THE EFFECTS OF ADRENERGIC AMINES AND THEOPHYLLINE ON CONTRACTILE FORCE AND CYCLIC AMP

by

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ABSTRACT

Time response studies of the effects of norepinephrine and phenylephrine revealed that both agonists caused an increase in cyclic AMP levels prior to increases in contractile force. Norepinephrine caused a nearly six fold increase in cyclic AMP, whereas phenylephrine produced only a 50% increase in the nucleotide. Pretreatment with reserpine did not affect the norepinephrine cyclic AMP response; however, the phenylephrine cyclic AMP response was abolished. Reserpine pretreatment did not significantly affect the contractile responses of either amine. In the presence of propranolol, norepinephrine was found to have the ability to produce an increase in contractile force in which cyclic AMP was apparently not involved. The time course of the contractile response induced by adrenergic amines was found to be remarkably influenced by the chronotropic response in spontaneously beating preparations while the cyclic AMP response was not greatly affected. This difference in the contractile response may be due to the ability of the chronotropic response to influence the flux of calcium through the cell membrane.

At 37° C phentolamine was found to have no effect on the isoproterenol dose-response curve. Phentolamine did, however, cause the norepinephrine log dose-response (LDR) curve to shift to the right and the maximum response was attenuated. Phentolamine competitively antagonized the phenylephrine LDR curve. Propranolol caused a shift to the right of the isoproterenol LDR curve. In the presence of propranolol the efficacy of

of isoproterenol was increased, which may be related to the ability of propranolol to antagonize binding and sequestration of internal free calcium. Propranolol competitively antagonized only the second component of phenylephrine activity which was probably due to catecholamine release.

At 22° C phentolamine was found to produce an apparent nonspecific, noncompetitive antagonism of the inotropic response to isoproterenol, norepinephrine and phenylephrine. This apparent blockade was found to be related to the ability of phentolamine to increase the inotropic effect of low temperature so as to leave little room within the limits of contractility for the agonist to produce a positive inotropic response. The efficacy of all the amines appeared to be increased in the presence of propranolol which was found to antagonize the inotropic effects of low temperature and thus leave more room within the limits of contractility for an amine to produce an inotropic response. The cyclic AMP response was found to be blocked by propranolol at 37° C, 22° C, and 17° C. Phentolamine did not block the cyclic AMP response at any temperature tested. Exposure to phenoxybenzamine 17° C for 45 minutes before testing at 37° C did not significantly affect either the contractile response or the cyclic AMP response from control experiments. It is therefore concluded that there is no interconversion of alpha and beta adrenergic receptors mediated by temperature. The interpretation of the effects of adrenergic antagonists at low temperature is complicated by their ability to modify the inotropic effect of temperature alone.

Theophylline alone produced a 50% increase in cyclic AMP levels, however, this response was abolished in reserpine pretreated tissue. In addition, theophylline was found to exert a direct contractile effect which was unrelated to cyclic AMP. The effect of theophylline on cyclic AMP appeared to be additive with the norepinephrine and phenylephrine responses. The effect of theophylline on amine-induced cardiac cyclic AMP and contractile force showed no correlation between the contractile and the cyclic AMP effects at the different times tested. It therefore seems logical that the cardiac effects of theophylline are not mediated through cyclic AMP. These results support the view that the methylxanthines exert their effects on heart through changes in calcium metabolism.

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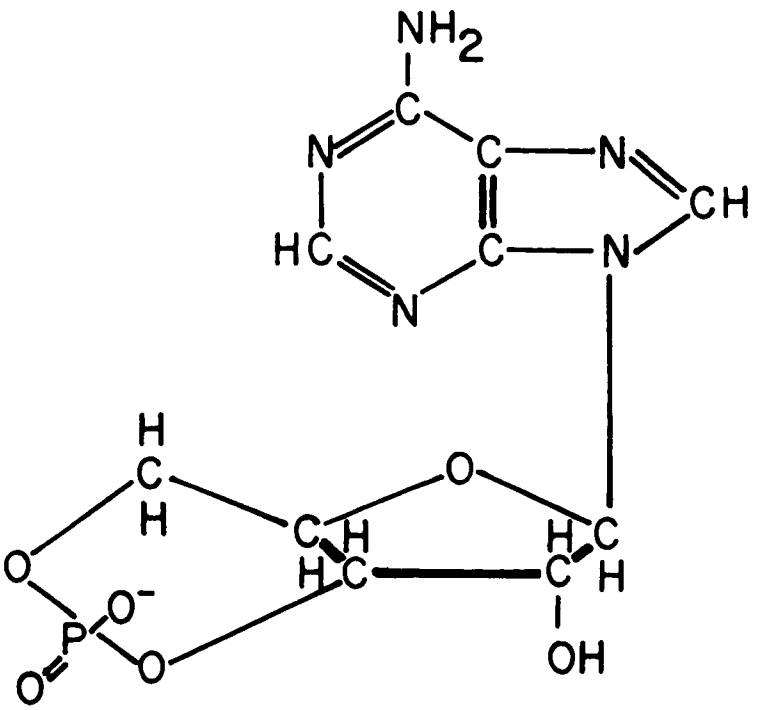
CHAPTER I
INTRODUCTION

The Role of Cyclic AMP in the Regulation of Cellular Processes

Cyclic AMP was discovered in the course of investigations on the mechanism of the glycogenolytic action of epinephrine and glucagon in liver (Rall et al., 1957: Sutherland and Rall, 1957). It was found that the response of liver homogenates to the hormones occurred in two stages. In the first stage, a particulate fraction of homogenates produced a soluble, heat-stable factor when incubated with hormones, Mg^{2+} ions, and ATP. In the second stage, this factor stimulated the formation of active phosphorylase in supernatant fractions of homogenates in which the hormones themselves were inactive. This heat-stable "factor" was isolated and eventually was determined to have the nucleotide structure depicted in figure 1 (Sutherland and Rall, 1957, 1958; Lipkin et al., 1959).

The proposition was then advanced that cyclic AMP was an intracellular "mediator" of the glycogenolytic action of epinephrine in liver and other tissues by virtue of increasing the concentration of the physiologically active species of glycogen phosphorylase (Rall and Sutherland, 1961; Sutherland and Rall, 1960). Subsequent investigations have provided evidence for the involvement of cyclic AMP in the actions of



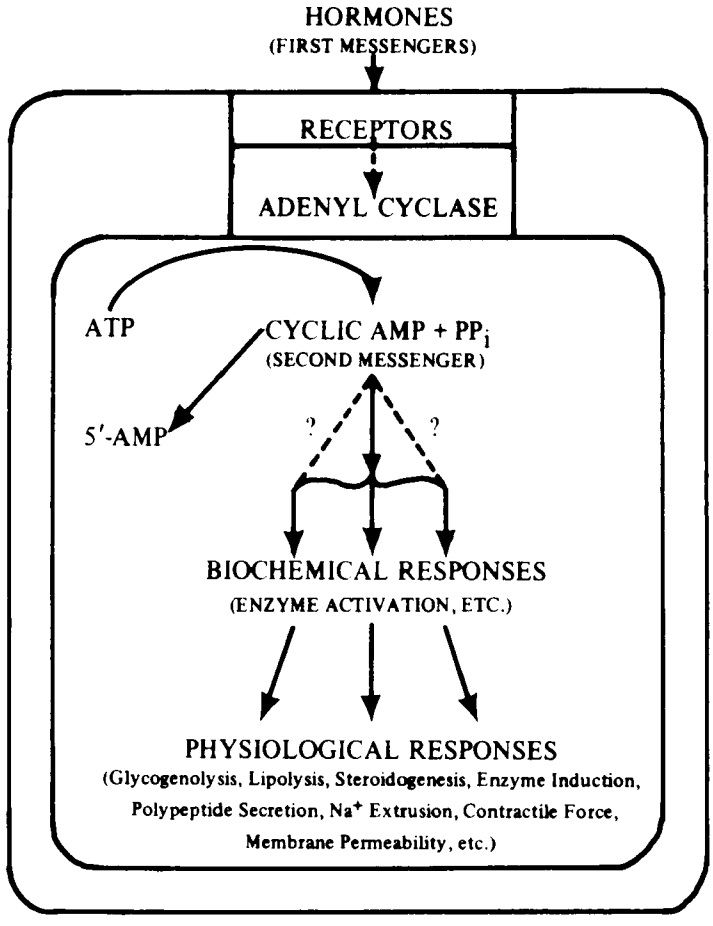
Adenosine 3',5'-phosphate (cyclic AMP)

Figure 1

a large number of polypeptide and amine hormones in a wide variety of tissues from a diverse array of animal species (Breckenridge, 1970; Gilman and Rall, 1971; Hardman et al., 1971; Liddle and Hardman, 1971; Robinson et al., 1968; Sutherland et al., 1957).

In considering the evidence linking cyclic AMP to hormonal regulation, Sutherland et al., 1965, formulated what has become known as the "second messenger concept" which is illustrated in figure 2 (Rall and Gilman, 1970). According to this concept, the first messengers, the hormones themselves, would interact with tissue-specific sites in the plasma membrane and produce an activation of the enzyme adenylate cyclase, also in the plasma membrane. The augmented level of cyclic AMP produced would then proceed to influence a variety of cell structures through a sequence of events that are largely unknown. Upon removal of the hormone the system would return to normal activity owing in part to the conversion of cyclic AMP to 5'AMP by one or more of a family of cyclic nucleotide phosphodiesterases, of which certain of the known members are susceptible to inhibition by the methyl-xanthines. Thus, cyclic AMP could be viewed as a kind of "trigger", setting in motion responses determined by the programming in the individual cell (Rall, 1972).

Subsequent investigations have served both to expand the number of hormones, tissues and cellular processes that can be linked to the regulatory function of cyclic AMP and



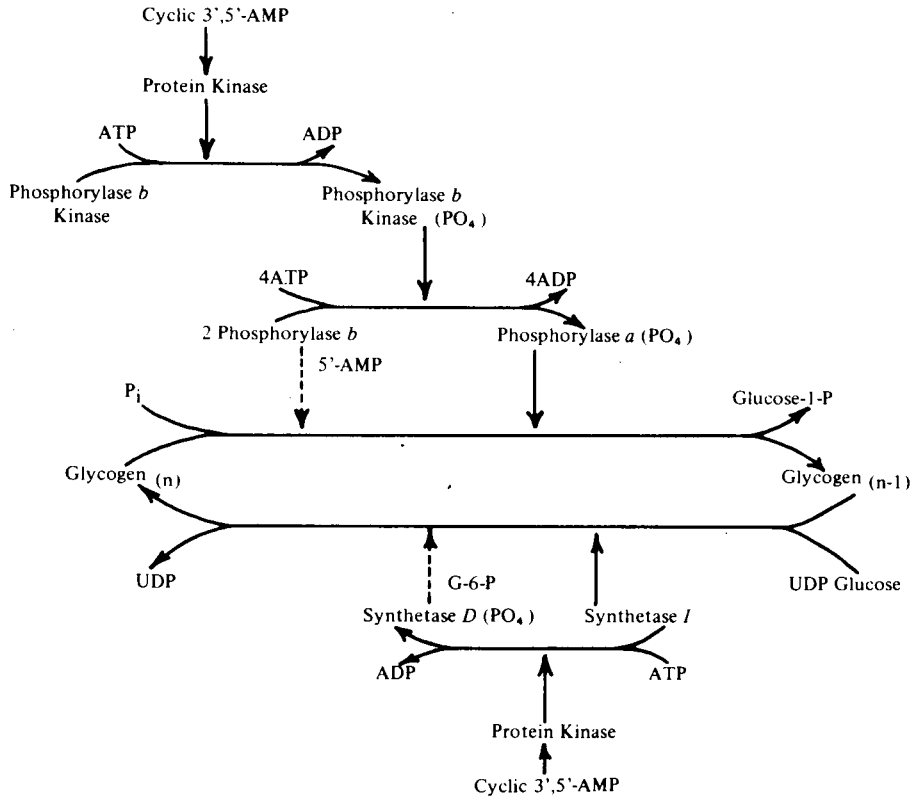
Second Messenger Concept

Figure 2

to provide greater insight into the mechanism of action of cyclic AMP. These investigations have revealed the existence of a family of protein kinases in a variety of tissues that are stimulated by cyclic AMP and that transfer phosphate groups to a variety of cellular proteins resulting in some cases in a marked change in biological properties (Greengard and Kuo, 1970; Langan, 1970; Walsh et al., 1970).

The mechanism of action of cyclic AMP in regulating glycogen metabolism is the best understood. Cyclic AMP is thought to interact with a phosphoprotein kinase (phosphorylase kinase kinase), causing an increased conversion of the inactive species of phosphorylase kinase to the active species by the transfer of phosphate from ATP to serine residues (Walsh et al., 1968). The phosphorylase kinase, in turn, converts the less active species of phosphorylase (e.g., phosphorylase b in muscle) to the active species (e.g., phosphorylase a in muscle) by an analogous phosphorylation reaction. On the other hand, cyclic AMP accelerates the conversion of the active species of glycogen synthetase to the less active species by a similar phosphorylation reaction catalyzed by an enzyme known as glycogen synthetase I kinase (Rosell-Perez and Larner, 1964) (figure 3).

Other investigations have also served to provide warning that rigorous application of the second messenger concept will not allow adequate explanation of certain observations. There have been a number of instances in which the application of cyclic AMP or its derivatives on intact cell preparations



The Mechanism of Action of Cyclic AMP in Regulating Glycogen Metabolism

Figure 3

has not faithfully reproduced the effects of the hormone in question. One example is the failure of N^6 butyryl cyclic AMP to bring about release of K^+ ions while efficiently inducing amylase release from rat parotid slices (Batzri et al., 1971a). The release of amylase is mediated by stimulation of beta-adrenergic receptors (and by cyclic AMP) while the release of K^+ ions involves stimulation of alpha-adrenergic receptors and thus, would not be expected to be mediated by increased levels of cyclic AMP (Batzri et al., 1971b). These data call to attention the possibility that hormones may exert effects not mediated by cyclic AMP by setting in motion parallel sequences of events that may or may not interact with those initiated by the formation of cyclic AMP. In some instances it may be extremely difficult to determine whether two different populations of receptors are involved (Rall, 1972).

In summary, the second messenger concept has been enormously useful in suggesting experimental approaches for the dissection of regulatory effects of a large number of hormones and in suggesting ways in which chemical agents and other environmental factors might influence hormone action. We realize, however, that cyclic AMP is only one of many second messengers, and that the "classical" second messenger view will not allow adequate explanation of all biological phenomenon.

The Positive Inotropic Effects of Catecholamines and Cyclic AMP

Considerable evidence suggests that the positive inotropic effects of catecholamines in the heart are mediated by cyclic AMP. Following administration of catecholamines, myocardial

cyclic AMP levels rise before or at least simultaneously with the positive inotropic response.(Robison et al.,1965; Drummond et al., 1966; Wastila et al., 1972). Effects on phosphorylase transformation and glycogenolysis occur later after changes in contractility are evident (Williamson, 1965; Mayer, 1963). Catecholamine agonists exhibit the same general order of potency in stimulating adenylate cyclase in vitro and in increasing contractility of the intact heart (Sutherland et al., 1968; Mayer, 1972). Other agents with positive inotropic effects, such as glucagon, prostaglandins, and histamine also stimulate myocardial adenylate cyclase (Farah and Tuttle, 1960; Murad and Vaughn, 1969; Sobel and Robinson, 1969; Klein and Levey, 1971).

Whereas the effects of epinephrine, norepinephrine, and isoproterenol on cyclic AMP levels of cardiac muscle are well documented, previous reports on the effects of phenylephrine are controversial. McNeill and Verma. 1973, have reported that phenylephrine increases cyclic AMP levels in perfused guinea pig hearts, and Drummond and Hemmings, 1973, have reported similar findings in rat heart. On the other hand previous studies by Benfey, 1971, and Benfey and Carolin, 1971, on formation of cyclic AMP by particle preparations from chicken and rabbit heart, as well as rabbit heart slices, did not reveal increased cyclic AMP formation on the addition of phenylephrine. McNeill et al., 1972, similarly did not find an increase in cyclic AMP formation with phenylephrine in a guinea pig cardiac

particle preparation. Lack of stimulation of cyclic AMP formation in broken cell preparation or tissue slices does not, however, exclude an effect in the intact heart. The response of adenylate cyclase to drugs and hormones may disappear on homogenation of the tissue (Oye and Sutherland, 1966).

The adrenergic receptors of cardiac muscle have been generally classified as beta-receptors on the basis of reports by some investigators that they could find no alpha-adrenergic antagonism of the positive inotropic response to adrenergic agonists (Nickerson and Chan, 1961; Moran and Perkins, 1961). Other investigators have reported evidence supporting the existence of alpha-adrenergic receptors in the myocardium. Govier, 1968, using guinea-pig atria, observed that a portion of the positive inotropic response to adrenergic agonists was blocked by alpha-antagonists and this portion was greater in response to epinephrine and norepinephrine than to isoproterenol. Similar observations were made using a second parameter of myocardial function, increased functional refractory period. Wenzel and Su, 1966, observed that phentolamine potentiated the contractile response of rat myocardium to epinephrine and norepinephrine and that complete blockade of the positive inotropic response to adrenergic agonists could only be achieved using both alpha and beta antagonists. Osnes and Oye, 1975, have reported that when phenylephrine was combined with the beta-blocker propranolol, a dissociation between cyclic AMP levels and contractile activity was found.

Cyclic AMP accumulation did not seem to be required for the inotropic response caused by alpha stimulation.

