTP-PTP, therefore, would depend on several factors e.g., the fraction of total sites occupied at equilibrium, the average length of time between nucleotide-binding and the termination of the reaction, the mean time required for the slow transition to be completed, the difference in the specific activity

of the enzyme when in the different transition forms and the variance of these parameters within the population of enzyme molecules. A significant proportion of the enzyme reaches the more active State II only in the presence of high nucleotide concentrations.

The experiments that measured the time course of the adenylate cyclase reaction in the presence of GMP-PNP were designed to distinguish between the two models. If the two site model were correct, adenylate cyclase activity would remain linear with time as the rate of cyclic AMP production would depend only on the fraction of inhibitory and stimulatory sites occupied at that concentration of the analog. If the multiple transition site model were correct, adenylate cyclase activity would be expected to increase with time as the enzyme molecules complete the second transition. The results of the time course experiments support the multiple transition state model. In the absence of isoproterenol, the initial response to GMP-PNP is a severe inhibition of cyclic AMP production, which is followed by a second phase characterized by a less severe inhibition (Figure 23). Attempts to increase further the proportion of enzyme in State II by preincubation in the presence of GMP-PNP were unsuccesful as preincubation at 37°C in either the presence or absence of GMP-PNP resulted in the complete loss of adenylate cyclase activity. In the presence of isoproterenol the rate of transition to State II was greatly

increased (Figure 23), but even in the presence of the catecholamine the initial rate of adenylate cyclase activity was significantly lower than control. At nonsaturating concentrations of GTP-PiP, both the rate of the slow transition and the steady state velocity of cyclic AMP production increased with increasing nucleotide concentration (Figure 24).

Effects of Mg^{2+}

Studies of the Mg^{2+} dependency of the sarcolemmal adenylate cyclase (Figure 25) have demonstrated that both the extent of the inhibition of basal activity by GTP and the GTP-induced enhancement of the response to isoproterenol are decreased as the Mg^{2+} concentration is raised. This implies that the Mg-GTP complex has no major effect on adenylate cyclase. Thus, the observed effects of GTP on both basal and isoproterenol stimulated adenylate cyclase appear to depend on the concentration of free Mg^{2+}.

In the absence of GTP, the general effect of raising the Mg^{2+} concentration is to increase sarcolemmal adenylate cyclase activity without markedly changing the response to catecholamines. This finding agrees with the earlier report of Drummond et al, (168). These investigators, however, consistently observed saturation at Mg^{2+} levels between 10 and 18 mM, whereas in this study no evidence of saturation was seen even at 40 mM except when NaF was present. Both the interaction of F^- and Mg^{2+} and the difficulties in comparing the work of Drummond with the present study have been

discussed previously.

Several mechanisms have been advanced to explain the effects of Mg^{2+} on adenylate cyclase activity. Drummond et al, (168) suggested that cardiac adenylate cyclase possesed a Mg^{2+}-binding site independent of the catalytic site for the substrate, MgATP. When this site is occupied by Mg^{2+}, the affinity of the enzyme for the substrate is not changed, but once formed the metal-enzyme complex has a greater catalytic reactivity than the Mg^{2+}-free enzyme. Bellaen reanalyzed the data of Drummond et al, (169) and concluded that the stimulatory effect of Mg^{2+} could be accounted for by a model in which free ATP (ATP^{4-}) is a potent inhibitor of cyclase activity. As the Mg^{2+} concentration is raised and ATP^{4-} is converted to the chelate MgATP, enzyme activity increases due to the removal of an inhibitory species.

A more rigorous study of hepatic adenylate cyclase was carried out by Rodbell and co-workers (138) who concluded that the inhibitory species was $HATP^{3-}$ (protonated substrate). This conclusion was based on the difference in the pH dependency of the hepatic enzyme when ADP-PNP was used as substrate instead of ATP. Since the two purines differ in the pK_a of their terminal phosphates, the proportion of protonated substrate at a given pH would depend on which substrate was used. Therefore, if protonated substrate were the inhibitory species, the difference in pK_a would be reflected in a change in the optimal pH for the enzyme. The

data presented by the Rodbell group supports this hypothesis as the difference in optimal pH for cyclase activity is in good agreement with the difference in pK_a values for the two substrates.

The analyses of Dellhen and the Rodbell group have been subjected to criticism. Garbers and Johnson (167) have noted that the experiments on which these analyses were based could not distinguish between stimulation by Mg^{2+} and inhibition by unchelated nucleotide, as both were allowed to vary in the substrate dependency experiments. In their own experiments, Garbers and Johnson avoided this problem by keeping the free Mg^{2+}, rather than total $MgCl_2$, constant as the total ATP was varied; in this way unchelated ATP was maintained in a fixed proportion to total nucleotide throughout the entire concentration range. The resulting double reciprocal plots of the substrate dependency of cardiac adenylate cyclase obtained at a variety of free Mg^{2+} concentrations indicated that free ATP was a hyperbolic (partial) inhibitor but that Mg^{2+} acted as an activator as well. The ability of unchelated ATP to act as a partial inhibitor can be deduced in the present study from the action of GTP, which acts as a partial inhibitor, to compete for a non-catalytic ATP binding site. The present data do not, however, define whether the inhibition by unchelated ATP is competitive or noncompetitive.