Phenylephrine was shown by Trendelenburg et al., 1962, to be a direct-acting amine, as judged by dose-response curves on the nictitating membrane of reserpine-treated cats. Daley et al., 1966, however, found that this compound released tritiated norepinephrine from mouse heart and Govier, 1968, reported that phenylephrine has not only a direct effect, but also an indirect action through the release of stored catecholamines. Yoo and Lee, 1970, have reported that in isolated rabbit atria in which catecholamines had been almost completely depleted by reserpine, phenylephrine exerted a positive inotropic action which was not significantly different from that observed on normal atria.

The first part of this study was undertaken to determine the time course of cyclic AMP changes in response to norepinephrine and phenylephrine. It was also intended to separate the direct and indirect effects through the release of stored catecholamines by phenylephrine on cyclic AMP.

The Dissociation of Cardiac Inotropic and Adenylate Cyclase Activating Adrenoceptors

Observations from several laboratories have suggested that the classification of adrenergic receptors into alpha and beta subtypes may be susceptible to change. Several groups have reported that in isolated perfused frog hearts, stimulation of cardiac rate and contractility by catecholamines has the properties of a classical beta adrenergic response when

experiments are preformed at warm temperatures (25-37° C) but of an alpha adrenergic response when experiments are performed at cold temperatures (5°-15° C) (Kunos and Szentivanyi, 1968; Buckley and Jordan, 1970; Kunos et al., 1973; Benfey et al., 1974). At warm temperatures, the order of potency of agonists in stimulating these preparations--isoproterenol > adrenaline > noradrenaline--is classical for a beta adrenergic receptor. Similarly, effects of the catecholamines at warm temperatures are blocked by propranolol but not by the alpha adrenergic antagonist phentolamine. When the same experiments are performed at temperatures below 25° C, the order of potency of agonists is reversed to that characteristic of alpha adrenergic receptors. Also at lower temperatures alpha adrenergic antagonists such as phenoxybenzamine and phentolamine block the effects of adrenaline, whereas beta adrenergic antagonists such as propranolol are ineffective. Graduations of response can be achieved by varying the temperature between 37° C and 10° C. Similar observations have been reported for the rat heart. (Kunos and Szentivany, 1968; Benfey et al., 1974). On the basis of such observations, Kunos et al., 1973, proposed that alpha and beta adrenergic receptors may represent allosteric configurations of the same active site which could be modulated by among other factors, temperature.

Caron and Lefkowitz, 1974, however, were not able to confirm the interconversion of receptors using the adenylate cyclase system model for study of the molecular properties of

beta adrenergic receptors. They reasoned that if, in fact, alpha and beta adrenergic receptors merely represent temperature sensitive transitions in the state of single macromolecule, then the ability of catecholamines to stimulate and of adrenergic antagonists to block stimulation of adenylate cyclase should vary with temperature in a fashion analogous to that reported above. In each case they found that stimulation of the adenylate cyclase activity by catecholamines had the characteristics of a beta adrenergic response at high (37° C) and low (15° C) temperatures. The beta antagonist, propranolol was an effective inhibitor of catecholamine-induced stimulation of the enzyme and the alpha adrenergic antagonist phentolamine was ineffective under all the conditions examined.

Subsequently Benfey, Kunos, and Nickerson, 1974, reported the dissociation of cardiac inotropic and adenylate cyclase activating adrenoceptors. They found that when the ambient temperature was reduced, the adrenoceptors mediating cyclic AMP production changed very little; they were blocked as effectively as at the higher temperature by propranolol and were not blocked by phenoxybenzamine. However, the adrenoceptors mediating the inotropic response were markedly changed by the decrease in temperature; phenoxybenzamine now inhibited this response and the inhibitory activity of propranolol was reduced about tenfold. It was concluded that the adrenoceptors that mediate cardiac inotropic responses at physiological temperatures are distinct from those that mediate the production of cyclic AMP, and that the activation of adenylate cyclase

and the accumulation of cyclic AMP are probably not intermediate steps in cardiac inotropic responses to catecholamines.

The inotropic response in cardiac muscle may be mediated through either alpha or beta receptors, However, and inotropic response mediated by an alpha receptor would not be expected to be associated with increased levels of cyclic AMP. A dissociation between the beta receptor, cyclic AMP, and the inotropic response would cast serious doubt on the cyclic AMP-second messenger concept. In these studies (Kunos and Szentivany, 1968; Kunos et al., 1973; Benfey et al., 1974), the inotropic and cyclic AMP responses have been consistently reported in dose ratios and not as complete dose or time response curves which would have been more revealing as to the mechanisms involved and which might in fact change the interpretation. It was therefore decided, as the second part of this project, to throughly investigate the biochemical and physiological changes induced by lower temperature in mammalian cardiac muscle to determine if more complete data would substantiate or refute the cyclic AMP-second messenger view concerning the beta-adrenergic receptor.

The Effect of Interaction Between Catecholamines and Theophylline on Contractility and Cardiac Cyclic AMP

It has been suggested that one of the criteria for involving cyclic AMP in the mechanism of action of cardiac-stimulant drugs would be to demonstrate an enhancement of the drug effect by the addition of a phosphodiesterase inhibitor to the system. The compounds usually used to demon-

strate this interaction are the methylxanthines, and more specifically, theophylline. Theophylline is known to have a positive inotropic effect and to inhibit phosphodiesterase. (Butcher and Sutherland, 1962). The addition of a phosphodiesterase inhibitor should enhance the effect of drugs which increase the synthesis of cyclic AMP by decreasing the degradation of the cyclic nucleotide. It has further been suggested that the positive inotropic effect of the methyated xanthines, themselves, may be mediated through cyclic AMP (Sutherland et al., 1968).

In support of this concept, it has been shown that theophylline will enhance the inotropic effect of catecholamines and histamine (Rall and West, 1963; McNeill and Muschek, 1972), and will also enhance the phosphorylase-activating effect of epinephrine (Hess et al., 1963). In some studies, however, the dose of theophylline required to potentiate the phosphorylase-activating effect of epinephrine or to induce cardiac glycogenolysis was found to produce a negative rather than a positive, inotropic effect (Hess et al., 1963; Vincent and Ellis, 1963). Kukovetz et al., 1973, were able to produce an elevation in cyclic AMP using a perfusion of theophylline, however, McNeill et al., 1974, found that when theophylline was injected into the heart, an increase in contractility and phosphorylase 'a' was noted, but cyclic AMP values were not affected. It was further reported that while theophylline did enhance the inotropic and phosphorylase-activating effect of both amines, cyclic AMP, increased by the injection of the

amines, was not further increased when the hearts were perfused concomitantly with theophylline.

Thus, we see that the literature dealing with the interaction between catecholamines and theophylline on contractility and cyclic AMP in the heart is controversial. It was therefore decided, as the third part of this project, to investigate this interaction, introducing as a new variable the time of exposure to theophylline before the addition of catecholamines. We hoped by this method to be able to resolve some of the differences in the literature and at the same time to determine the importance of cyclic AMP in mediating the cardiac effects of the methylxanthines in rate controlled preparations.

CHAPTER II

MATERIALS AND METHODS

I. Animals

Wistar rats (200-300 g.) of either sex were used throughout the investigation. They received food and water *ad. libitum*.

II. Preparation of Tissues

All animals were pretreated with heparin (8mg/kg, subcutaneously) one to two hours prior to sacrifice.

A. Atria

The animal was stunned by a heavy blow at the base of the skull and the neck was quickly broken. The chest was then opened with scissors by a parasternal incision. The heart was exposed, lifted gently by grasping the apex, and removed by cutting the great vessels which suspend it from above and behind. The heart was immediately placed in oxygenated Chenoweth-Koelle solution (Chenoweth and Koelle, 1946) at 20° C and was gently compressed a few times to express residual blood. This phase of the preparation, from sacrifice to cooling of the excised heart, consumed 30 seconds of less. At this time, the left or right atrium can be recognized and removed directly from its position above the ventricles by cutting with small scissors. The

two atria are easily differentiated on the basis of rhythm and shape, since the right atrium beats spontaneously and has an irregular, asymmetrical shape. The left is quiescent after isolation and has a regular triangular shape with smoothly rounded corners and sides. However, speed is essential. At no time should the tissue be allowed to remain out of the dissecting dish for more than 20 seconds. Threads were then placed at one corner and the opposite side of the atrium and one was made fast to the muscle holder; then, after tranfer from the dissecting dish to the muscle chamber, the other was made fast to the transducer as shown in figure 4. Using a Harvard isometric tension clamp, tension on the atrium was adjusted to one gram. Prior to the addition of any drugs, the atria were allowed to stabilize for periods of fifteen to thirty minutes.

With appropriate experience, the entire procedure from sacrifice to mounting in the chamber should not take longer than three or four minutes. Experience has indicated that the longer this period, the less viable a preparation is obtained, even though the preparation is well oxygenated in the dissecting dish. This is particularly evident for the quiescent left atrium, but less critical for the spontaneously beating right atrium. (Katzung, 1968; Levy, 1971)

B. The Isolated Perfused Whole Heart

For the isolated whole heart preparations, the animal was killed and the heart, with at least one cm. of aorta attached, was quickly removed and placed in dissecting dish

Figure 4. (A) Typical configuration of a vertical chamber and muscle holder (MH) for *in vitro* atrial and ventricular preparations. (B) Detail of a muscle holder incorporating punctate electrodes. The entire holder is fabricated from a single piece of 0.65- or 0.97-cm acrylic plastic. MC, Muscle chamber, jacketed or immersed in a constant temperature bath; FD, fritted glass disk for dispensing O_2-CO_2 bubbles (a fine polyethylene capillary inserted from the top was used in the present series of experiments; M, isolated myocardial tissue tied down with the thread T, and connected at its upper end by a second thread, or fine wire to the transducer "Trans"; S, wires to stimulator; G, groove for anchoring thread used to tie down the muscle; E, punctate electrodes (silver or platinum); W, insulated wires to stimulator.

(Ref. Katzung, 1968)

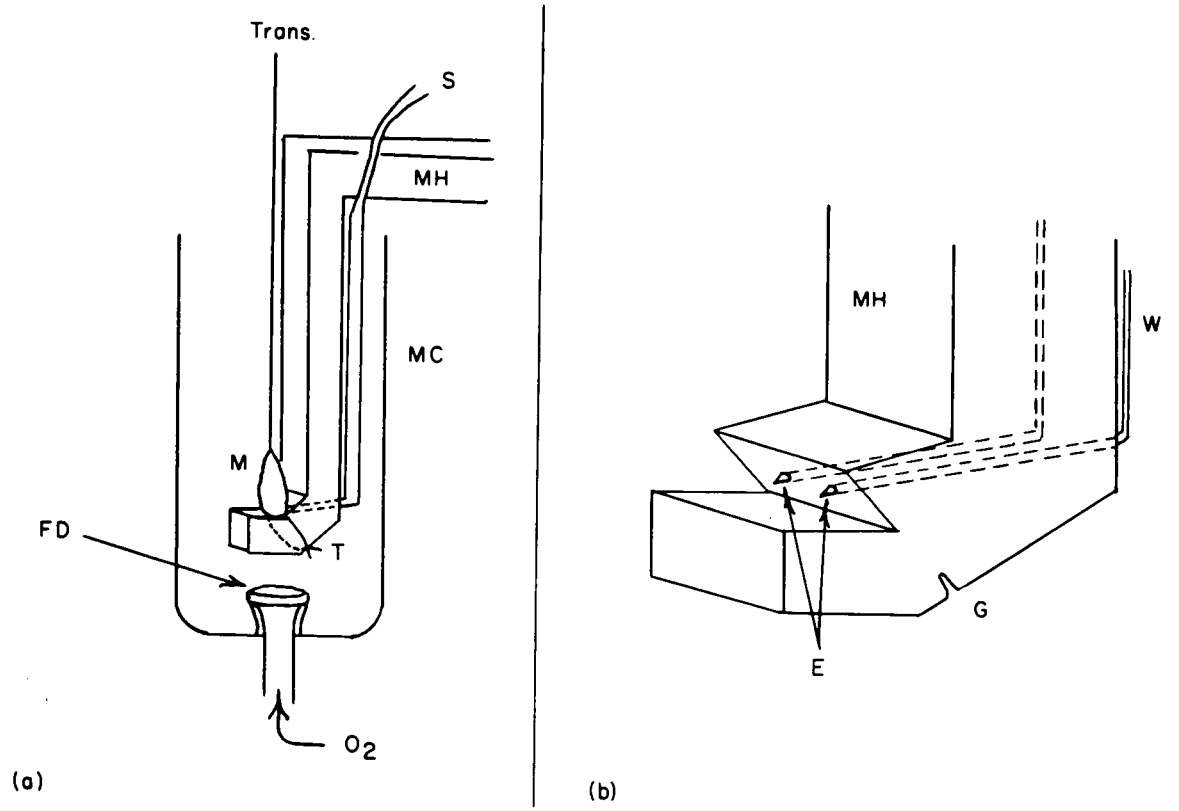


Figure 4

containing oxygenated Chenoweth-Koelle solution exactly as described for the atria preparation. The aorta was located and dissected free and all other vessels connected to the heart were trimmed away. The aorta was cut just below the point where it divides and the heart was transferred to the perfusion apparatus where the aorta was tied onto the glass cannula. Care must be taken to see that air bubbles do not enter the aorta and any bubbles which have formed in the cannula should be removed. A Palmer clip was placed in the apex of the ventricles and an attached string was connected to the force-displacement transducer via a pair of pulleys. Using a Harvard isometric tension clamp, tension on the ventricles was adjusted to one gram. Prior to the addition of any drugs, the hearts were allowed to stabilize for periods of fifteen to thirty minutes.

III. Apparatus

A 100 ml. tissue bath was used for the experiments with isolated atria. Water was circulated through the outer jacket to maintain the solutions in the bath at the desired temperature. A mixture of oxygen (95%) and carbon dioxide (5%) was bubbled through the solutions by means of a fine polyethylene capillary inserted from the top. A needle and thread were used to tie the atria to a muscle holder which incorporated punctate electrodes as shown in figure 4. A second thread attached to apex of the atria was connected to a Grass force-displacement transducer, and isometric contractions were recorded on a Grass model 79 polygraph (Grass Instruments Quincy, Mass., U.S.A.). The left atria were electrically driven at one Hz.

with three millisecond square wave pulses at four volts by a Grass model S 6 stimulator. A mechanical beater was used to assure rapid equilibration of all parts of the bath. Tension on the atria was adjusted by means of a Harvard isometric tension clamp. At various times after addition of drug, the bath was rapidly lowered and the atria were frozen with chilled Wollenberger tongs (Wollenberger et al., 1960) which had been kept in a beaker of 2-methylbutane immersed in dry ice. All tissues were stored at -80° C until assayed for cyclic AMP.

The isolated heart system was essentially that described by Fallen, et al., 1967. It consists of a modified Langendorff apparatus in which the perfusion of the heart via the aorta was maintained at a constant flow rate of four ml per minute rather than at a constant pressure. This was achieved by use of a Holter Microinfusion roller pump (Extracorporeal Medical Specialties, King of Prussia Pa., model RL 175). Oxygenated solutions were pumped into the heart from a reservoir. Water was circulated through the outer jacket of the reservoir and a water jacket surrounding the final section of tubing to maintain the heart and solutions at 37° C. Contractility was monitored by means of a Palmer clip placed in the apex of the heart and connected to a Grass force-displacement transducer and recorded on a Grass model 7 polygraph. At various times the hearts were frozen and stored as described for the atria preparation.

IV. Solutions

A modified Locke-Ringer solution described by Chenoweth and

Koelle, 1946, was used exclusively in all experiments. The buffer was prepared by dissolving the following amounts of reagents in one liter of distilled water (all weights are expressed as grams of the anhydrous compound): Glucose, 1.9; NaCl, 7.0; KCl, 0.42; $CaCl_2$, 0.24; $MgCl_2$, 0.20. A pH of 7.4 was obtained by adding $NaHCO_3$ (2.0 g/liter for the atria experiments or 1.75 g/liter for the whole heart experiments) and bubbling a mixture of 95% oxygen and 5% carbon dioxide through the solution.

In the isolated atria experiments, concentrated solutions of drugs were pipetted into the tissue bath in amounts required to achieve the desired molar concentration. The cumulative method of determining dose response curves was used. When the maximum response for a particular dose of a drug was reached, sufficient drug was added to achieve the next highest molar concentration of that drug. In some experiments, propranolol (10^{-6} M) or phentolamine (10^{-6} M) were added to the bath fifteen minutes prior to drug administration. Phenoxybenzamine was added forty five minutes before a drug treatment. Reserpine pre-treatment consisted of a dose of 3 mg/kg intraperitoneally twenty four hours prior to the experiment. In the case of isolated whole hearts, drugs dissolved in Chenoweth-Koelle solution were injected via a side-arm cannula. All doses were calculated as the free base.

V. Cyclic AMP Assay

The method of extraction has previously been described by Gilman, 1972. Frozen tissue samples (20-35mg) were homogenized

in 2 ml of cold 5% trichloroacetic acid (TCA). The samples were centrifuged on a bench centrifuge set at its maximum speed for ten minutes. TCA supernatants were then extracted five times with 5 ml of ether after the addition of 0.2 ml of 1 N HCl. The purpose of this extraction was to remove the TCA. Residual ether was removed byyblowing nitrogen gas over the surface of the samples for one to two minutes and the aqueous extracts were lyophylized and redissolved in 0.8 ml of Tris/EDTA buffer (pH 7.5) 0.05 M containing 4 mM EDTA. Two 50 µl portions of the sample were used in the cyclic AMP determination.

B. Cyclic AMP Determination

Cyclic AMP concentrations were determined using an Amersham/Searle Cyclic AMP Assay Kit code TRK 432. This kit is a commercial adaptation of a simplified competitive protein-binding assay for cyclic AMP in plasma which has been previously described by Latner and Prudhoe (1973). The method is based on the competition between unlabeled cyclic AMP and a fixed quantity of tritium labeled cyclic AMP for binding to a protein which has a high specificity for cyclic AMP (Gilman, 1970). The amount of labeled protein-cyclic AMP complex formed is inversely related to the amount of unlabeled cyclic AMP present in the assay. The concentration of cyclic AMP in the unknown is determined by comparison with a linear standard curve.

Separation of the protein bound cyclic AMP from the unbound nucleotides is achieved by adsorption of the free nucleotide on charcoal, followed by centrifugation on a

bench centrifuge set at its maximum speed for fifteen minutes at 4° C, as initially described for this assay by Brown et al., 1971. The only modification we made in the use of the kit is that the entire supernatant is removed for liquid scintillation counting by decanting instead of a 200 µl sample as is recommended. After centrifugation, even if the tubes are maintained at 4° C, the samples must be removed for liquid scintillation counting within ten minutes if there is to be no significant change in the radioactivity of the supernatant. Attempts at removing the 200 µl sample required excess time and, in addition, caused disturbances to the charcoal pellet. Both of these factors contributed to a loss of accuracy.

The alternative procedure for calculation of results which is presented in the booklet accompaning each cyclic AMP kit was used. By this method the standard curve in competitive protein binding assays is presented as percent radioactivity bound to the binding protein, plotted against the concentration of the standard. This was found to give a better separation of values on the calibration curve in the range of interest (0.25 to 4.0 pmole/incubation tube).

The use of a high viscosity scintillation gel is essential (Latner and Prudhoe, 1973), and the recommended cocktail, PCS Solubilizer (Amersham/Searle's catalog number 196097) was used. Counts per minute were used directly for calculation in all assays in accordance with the manufacturer's recommendation. Variations in the efficiency of counting between samples are not normally great enough to necessitate conversion of the

counts per minute to disintegrations per minute. A counting time of four minutes was used for all samples in this assay. At no time was the count rate/sample less than at least three times the background count.

VI. Drugs and Chemicals

The drugs used in these experiments were l-phenylephrine hydrochloride (Sigma Chemical Co.), l-norepinephrine hydrochloride (Sigma Chemical Co.), l-isoproterenol hydrochloride (Winthrop Pharaceutical Co.), propranolol (Ayerst Laboratories, Inc.), phentolamine hydrochloride (Ciba Pharmaceutical Co.), phenoxybenzamine hydrochloride (Ciba Pharmaceutical Co.), theophylline (Merck and Company, Inc.), reserpine (Sigma Chemical Co.), and heparin sodium (Nutritional Biochemicals Corporation). All other chemicals, solvents, and reagents were analytical reagent grade. They were used as they were recieved without further purification.

VII. Calculations and Statistical Methods

The cyclic-AMP results are expressed as picomoles of cyclic AMP per milligram wet weight of tissue. Contractility is presented as percentage increase over control or as change in contractile force (g). The results were pooled and averaged and the standard error of the mean was determined at each experimental point. The data were compared by means of the Students t-test for unpaired data. A probability of less than 0.05 was chosen as the criterion of significance.

CHAPTER III

RESULTS

Time-Response Effects of Norepinephrine and Phenylephrine on Cardiac Cyclic AMP and Contractility

Preliminary experiments (figure 12) established that 10^{-5} M norepinephrine or 10^{-4} M phenylephrine produced the maximum positive inotropic effect that could be obtained with these drugs. With the use of these maximally effective doses, time-response studies were carried out to determine the effects of the agonists on contractility and cyclic AMP.

In driven left atria, norepinephrine (figure 5) significantly increased cyclic AMP from a control value of 0.26±0.03 pmole/mg wet weight to a peak of 1.16±0.13 at 10 seconds and 1.18±0.15 at 15 seconds. Cyclic AMP then decreased to 0.67±0.09 pmole/mg at 60 seconds. Contractile force was significantly elevated at 10 seconds and peaked at 60 seconds (86.9±12.2% increase over control). Phenylephrine (figure 6) significantly increased cyclic AMP from a control value of 0.26±0.03 pmole/mg to 0.35±0.03 at 10 seconds. Cyclic AMP values remained constant at this level for 100 seconds. Contractile force was significantly elevated at 20 seconds and peaked at 100 seconds (58.3±7.3% increase over control).

Reserpine pretreatment did not significantly affect the contractile response to either amine or to the cyclic AMP increase produced by norepinephrine. However, the treatment abolished the phenylephrine induced cyclic AMP change.

Similar results were obtained in the spontaneously beating rat atrium (figure 7). Norepinephrine significantly increased cyclic AMP from a control value of 0.26±0.01 pmoles/mg to 1.02±0.06 at 10 seconds and a peak of 1.38±0.08 at 15 seconds. Cyclic AMP then decreased to 0.87±0.05 at 60 seconds. Contractile force was significantly elevated at 10 seconds and peaked at 35 seconds (0.50±0.07 g increase). Phenylephrine significantly increased cyclic AMP from a control value of 0.28±0.01 p mole/mg to 0.35±0.02 at 10 seconds. Cyclic AMP values remained constant at this level for 60 seconds. Contractile force was significantly elevated at 10 seconds and peaked at 40 seconds (0.58±0.06 g increase). Again reserpine pretreatment did not significantly affect the contractile response to either amine or to the cyclic AMP produced by norepinephrine. The treatment abolished the phenylephrine induced cyclic AMP change.

Attempts to measure the time-response effects of norepinephrine and phenylephrine in driven right ventricle strips were not successful. Norepinephrine (10^{-5} M) increased cardiac cyclic AMP from a control value of 0.30±0.02 pmole/mg to 0.36±0.03 at 10 seconds, 0.39±0.03 at 15 seconds, and 0.31±0.03 at 20 seconds. This small cyclic AMP response may indicate that the ventricle strips had been extensively

damaged during dissection. Dobson et al., 1974, have recently reported on the problems encountered with this preparation. No attempts were made to measure the cyclic AMP response to phenylephrine in the driven ventricle strips.

The time-response effects of norepinephrine and phenylephrine on cardiac cyclic AMP were next determined in the perfused rat heart (figure 8). Norepinephrine (1 ug) significantly increased cyclic AMP from a control value of 0.24±0.02 pmole/mg to 0.45±0.05 at 5 seconds and a peak value of 0.71±0.08 at 10 seconds. Cyclic AMP then decreased to 0.48±0.04 at 15 seconds. Norepinephrine (2 ug) increased cyclic AMP to 1.18±0.06 pmole/mg at 10 seconds (a value near the maximum response obtained in atria). Since a previous report from this laboratory (McNeill and Verma, 1973) had indicated that the dose of phenylephrine needed to elicit an increase in cyclic AMP in the perfused heart was 1000 times greater that for norepinephrine, a 2 mg dose of phenylephrine was used. This dose of phenylephrine increased cardiac cyclic AMP from a control value of 0.24±0.02 pmole/mg to 0.38±0.04 at 10 seconds. Pretreatment with reserpine abolished the phenylephrine induced cyclic AMP response.

As phenylephrine had been shown to be capable of increasing contractile force without causing a change in cyclic AMP levels in reserpine pretreated tissue, it was decided to see if norepinephrine was capable of a similar action in the presence of propranolol. Time-response effects of norepinephrine (10^{-5} M) on cardiac cyclic AMP and

contractility in the driven rat atrium in the presence of propranolol (10^{-6} M) and phentolamine (10^{-6} M) were determined (figure 9). In the presence of phentolamine, norepinephrine significantly increased cyclic AMP from a control value of 0.26±0.03 pmole/mg to 0.85±0.07 at 15 seconds. Contractile force was significantly elevated at 10 seconds and peaked at 70 seconds (0.70±0.08 g). In the presence of propranolol, norepinephrine did not increase cardiac cyclic AMP from control values although contractile force was significantly increased at 15 seconds and peaked at 180 seconds (0.53±0.06 g increase).

It is well known that suprathreshold stimulation of the order of two or three times threshold intensity may produce detectable release of catecholamines. This effect is more apparent at higher stimulus frequencies (e.g. 100 to 200 beats per minute) (Levy, 1971). Since threshold voltage was 1-2 volts and the left atria preparations were being stimulated at 4 volts, it was decided to test the effect of different voltages and rates of stimulus on cardiac cyclic AMP. The effects of different voltage stimuli (4,10,15, and 20 volts) can be seen in figure 10. No significant difference was detected for any of the voltages tested. Likewise, different rates of stimulus (2 Hz vs 1 Hz) had no effect on cardiac cyclic AMP levels (figure 11).

The Effect of Temperature on Cardiac Inotropic and Adenylate Cyclase Activating Adrenoceptors

As a preliminary experiment it was necessary to establish the cumulative dose-response curves of isoproterenol, nor-

epinephrine, and phenylephrine for driven left atria and spontaneously beating right atria at 37° C (figure 12). As expected, the order of potency of agonists in stimulating these preparations was isoproterenol > norepinephrine > phenylephrine. The order of efficacy was norepinephrine > phenylephrine > isoproterenol. The amines produced equally potent effects on the right and left atria respectively. However, because the spontaneously beating right atria were subject to increasing rate, the efficacy was less than in the left atria.

The cumulative dose-response curves of isoproterenol, norepinephrine, and phenylephrine for driven left atria were next determined at 17° C (figure 13). The positive inotropic effect of all the amines was greatly reduced by lowering the temperature from 37° C to 17° C. Phenylephrine, produced a significant negative inotropic effect (-0.07±0.04 g) at 10^{-4} M. The order of efficacy was now norepinephrine > isoproterenol > phenylephrine. The order of potency of agonists in stimulating these preparations at 17° C was norepinephrine=isoproterenol > phenylephrine.

The effect of temperature changes on the strength of contraction of driven rat left atria is shown in figure 16. Contractile force first decreased from 0.78±0.08 g at 37° C to 0.61±0.07 g at 30° C, then increased to 1.36±0.13 g at 18° C and 1.34±0.12 g at 17° C. The effects of phentolamine (10^{-6} M) and propranolol (10^{-6} M) on the inotropic effect of temperature change at 22° C are also shown in figure 16.

Phentolamine increased the inotropic effect of temperature change from 0.99±0.10 g to 1.25±0.13 g. Propranolol decreased the strength of contraction from 0.99±0.10 g to 0.74±0.08 g.

Figure 14 shows the cumulative dose-response curves of isoproterenol, norepinephrine, and phenylephrine at 37° C; in the absence of antagonists, in the presence of propranolol (10^{-6} M), and in the presence of phentolamine (10^{-6} M) for driven left atria at 37° C. The concentration of the agonist causing 50% of the maximum effect is marked on each curve. Phentolamine had no effect on the isoproterenol dose-response curve. Phentolamine caused a shift of the norepinephrine and phenylephrine curves to the right and suppressed the maximal response of norepinephrine. Propranolol caused a shift to the right of both the isoproterenol and the norepinephrine curves. An increase in efficacy was observed with high doses of isoproterenol in the presence of propranolol. Propranolol reduced the slope and diminished the maximum response of the phenylephrine log dose-response curve, but the curve was not shifted to the right. Reduction of the propranolol concentration to 10^{-7} M resulted in a phenylephrine LDR curve intermediate between the control and the 10^{-6} M propranolol curve, however, increasing propranolol to 5×10^{-6} M did not further suppress the inotropic effect.

Figure 15 shows the cumulative dose-response curves of isoproterenol, norepinephrine, and phenylephrine; in the absence of antagonists, in the presence of propranolol (10^{-6} M),

and in the presence of phentolamine (10^{-6} M) for the driven left atria at 22° C. Phentolamine reduced the slope and diminished the maximum response for all the amines. The LDR curves were not, however, shifted to the right as can be seen by the concentration of the agonists causing 50% of the maximum effect. Propranolol caused the isoproterenol and norepinephrine LDR curves to shift to the right. Propranolol did not shift the phenylephrine LDR curve to the right. The apparent maximum effect of all the amines was increased in the presence of propranolol.

The time response effects of norepinephrine (10^{-5} M) and phenylephrine (10^{-4} M) on cardiac cyclic AMP in the driven left atrium at 22° C are shown in figure 17. Norepinephrine significantly increased cyclic AMP from a control value of 0.30±0.03 pmole/mg to 0.63±0.06 at 30 seconds. Cyclic AMP remained constant at this level for 90 seconds. Phenylephrine did not increase cyclic AMP over control levels at this temperature.

Figure 18 shows the time-response effects of norepinephrine (10^{-5} M) and phenylephrine (10^{-4} M) on cardiac cyclic AMP in the driven left atrium at 17° C. Norepinephrine significantly increased cyclic AMP from a control value of 0.30±0.02 pmole/mg to 0.49±0.05 at 90 seconds. Phenylephrine did not increase cyclic AMP over control levels at this temperature.

The effects of propranolol (10^{-6} M) and phentolamine (10^{-6} M) on cardiac cyclic AMP in response to norepinephrine (10^{-5} M) at 17° C (90 seconds), 22° C (30 seconds), and 37° C

(15 seconds) are seen in figure 19. Propranolol blocked the cyclic AMP response to norepinephrine at all temperatures tested while phentolamine did not.

The effect of exposure to phenoxybenzamine (10^{-6} M) for forty-five minutes at 17° C and 37° C on cardiac cyclic AMP and contractility in response to norepinephrine (10^{-5} M) is shown in figure 20. Driven left atria which had been treated with phenoxybenzamine at 17° C and 37° C were first raised to 37° C before addition of norepinephrine. Contractile response and cyclic AMP were determined at their maximum response at 60 and 15 seconds respectively. If alpha and beta adrenergic receptors represent allosteric configurations of the same active site, phenoxybenzamine should irreversably block the alpha configuration at 17° C and the antagonism should remain in effect when the temperature was raised to 37° C. Phenoxybenzamine attenuated the contractile response ($P < 0.02$) for both temperatures of incubation. The cyclic AMP responses were not significantly changed by phenoxybenzamine.

The Effect of Theophylline on Amine-Induced Cardiac Cyclic AMP and Cardiac Contractility.

From figure 21 it can be seen that theophylline alone (5×10^{-4} M) increased the contractile response of the rat left atria (0.20±0.02 g) at three minutes. Reserpine pretreatment (3 mg/kg twenty-four hours before the experiment) did not significantly ($P=0.10$) decrease the maximum inotropic response (0.17±0.02 g).

The effect of exposure to theophylline for three minutes,

fifteen minutes, and sixty minutes on cardiac contractility in response to norepinephrine (10^{-5} M) in driven left atria is shown in figure 22. All increases in contractile force are calculated from the original base line before addition of theophylline. After fifteen minutes a concentration of $1x10^{-5}$ M theophylline did not alter the dose response curve of norepinephrine; $1x10^{-3}$ M theophylline produced fibrilation when combined with norepinephrine above 10^{-7} M; and $5x10^{-4}$ M significantly increased the inotropic response (0.86±0.08 g vs 0.72±0.07 g control; $P<0.05$). The same dose of theophylline ($5x10^{-4}$ M) was found to produce a negative inotropic effect at three minutes (0.41±0.05 g; $P<0.01$), and no response at all could be obtained with norepinephrine after 60 minutes exposure. Figure 23 shows that no concentration of theophylline tested was able to increase the inotropic response to phenylephrine at 15 minutes.

The effect of exposure to theophylline ($5x10^{-4}$ M) on cardiac cyclic AMP in the driven left atrium is shown in figure 24. Theophylline alone significantly increased cyclic AMP at three minutes exposure (0.38±0.04 pmole/mg vs 0.27± 0.03 control; $P<0.01$). Exposure to theophylline for three minutes did not significantly increase the peak cyclic AMP response to norepinephrine 10^{-6} M (0.73±0.09 pmole/mg vs. 0.66±0.06 control). The response of norepinephrine 10^{-5} M, however, was increased (1.34±0.14 pmole/mg vs. 1.13±0.11 control; $P<0.05$). This increase was not significant when the effect of theophylline alone was subtracted.

The effect of exposure to theophylline alone ($5x10^{-4}$ M) for 15 minutes significantly increased cyclic AMP (0.36±0.03 pmole/mg vs 0.24±0.01 control; $P<0.01$). Reserpine pretreatment with 3 mg/kg twenty-four hours before the experiment abolished the cyclic AMP increase seen with theophylline (0.27±0.04 pmole/mg vs 0.24±0.02 control). Theophylline significantly increased the cyclic AMP response to norepinephrine (10^{-6} M, 0.84±0.07 pmole/mg vs 0.66±0.06 control; $P<0.01$). Again neither increase was significant when the effect of theophylline alone was subtracted. Theophylline did not significantly increase the cyclic AMP response to norepinephrine 10^{-5} M (1.24±0.09 pmole/mg vs 1.17±0.08 control; $P>0.40$). The cyclic AMP response to phenylephrine 10^{-4} M (0.44±0.07 pmole/mg vs 0.37±0.04 control) was not significantly increased by theophylline. In all cases the effect of theophylline alone on cyclic AMP appeared to be additive with the adrenergic amine response.

Figure 5. Time-response effects of norepinephrine (10^{-5} M) on cardiac cyclic AMP and contractility in the driven left atrium at 37° C. Each point represents the mean of five to eight atria and the vertical bars represent one S.E.M.