The Mg^{2+}-dependency experiments shown in Figure 25 are also consistent with a direct interaction between Mg^{2+} and sarcolemmal adenylate cyclase. Double reciprocal plots of the Mg^{2+} data show three separate regions with different slopes. The right hand region corresponds to the small increase in activity seen at 1 or 2 mM free Mg^{2+}. This increase in activity can be explained by increased formation of the true substrate, $MgATP^{2-}$. The second region, extending from 2 to 10 mM, corresponds to the region examined by Drummond et al, (168) and Dellaen (169). Extrapolation of the asymptotes to these sections of the curves yields an average K_m value of approximately 3 mM, which is in good agreement with that reported by Drummond et al, (168). The increase in adenylate cyclase activity within this range of Mg^{2+} may be due to the chelation of ATP^{4-} or $HATP^{3-}$ as the concentrations of these species decrease significantly as the Mg^{2+} level is raised to 10 mM (see Appendix). This mechanism cannot account for the further rise in activity shown in the third region of the reciprocal plots (10-40 mM). It appears unlikely that if a common factor underlies the increase in activity in the second and third regions of the Mg^{2+} dependency, there would be a change in the slope of the double reciprocal plots. In addition, calculation of the levels of unchelated or protonated ATP (see Appendix) shows that the concentration of these species changes very little at high concentrations of Mg^{2+}. The increase in

activity at high Mg^{2+} concentrations thus can be explained most readily by a direct effect of the metal ion itself on the enzyme.

The data presented in this study are consistent with the following model. Catecholamines influence sarcolemmal adenylate cyclase activity by interacting with a beta adrenergic receptor site. The efficacy of a given concentration of an adrenergic agonist depends on the concentration of guanyl nucleotide present in the reaction medium. The terminal phosphate of GTP is required for the enhancement of the response of the enzyme to isoproterenol, but not for the inhibitory effect on basal activity. As only the unchelated form of the nucleotide interacts with adenylate cyclase, the effect of GTP will depend on the concentration of Mg^{2+} present.

GTP exerts its effects by interacting with a non-cooperative allosteric regulatory site contained within the adenylate cyclase system. Studies with the synthetic analog GTP-PNP indicate that subsequent to nucleotide binding at this site, the enzyme undergoes at least two conformational changes. The first is rapid and produces a less active enzyme. The second transition is slower but is associated with increased adenylate cyclase activity. The rate of this second transition is greatly increased by the catecholamines. In addition to modifying the effect of GTP, Mg^{2+} appears to stimulate adenylate cyclase activity

in three ways; by formation of the true substrate $Mg ATP^{2-}$, by removal of an inhibitory unchelated form of ATP, and by a direct interaction with the catalytic subunit of the enzyme.

The physiological significance of the interrelationship between the effects of guanyl nucleotides and the catecholamines may be to coordinate the response of the heart to catecholamines arriving at the cell exterior with events occuring within the cell. Goldberg et al, (176) proposed that increased utilization of GTP could explain the decreased adrenergic sensitivity of cardiac adenylate cyclase after exposure to cholinergic agents which activate guanylate cyclase (15, 171). The presently observed differences in the effects of GTP and GDP provide an additional regulatory mechanism that is sensitive to the GTP/GDP ratio. Thus GTP hydrolysis not only decreases the concentration of the stimulatory species GTP, but also increases the level of GDP, which is a competitive inhibitor of the GTP-induced stimulation of the action of beta agonists. Additionally, the lower Mg^{2+} affinity of nucleoside diphosphates could increase the effect of a change in GTP/GDP ratio on adenylate cyclase activity as the Mg^{2+}-dependency experiments indicate that MgGTP does not interact with the enzyme. By this mechanism, GTP would initially increase the response of the heart to circulating catecholamines. As the work output of the heart increases, however the cytoplasmic ATP/ADP ratio begins to fall (172) accompanied by a decreased GTP/GDP ratio as the proportions of guanyl and adenyl nucleotides

are maintained in equilibrium through the enzyme nucleoside diphosphokinase (173, 174). Thus, these responses to the guanyl nucleotides could increase the sensitivity of the heart to catecholamines while at the same time serving as negative feedback pathway that prevents excessive stimulation by these agents in the energy depleted heart.

APPENDIX

The concentrations of ATP^{4-}, $HATP^{3-}$, $MgATP^{2-}$, $MgATP^{1-}$ and Mg^{2+} present under the standard adenylate cyclase assay conditions were estimated by the sequential solution of the following equilibrium equations:

$$K_1 = [HATP]/[H][ATP]$$
$$K_2 = [MgATP]/[Mg][ATP]$$
$$K_3 = [MgHATP]/[Mg][HATP]$$

in addition to the two conservation equations for ATP and Mg^{2+}. The values used for K_1, K_2 and K_3 were $3.36 \times 10^6\ M^{-1}$, $1.74 \times 10^4\ M^{-1}$ and $1.8 \times 10^2\ M^{-1}$ respectively, as determined by Khan and Martell (175).

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