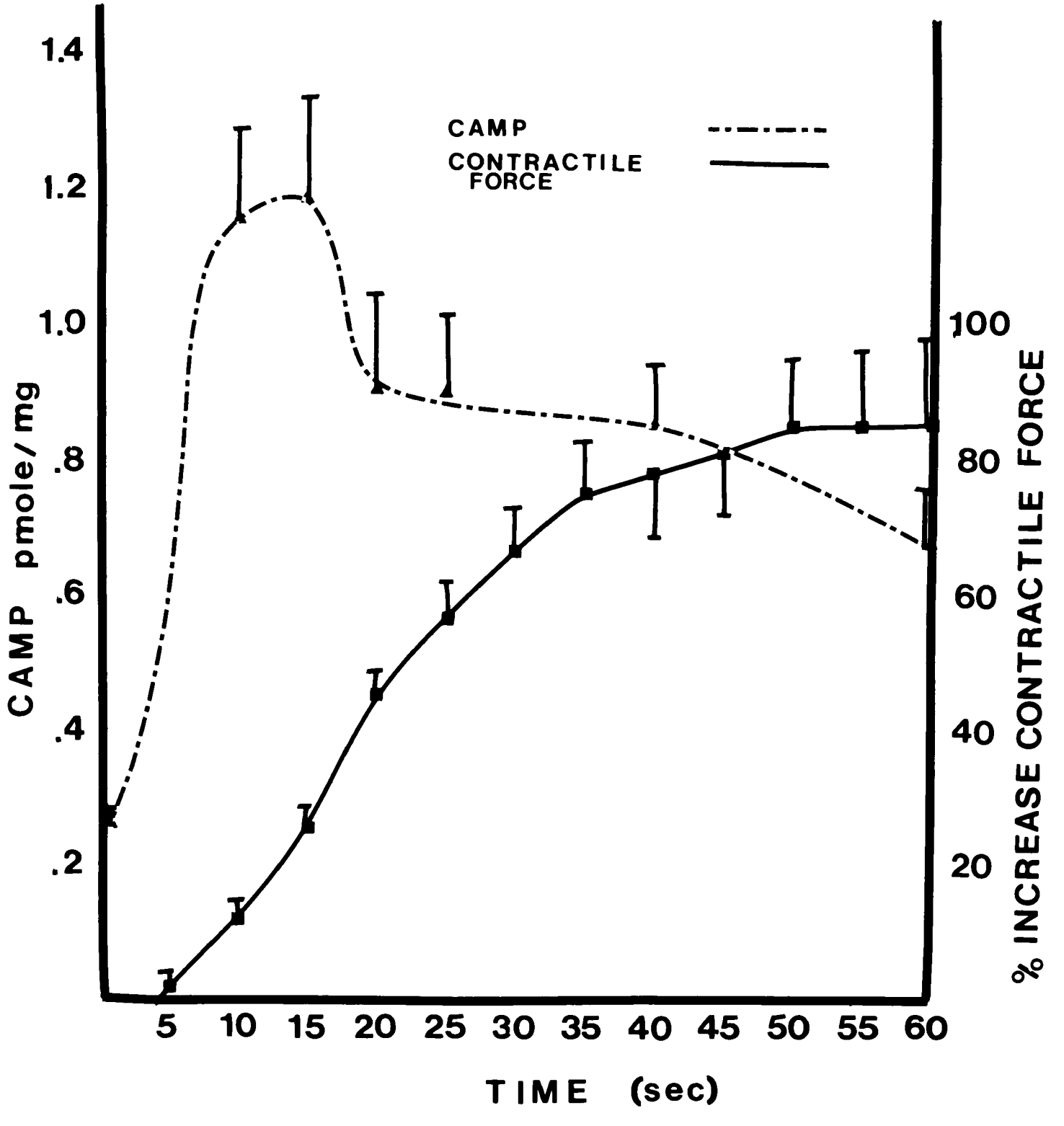

CAMP
CONTRACTILE FORCE
CAMP pmole/mg
% INCREASE CONTRACTILE FORCE
TIME (sec)
1.4
1.2
1.0
.8
.6
.4
.2
100
80
60
40
20
5
10
15
20
25
30
35
40
45
50
55
60

Figure 6. Time-response effects of phenylephrine (10^{-4} M) on cardiac cyclic AMP and contractility in the driven left atrium at 37^{0} C. Reserpine pretreatment was 3 mg/kg twenty-four hours before the experiment. Each point represents the mean of five to eight atria and the vertical bars represent one S.E.M.

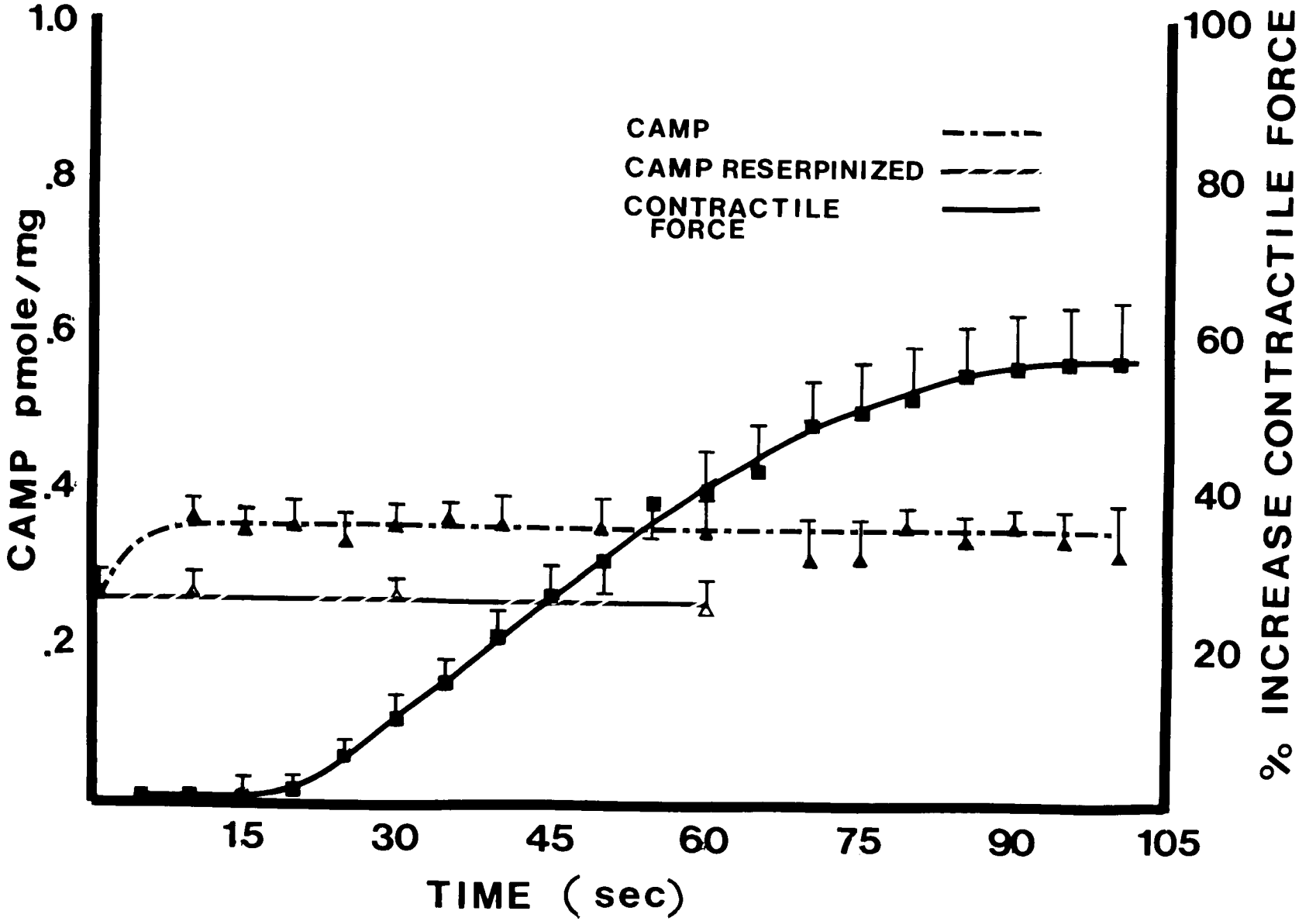

1.0
.8
.6
.4
.2
CAMP pmole/mg
100
80
60
40
20
% INCREASE CONTRACTILE FORCE
CAMP
CAMP RESERPINIZED
CONTRACTILE FORCE
15
30
45
60
75
90
105
TIME (sec)

Figure 7. Time-response effects of norepinephrine (10^{-5} M) and phenylephrine (10^{-4} M) on cardiac cyclic AMP and contractility in the spontaneously beating right atrium at 37° C. Reserpine pretreatment was 3 mg/kg twenty-four hours before the experiment. Each point represents the mean of five atria and the vertical bars represent one S.E.M.

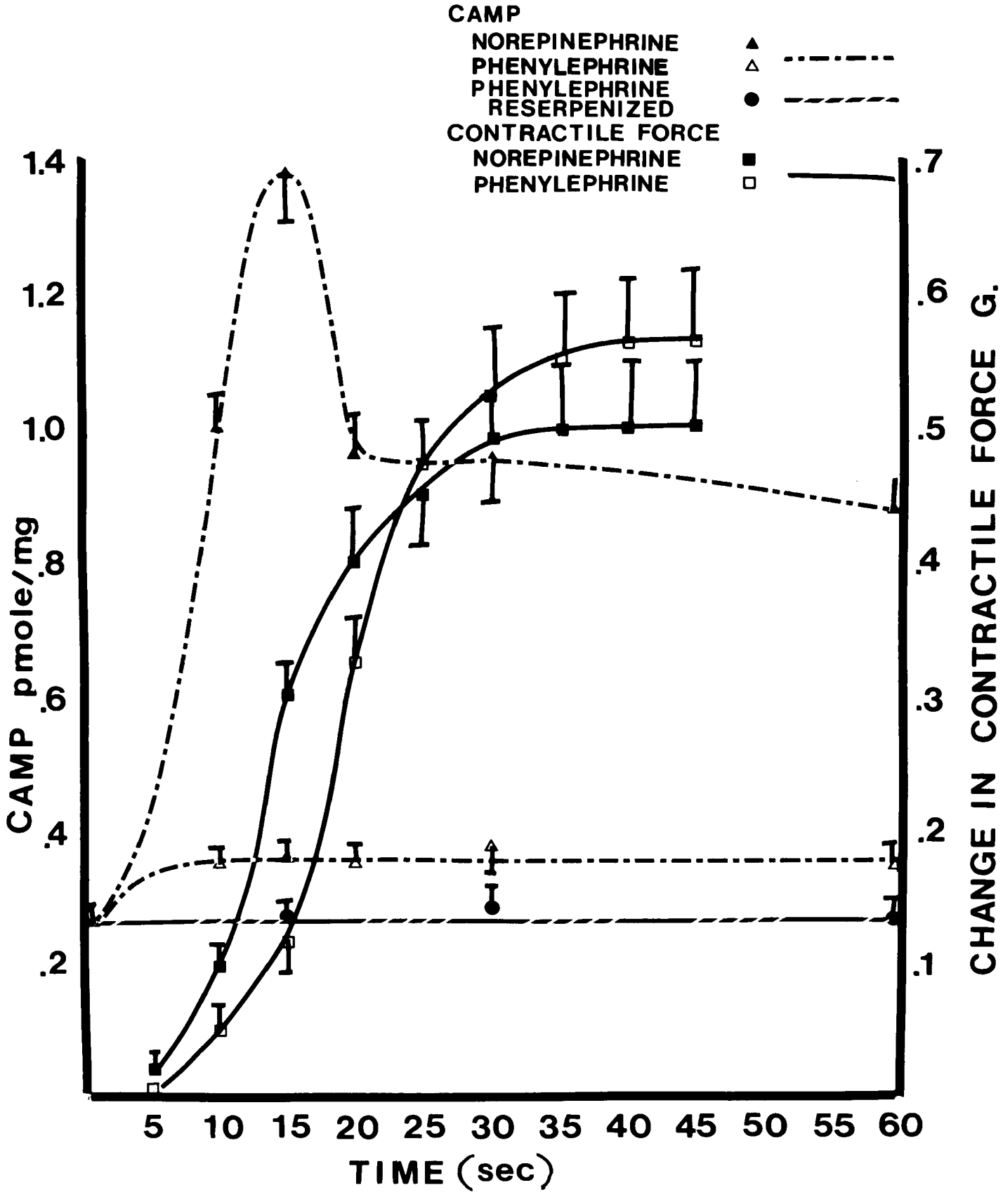

CAMP
NOREPINEPHRINE
PHENYLEPHRINE
PHENYLEPHRINE RESERPENIZED
CONTRACTILE FORCE
NOREPINEPHRINE
PHENYLEPHRINE
1.4
1.2
1.0
.8
.6
.4
.2
CAMP pmole/mg
.7
.6
.5
.4
.3
.2
.1
CHANGE IN CONTRACTILE FORCE G.
5 10 15 20 25 30 35 40 45 50 55 60
TIME (sec)

Figure 8. Time-response effects of norepinephrine and phenylephrine on cardiac cyclic AMP in theperfused rat heart at 37° C. Reserpine pretreatment was 3 mg/kg twenty-four hours before the experiment. Each point represents the mean of five to eight atria and the vertical bars represent one S.E.M.

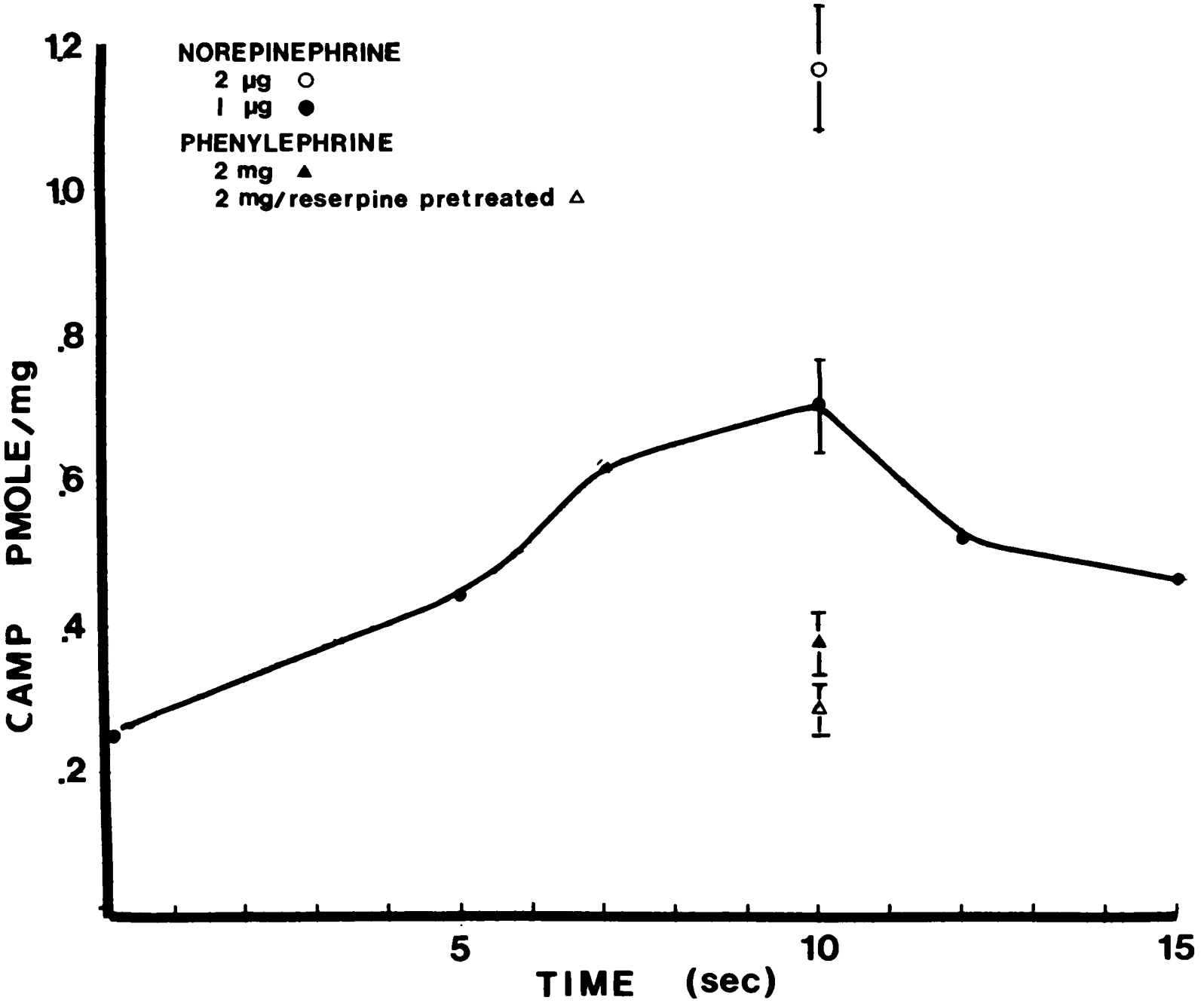

NOREPINEPHRINE
2 µg ○
1 µg ●
PHENYLEPHRINE
2 mg ▲
2 mg/reserpine pretreated △
1.2
1.0
.8
.6
.4
.2
CAMP PMOLE/mg
5
10
15
TIME (sec)

Figure 9. Time-response effects of norepinephrine (10^{-5} M) on cardiac cyclic AMP and contractility in the driven left atrium in the presence of propranolol (10^{-6} M) and phentolamine (10^{-6} M) at 37° C. Each point represents the mean of five atria.

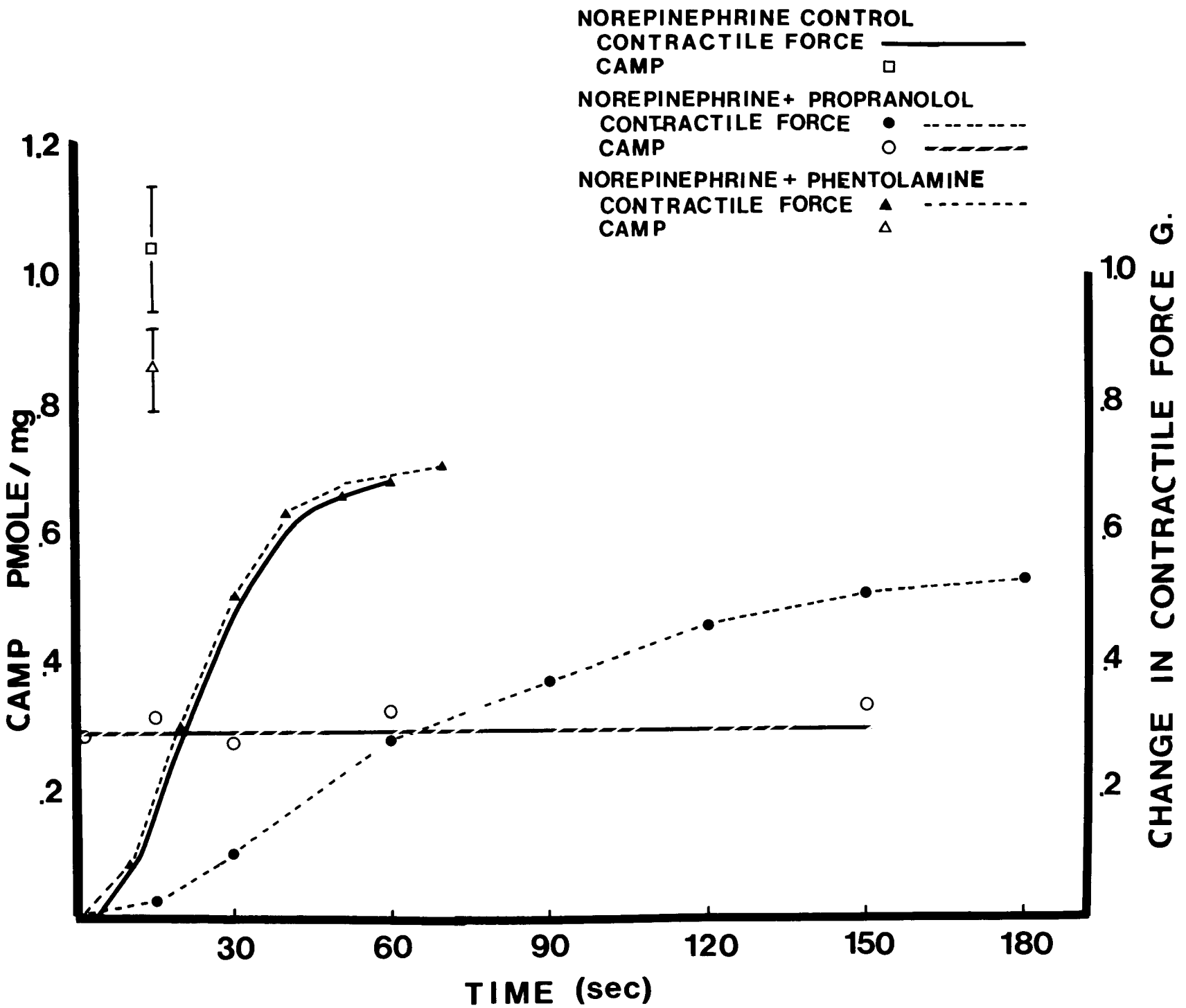
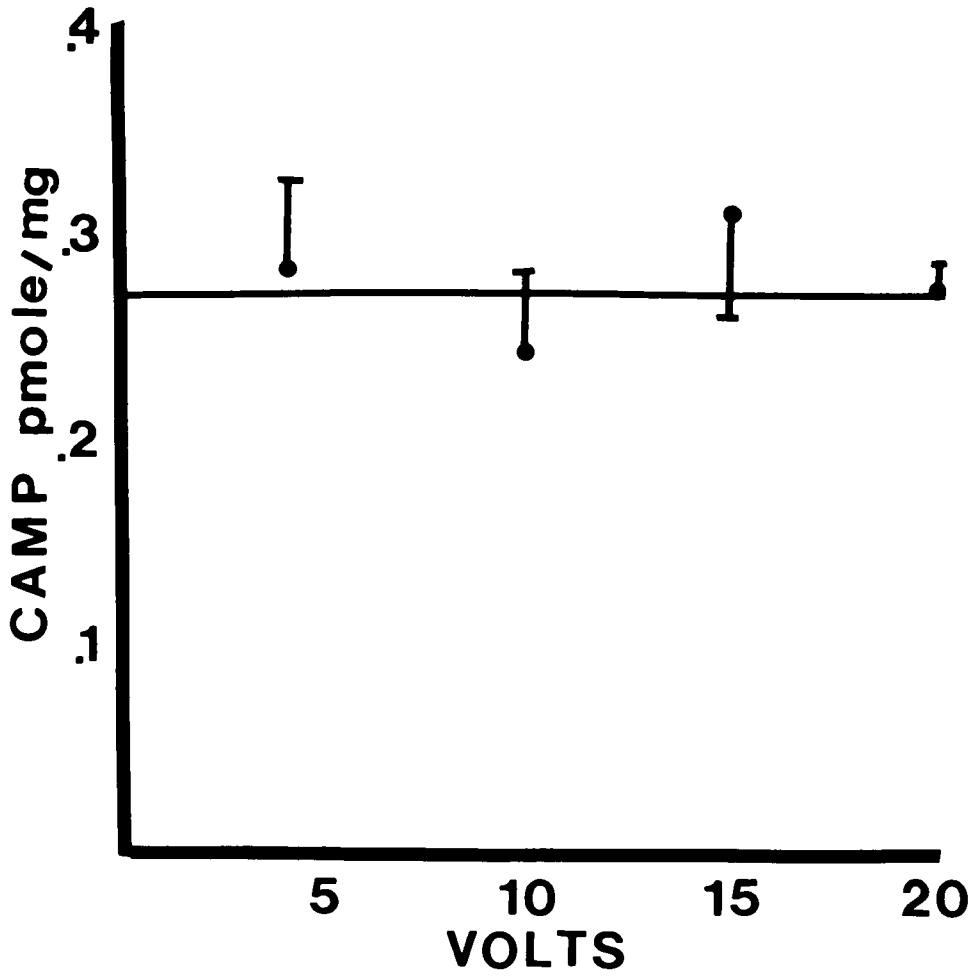

NOREPINEPHRINE CONTROL
CONTRACTILE FORCE
CAMP
NOREPINEPHRINE+ PROPRANOLOL
CONTRACTILE FORCE
CAMP
NOREPINEPHRINE + PHENTOLAMINE
CONTRACTILE FORCE
CAMP
CAMP PMOLE / mg
1.2
1.0
.8
.6
.4
.2
CHANGE IN CONTRACTILE FORCE G.
1.0
.8
.6
.4
.2
30
60
90
120
150
180
TIME (sec)

Figure 10. The effects of different voltage stimuli (4,10,15, and 20 V.) on cardiac cyclic AMP in the driven left atrium at 37° C. The muscles were stimulated at a frequency of 1 Hz. with square-wave pulses of 3 ms duration. Each point represents the mean of four atria and the vertical bars represent one S.E.M.

Figure 11. The effects of different rates of stimulus (2 Hz. vs 1 Hz.) on cardiac cyclic AMP in the driven left atrium at 37° C. The muscles were stimulated with square-wave pulses of 3 ms duration and 4 volts. Each bar represents the mean of five atria.


.4
.3
.2
.1
CAMP pmole/mg
5
10
15
20
VOLTS

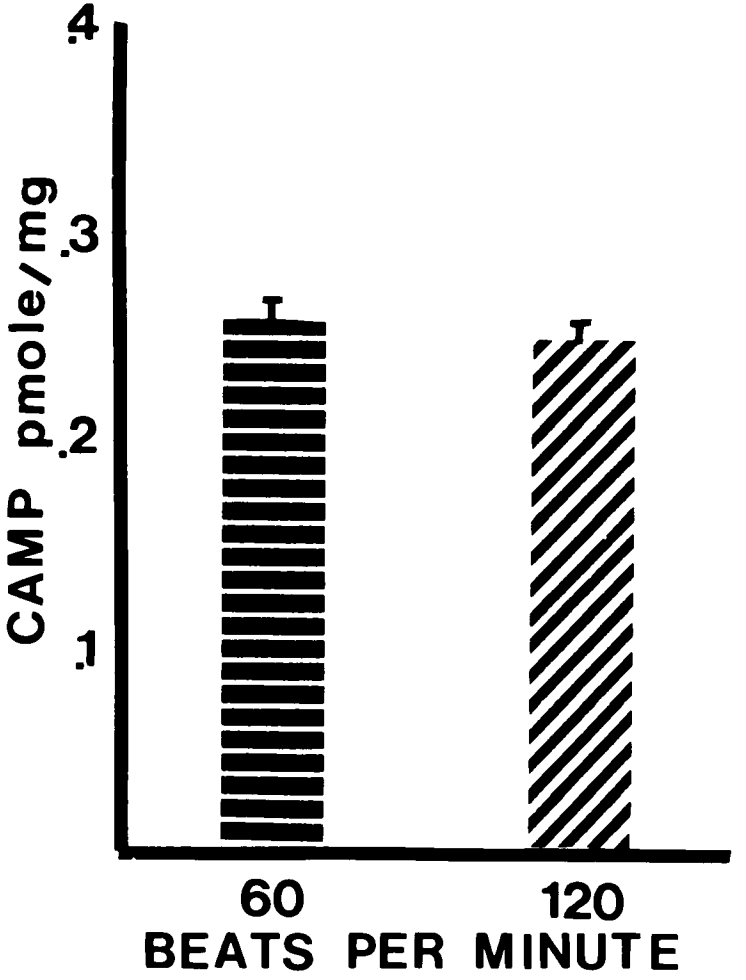

.4
.3
.2
.1
CAMP pmole/mg
60
120
BEATS PER MINUTE

Figure 12. Cumulative dose-response curves of isoproterenol, norepinephrine, and phenylephrine for driven left atria and spontaneously beating right atria at 37° C. Each point represents the mean of five to seven atria.

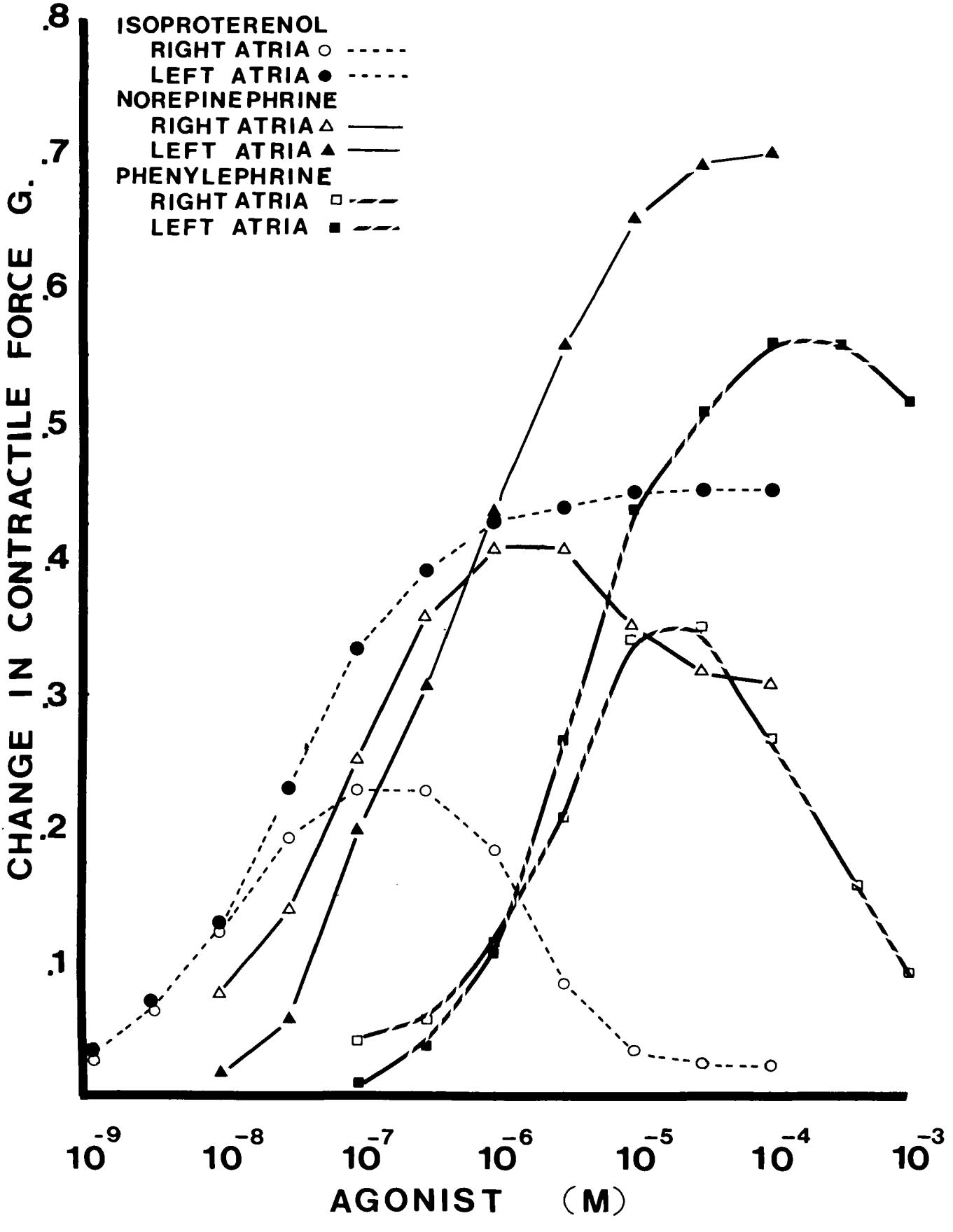

ISOPROTERENOL
RIGHT ATRIA
LEFT ATRIA
NOREPINEPHRINE
RIGHT ATRIA
LEFT ATRIA
PHENYLEPHRINE
RIGHT ATRIA
LEFT ATRIA
CHANGE IN CONTRACTILE FORCE G.
.8
.7
.6
.5
.4
.3
.2
.1
10^{-9}
10^{-8}
10^{-7}
10^{-6}
10^{-5}
10^{-4}
10^{-3}
AGONIST (M)

Figure 13. Cumulative dose-response curves of isoproterenol, norepinephrine, and phenylephrine for driven left atria at 17° C. Each point represents the mean of five atria.

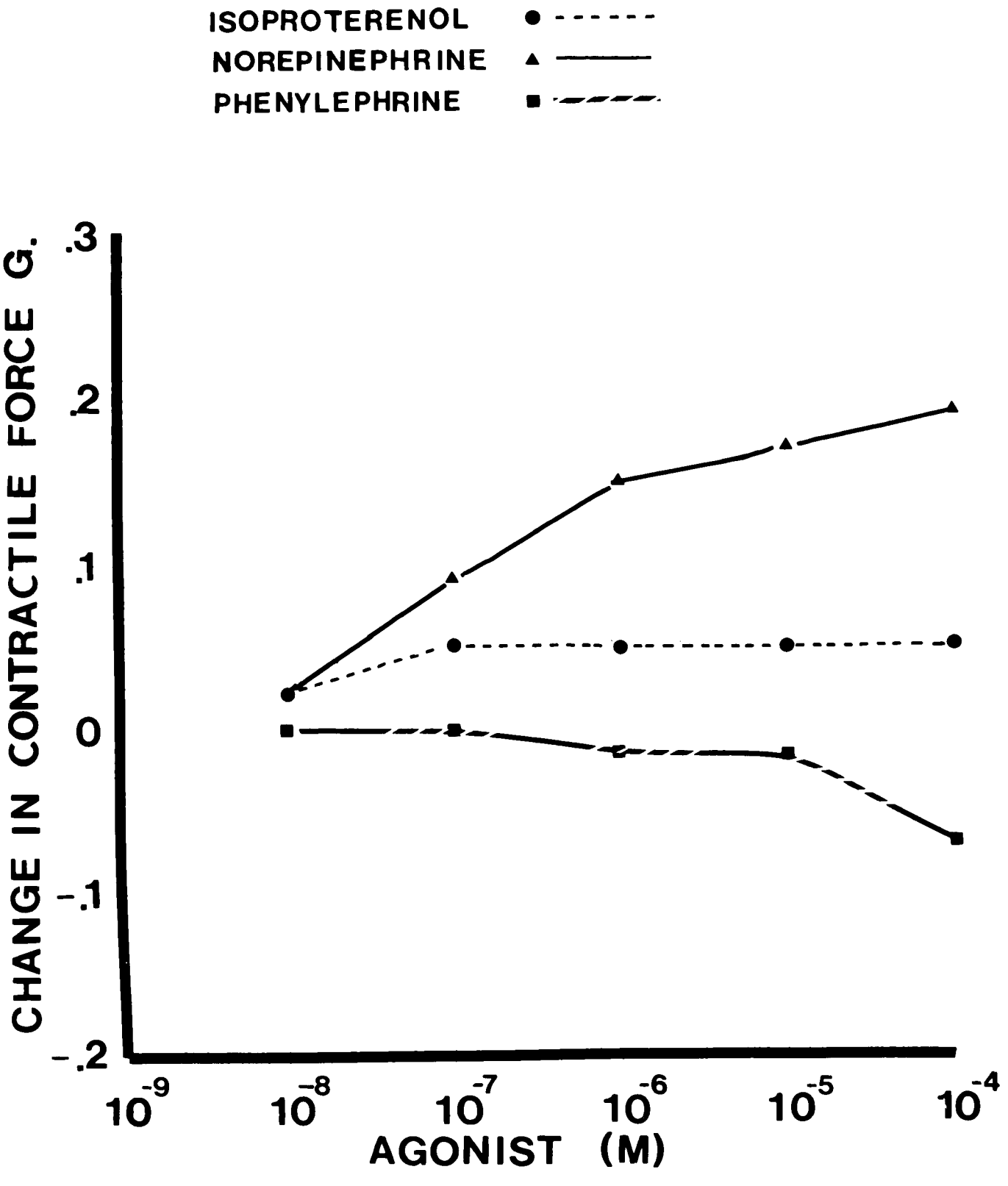

ISOPROTERENOL
NOREPINEPHRINE
PHENYLEPHRINE
CHANGE IN CONTRACTILE FORCE G.
.3
.2
.1
0
-.1
-.2
10^{-9}
10^{-8}
10^{-7}
10^{-6}
10^{-5}
10^{-4}
AGONIST (M)

Figure 14. Cumulative dose-response curves of isoproterenol, norepinephrine, and phenylephrine; in the absence of antagonists, in the presence of propranolol (10^{-6} M), and in the presence of phentolamine (10^{-6} M) for driven left atria. Each point represents the mean of five atria. The concentration of the agonist causing 50% of the maximum effect is marked on each curve with a star.

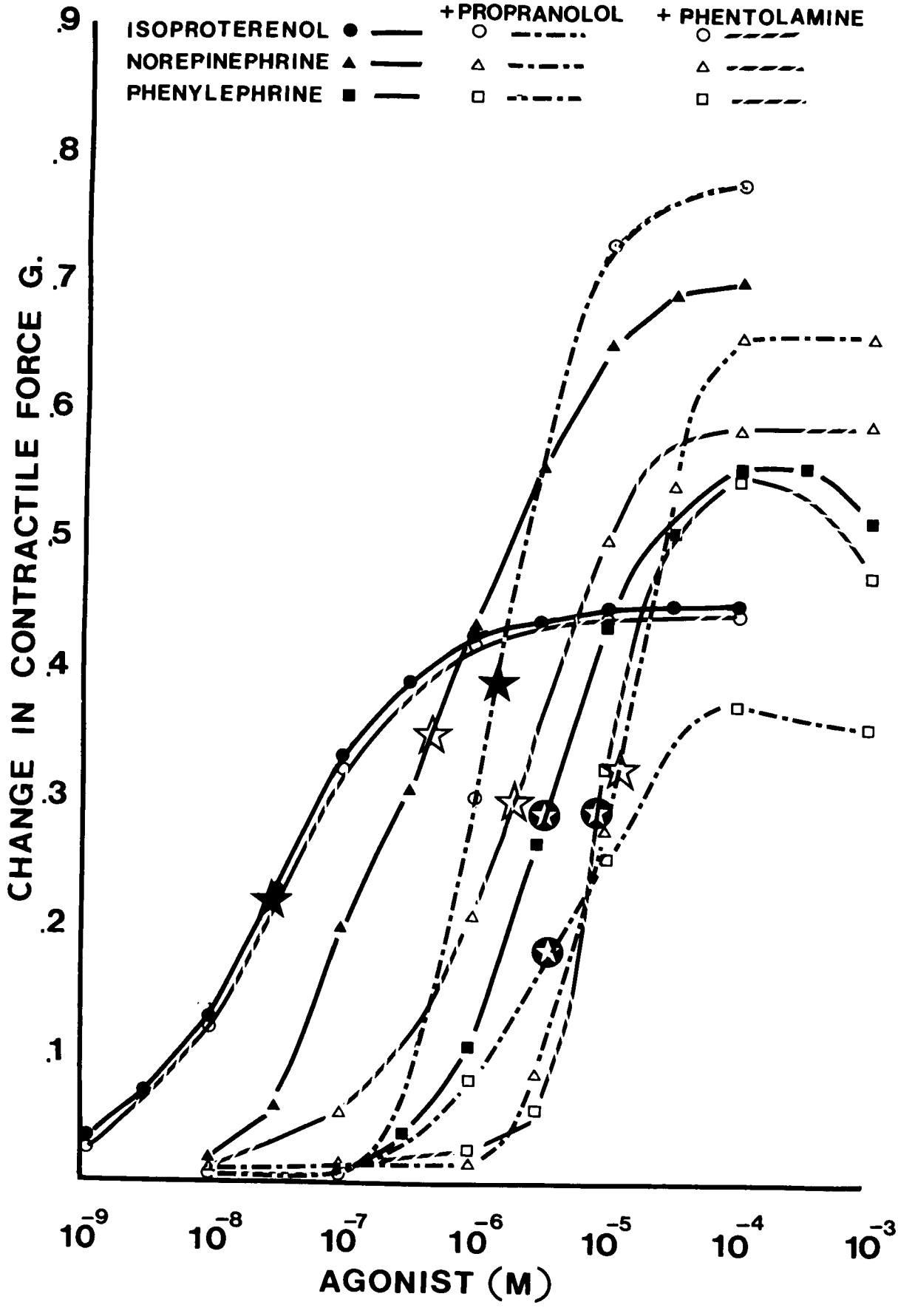

ISOPROTERENOL
NOREPINEPHRINE
PHENYLEPHRINE
+PROPRANOLOL
+ PHENTOLAMINE
CHANGE IN CONTRACTILE FORCE G.
.9
.8
.7
.6
.5
.4
.3
.2
.1
10^{-9}
10^{-8}
10^{-7}
10^{-6}
10^{-5}
10^{-4}
10^{-3}
AGONIST (M)

Figure 15. Cumulative dose-response curves of isoproterenol, norepinephrine, and phenylephrine: in the absence of antagonists, in the presence of propranolol (10^{-6} M), and in the presence of phentolamine (10^{-6} M) for driven left atria at 22^{0} C. Each point represents the mean of five atria. The concentration of the agonist causing 50% of the maximum effect is marked on each curve with a star.

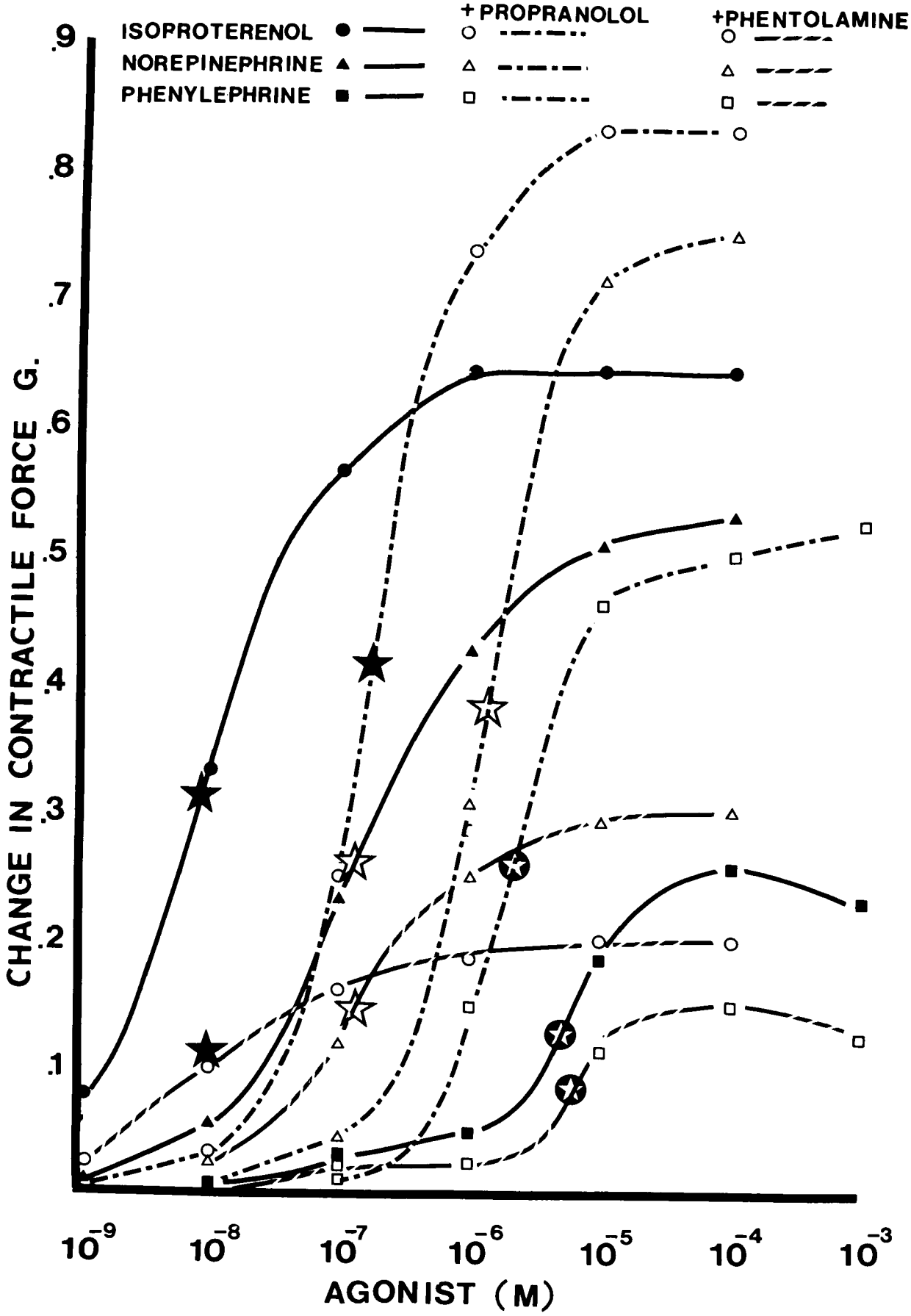

ISOPROTERENOL
NOREPINEPHRINE
PHENYLEPHRINE
+PROPRANOLOL
+PHENTOLAMINE
CHANGE IN CONTRACTILE FORCE G.
.9
.8
.7
.6
.5
.4
.3
.2
.1
10^{-9}
10^{-8}
10^{-7}
10^{-6}
10^{-5}
10^{-4}
10^{-3}
AGONIST (M)

Figure 16. The effect of temperature changes on the strength of contraction of driven left atria. The effect of phentolamine (10^{-6} M) and propranolol (10^{-6} M) on the inotropic effect of temperature change is shown at 22° C. Each point represents the mean of five atria and the vertical bars represent one S.E.M.

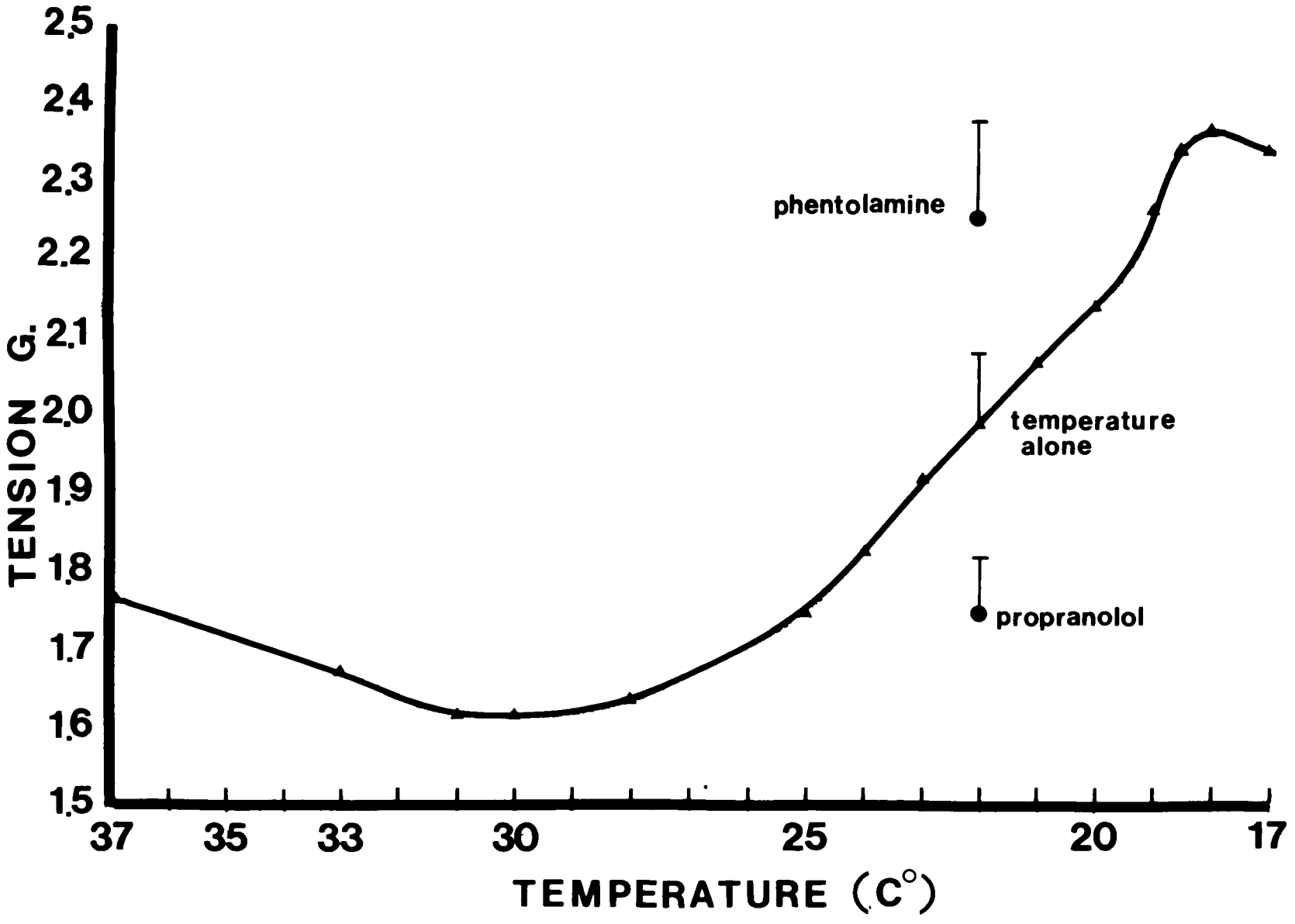

2.5
2.4
2.3
2.2
2.1
2.0
1.9
1.8
1.7
1.6
1.5
TENSION G.
phentolamine
temperature
alone
propranolol
37
35
33
30
25
20
17
TEMPERATURE (C°)

Figure 17. Time-response effects of norepinephrine (10^{-5} M) and phenylephrine (10^{-4} M) on cardiac cyclic AMP in the driven left atrium at 22° C. Each point represents the mean of five atria.

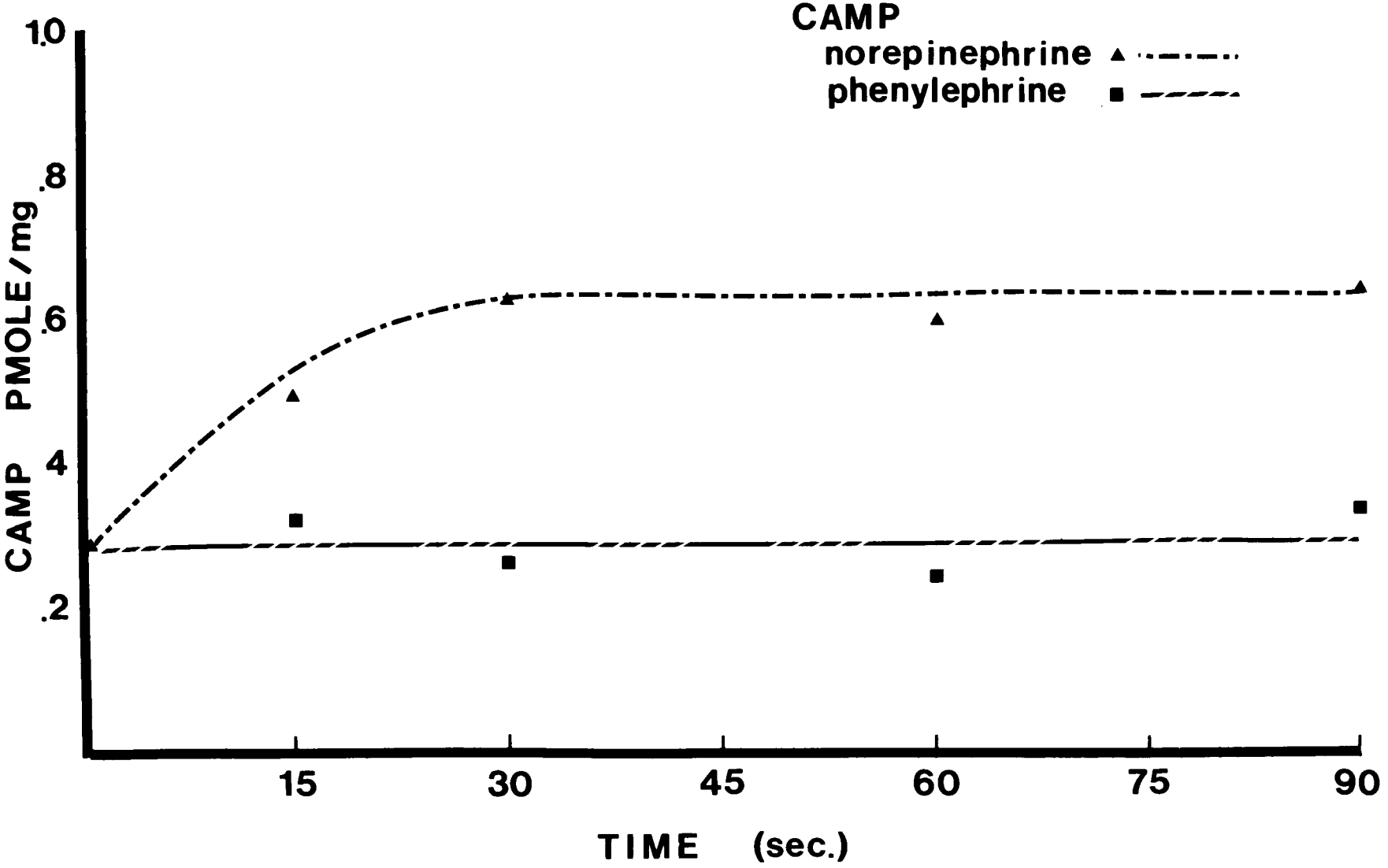

CAMP
norepinephrine
phenylephrine
CAMP PMOLE/mg
1.0
.8
.6
.4
.2
15
30
45
60
75
90
TIME (sec.)

Figure 18. Time-response effects of norepinephrine (10^{-5} M) and phenylephrine (10^{-4} M) on cardiac cyclic AMP in the driven left atrium at 17° C. Each point represents the mean of 5 atria.

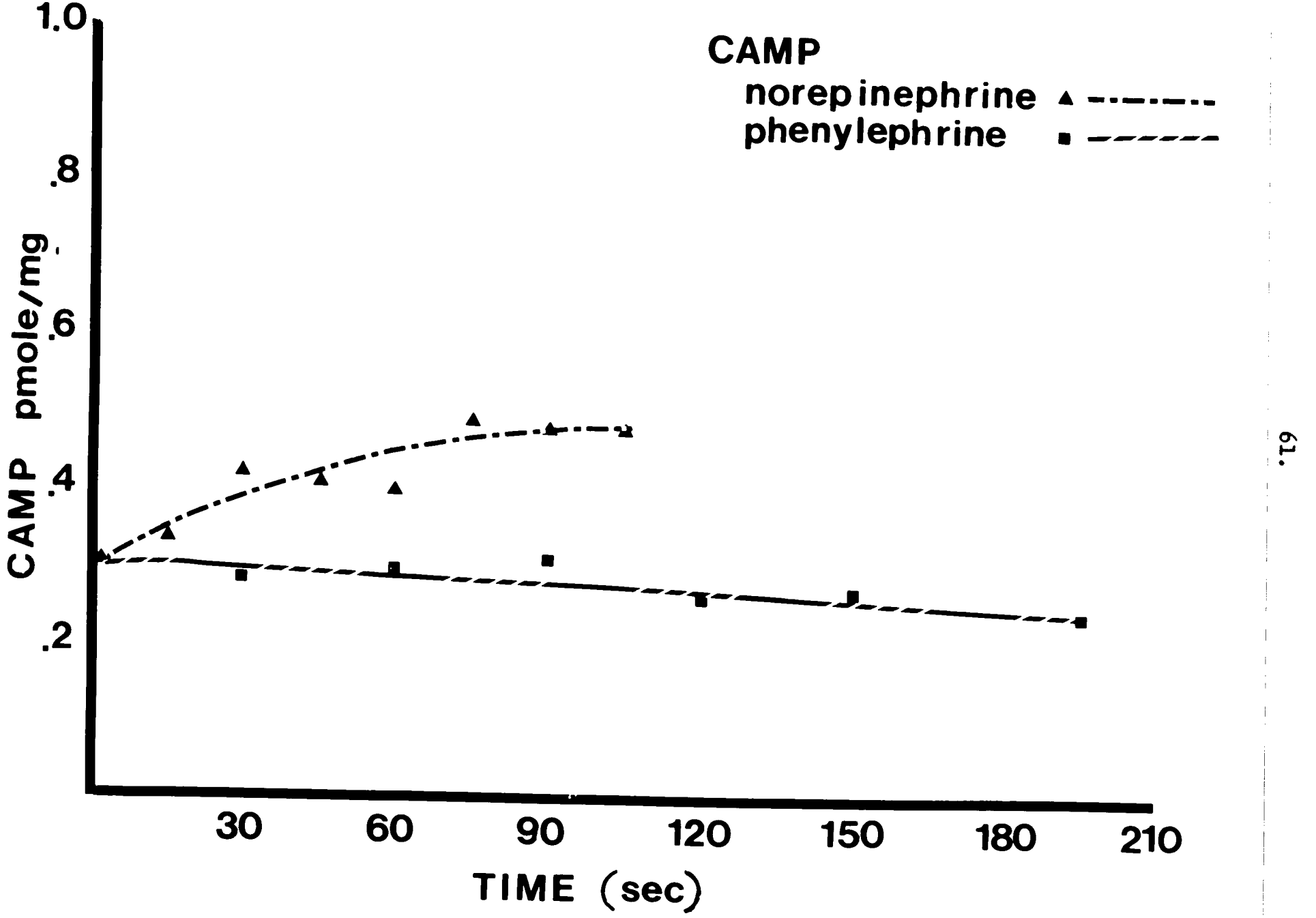

CAMP
norepinephrine
phenylephrine
CAMP pmole/mg
1.0
.8
.6
.4
.2
30
60
90
120
150
180
210
TIME (sec)

Figure 19. The effects of propranolol (10^{-6} M) and phentolamine (10^{-6} M) on cardiac cyclic AMP in response to norepinephrine (10^{-5} M) at 17° C (90 sec.), 22° C (30 sec.), and 37° C (15 sec.). Control values for cyclic AMP in response to norepinephrine (10^{-5} M) were as follows: 17° C (90 sec.), 0.49 pmole/mg; 22° C (30 sec.), 0.63 pmole/mg; 37° C (15 sec.), 1.18 pmole/mg. Each bar represents the mean of five atria.

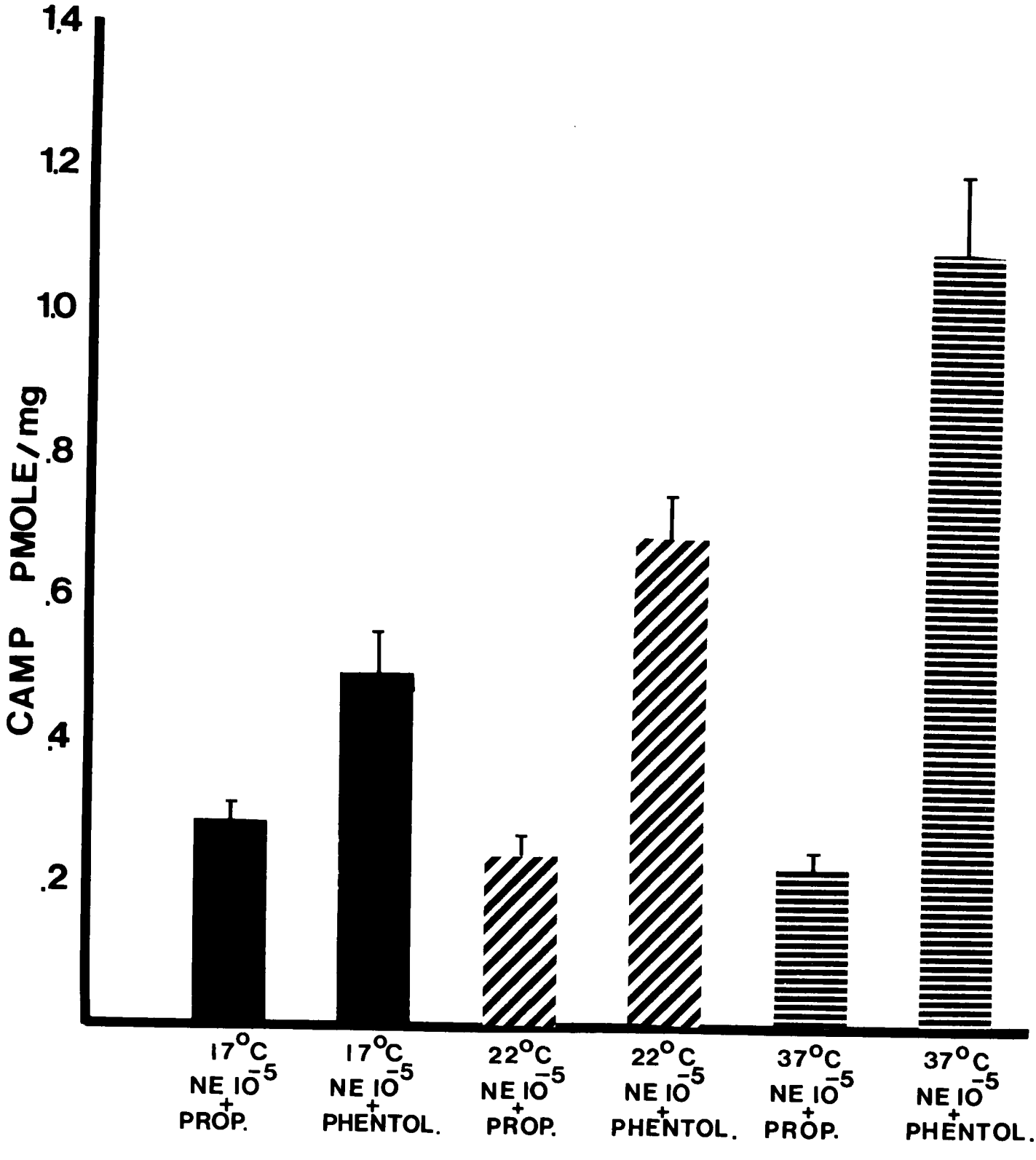
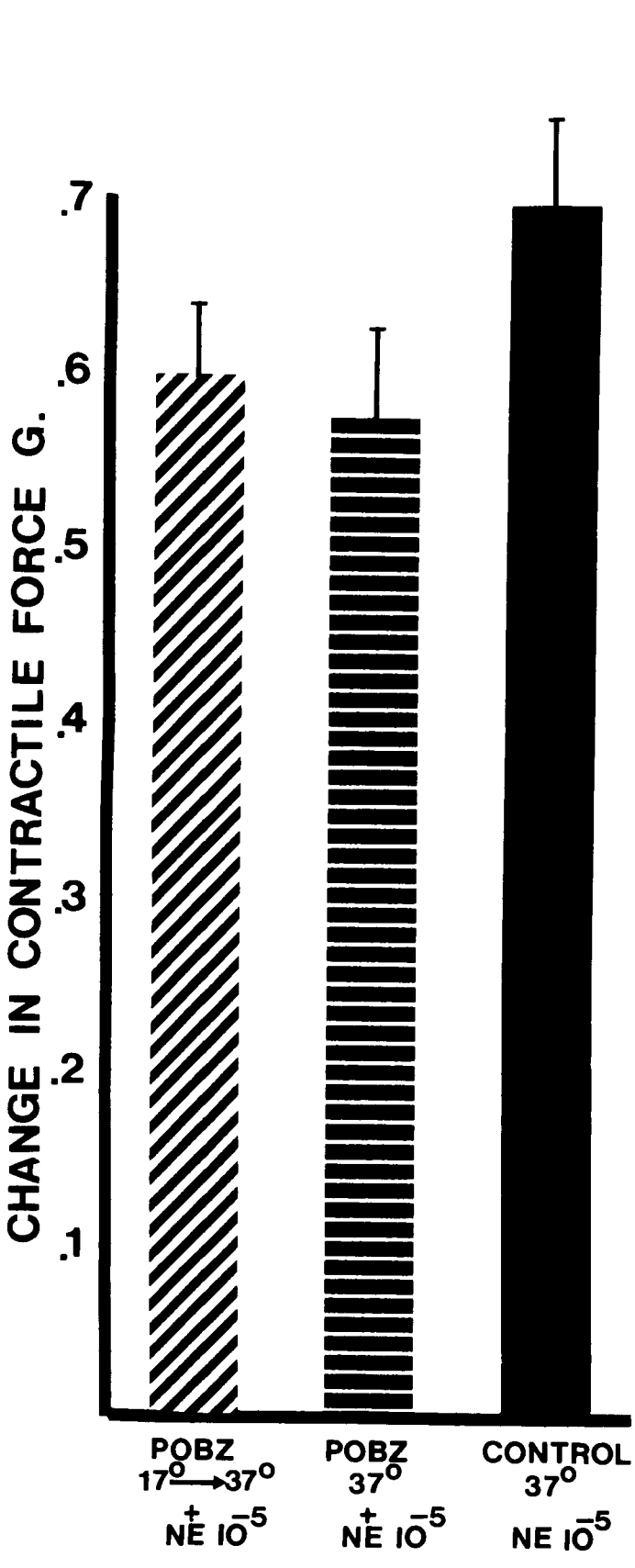

1.4
1.2
1.0
.8
.6
.4
.2
CAMP PMOLE/mg
17°C NE 10⁻⁵ + PROP.
17°C NE 10⁻⁵ + PHENTOL.
22°C NE 10⁻⁵ + PROP.
22°C NE 10⁻⁵ + PHENTOL.
37°C NE 10⁻⁵ + PROP.
37°C NE 10⁻⁵ + PHENTOL.

Figure 20. The effect of exposure to phenoxybenzamine (10^{-6} M) for forty-five minutes at 17° C and 37° C on cardiac cyclic AMP and contractility in response to norepinephrine (10^{-5} M). Driven left atria which had been treated with phenoxybenzamine at 17° C were first raised to 37° C before addition of norepinephrine. Contractile response and cyclic AMP were determined at their maximum response at 60 and 15 seconds respectively. Each bar represents the mean of five atria.


CHANGE IN CONTRACTILE FORCE G.
.7
.6
.5
.4
.3
.2
.1
POBZ 17°→37° + NE 10⁻⁵
POBZ 37° + NE 10⁻⁵
CONTROL 37° NE 10⁻⁵

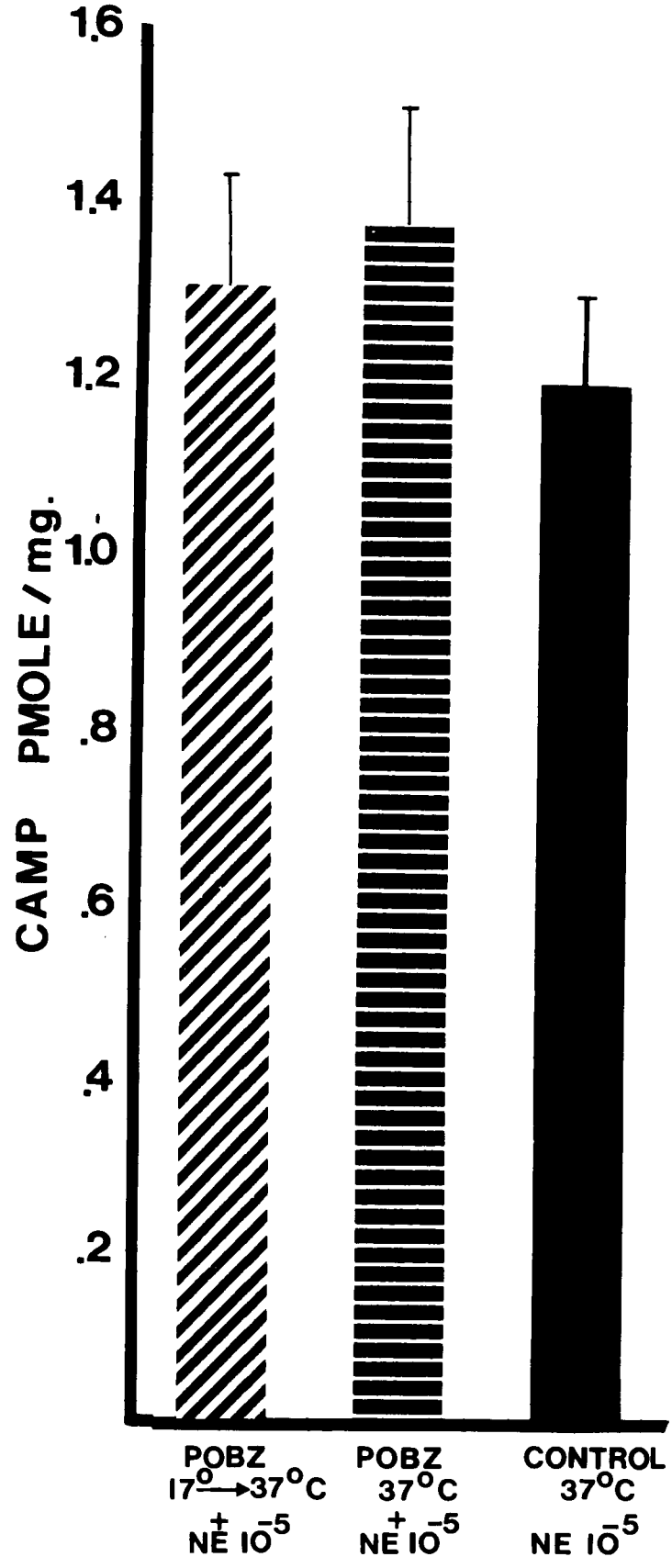

CAMP PMOLE/mg.
1.6
1.4
1.2
1.0
.8
.6
.4
.2
POBZ 17°→37°C + NE 10⁻⁵
POBZ 37°C + NE 10⁻⁵
CONTROL 37°C NE 10⁻⁵

Figure 21. Time-response effects of theophylline ($5x10^{-4}$ M) on cardiac contractility in the driven left atrium. Reserpine pretreatment was 3 mg/kg twenty-four hours before the experiment. Each point represents the mean of five atria.

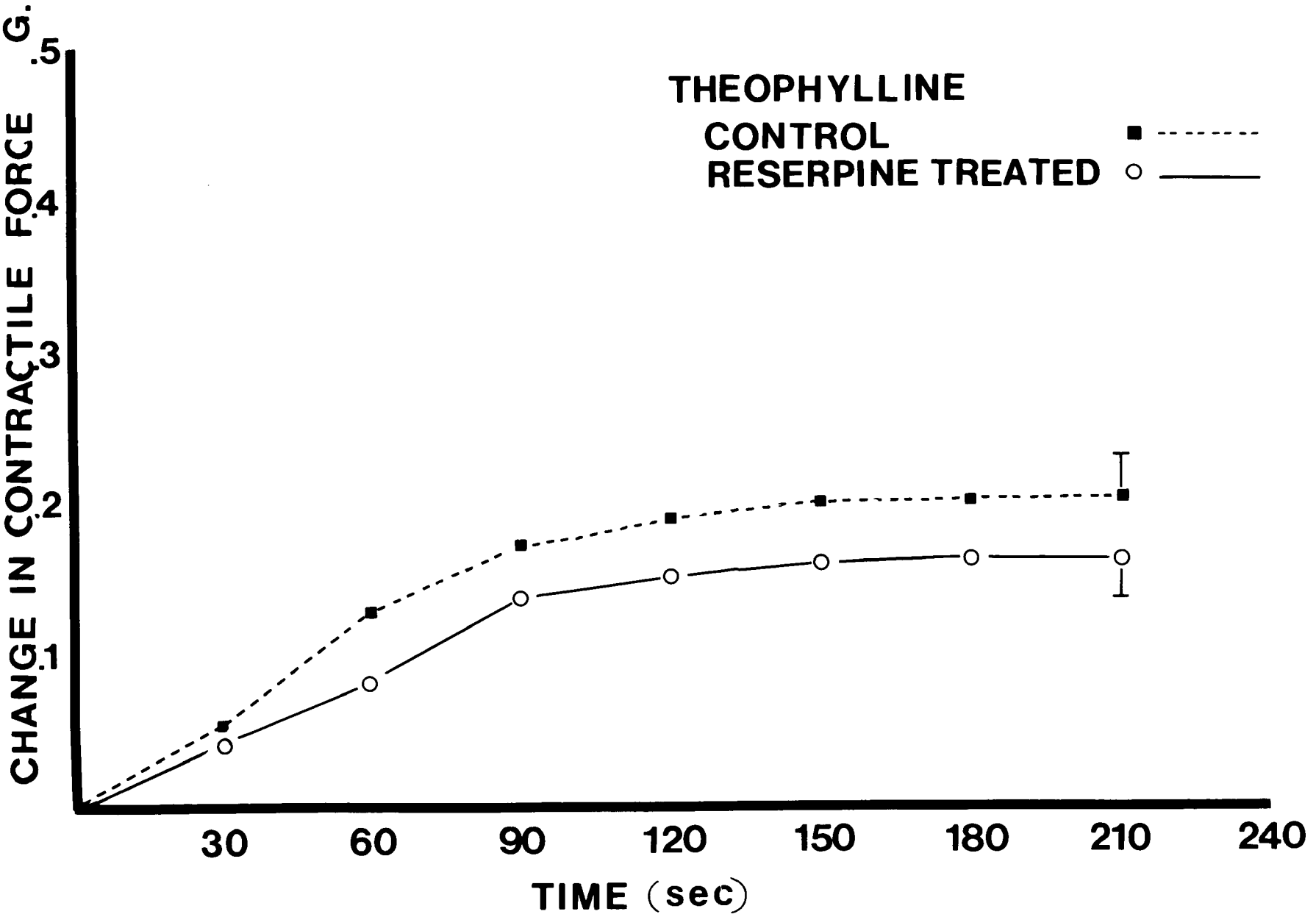

THEOPHYLLINE
CONTROL
RESERPINE TREATED
CHANGE IN CONTRACTILE FORCE G.
.5
.4
.3
.2
.1
30
60
90
120
150
180
210
240
TIME (sec)

Figure 22. The effect of exposure to theophylline for three minutes, fifteen minutes and sixty minutes on cardiac contractility in response to norepinephrine (10^{-5} M) in driven left atria. Each point represents the mean of five atria.

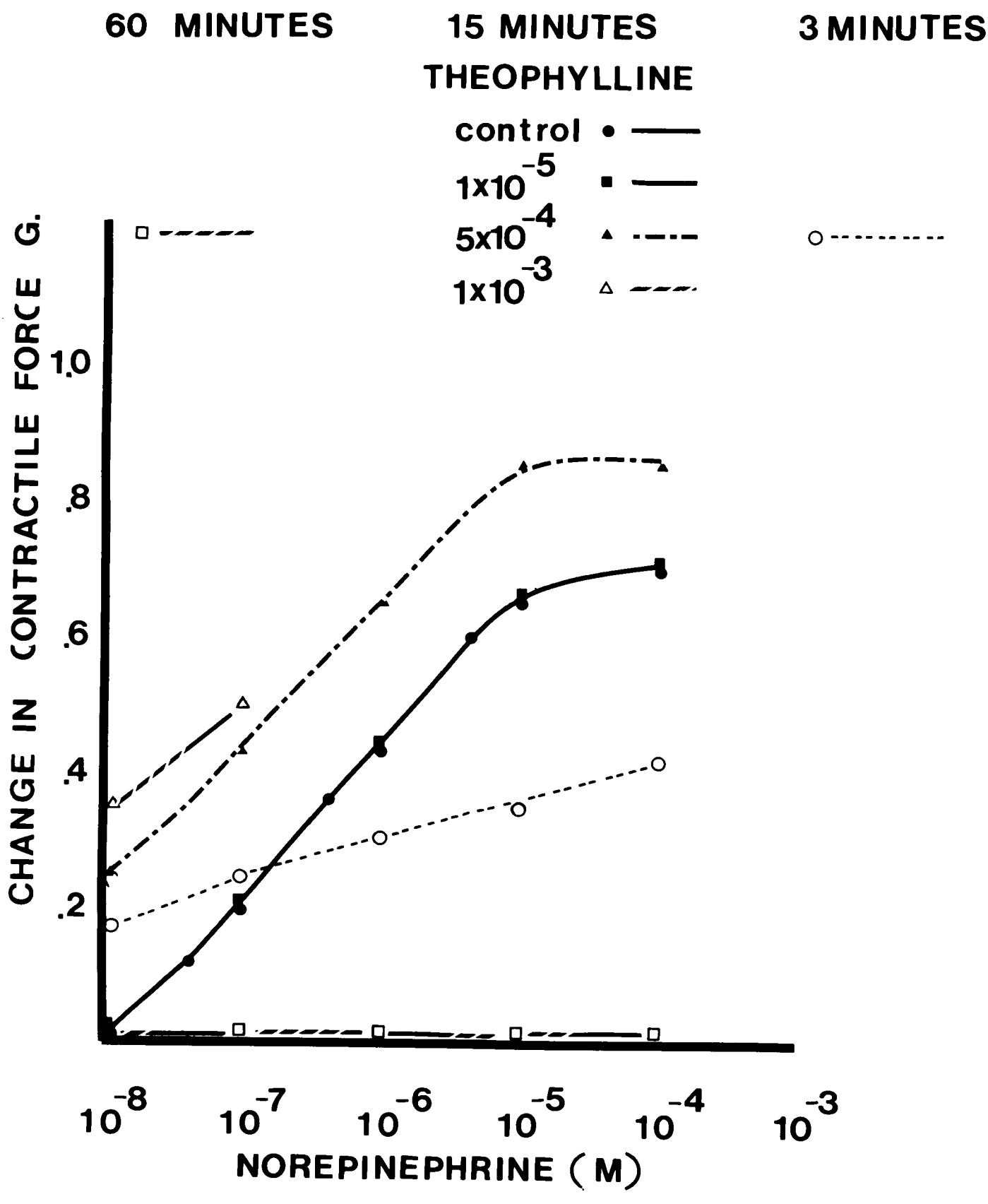

60 MINUTES
15 MINUTES
3 MINUTES
THEOPHYLLINE
control
1x10-5
5x10-4
1x10-3
CHANGE IN CONTRACTILE FORCE G.
1.0
.8
.6
.4
.2
10-8
10-7
10-6
10-5
10-4
10-3
NOREPINEPHRINE (M)

Figure 23. The effect of exposure to theophylline for fifteen minutes on cardiac contractility in response to phenylephrine (10^{-4} M) in driven left atria. Each point represents the mean of five atria.

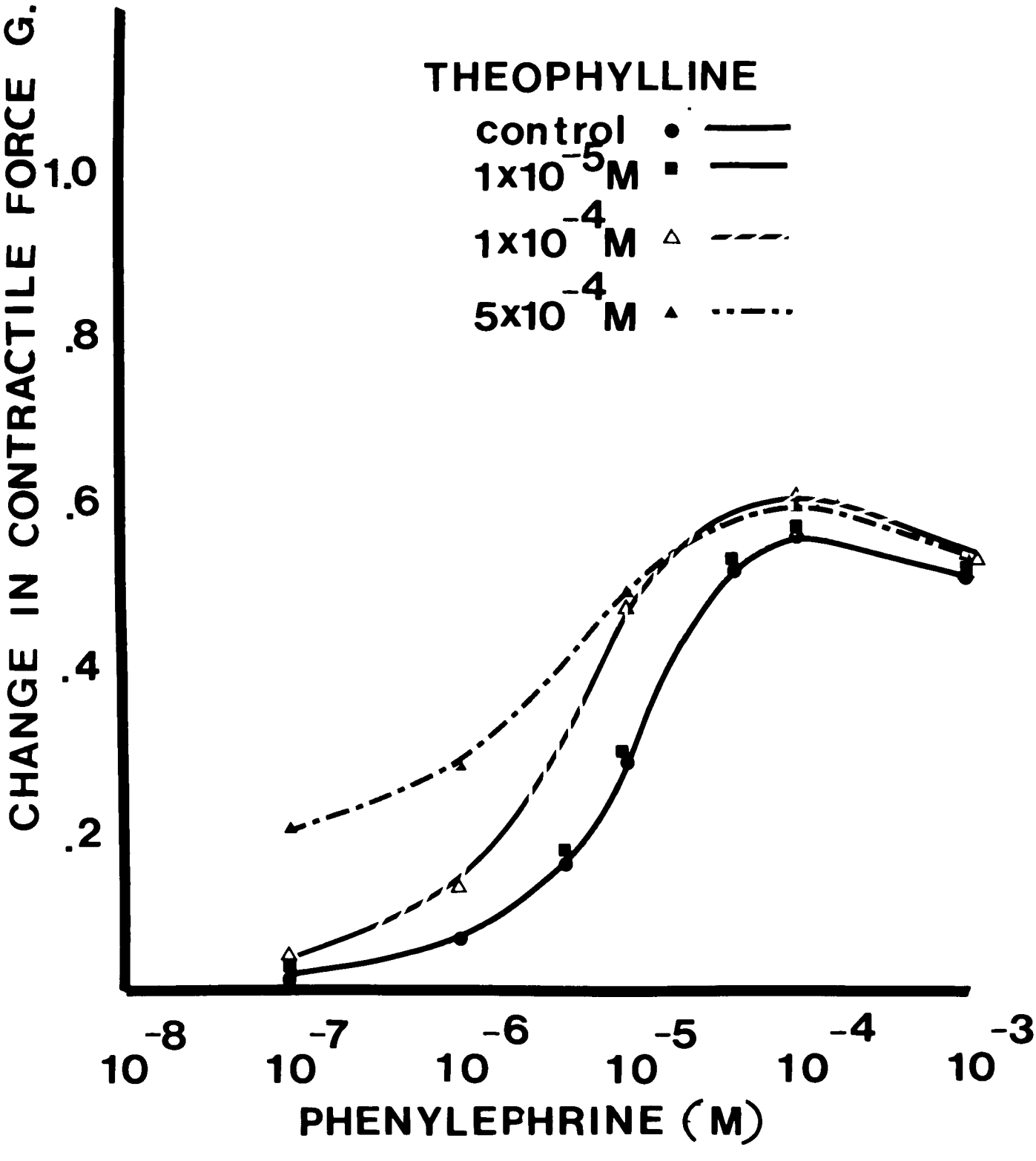

THEOPHYLLINE
control
1x10⁻⁵M
1x10⁻⁴M
5x10⁻⁴M
CHANGE IN CONTRACTILE FORCE G.
1.0
.8
.6
.4
.2
10⁻⁸
10⁻⁷
10⁻⁶
10⁻⁵
10⁻⁴
10⁻³
PHENYLEPHRINE (M)

Figure 24. The effect of exposure to theophylline ($5x10^{-4}$ M) on cardiac cyclic AMP in the driven left atrium. Theophylline alone increased cyclic AMP at three minutes exposure. Exposure to theophylline for three minutes increased the peak cyclic AMP response to norepinephrine (10^{-6} and 10^{-5} M) in an additive manner. Each bar represents the mean of five to eight atria. One S.E.M. is shown.

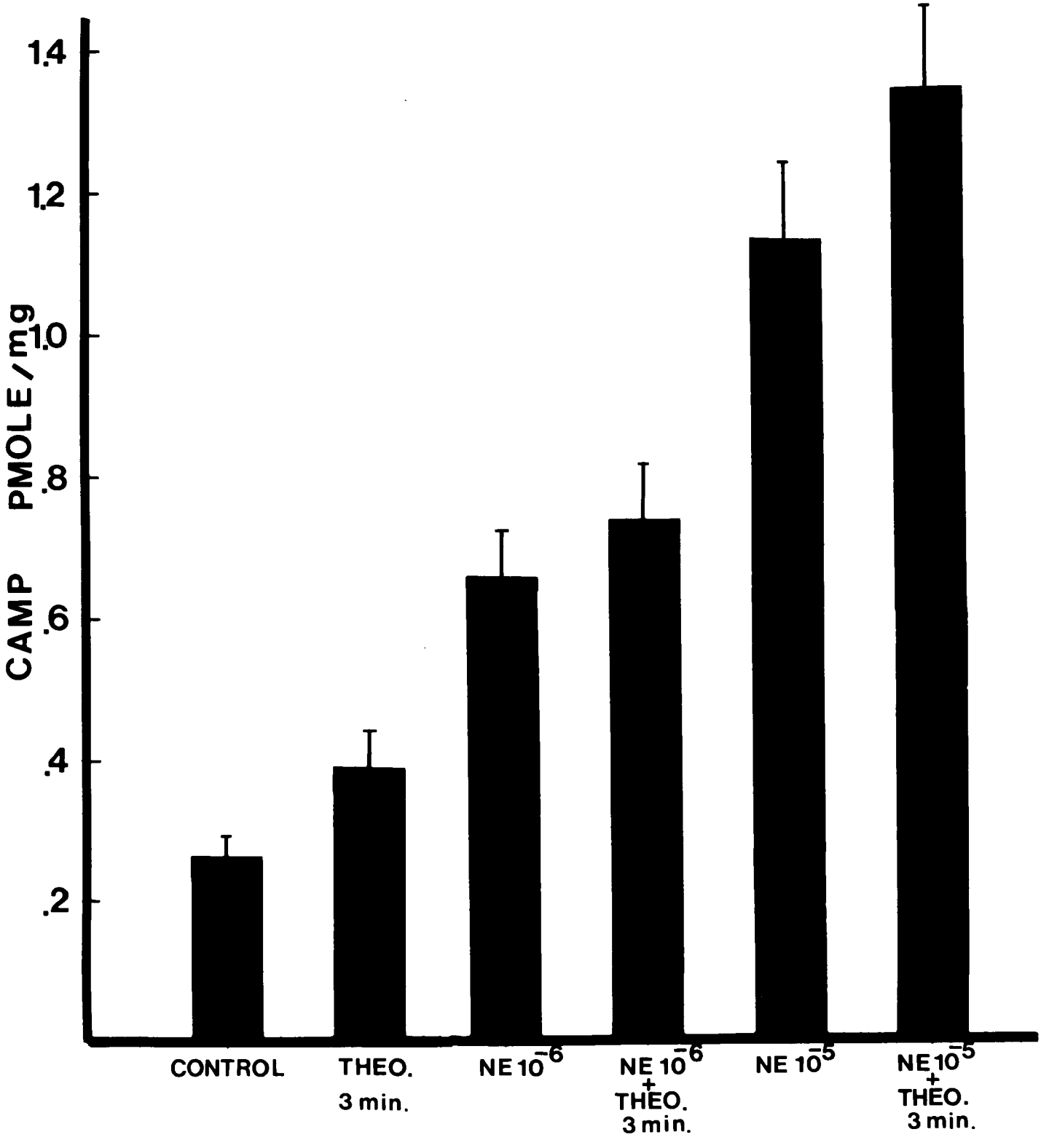
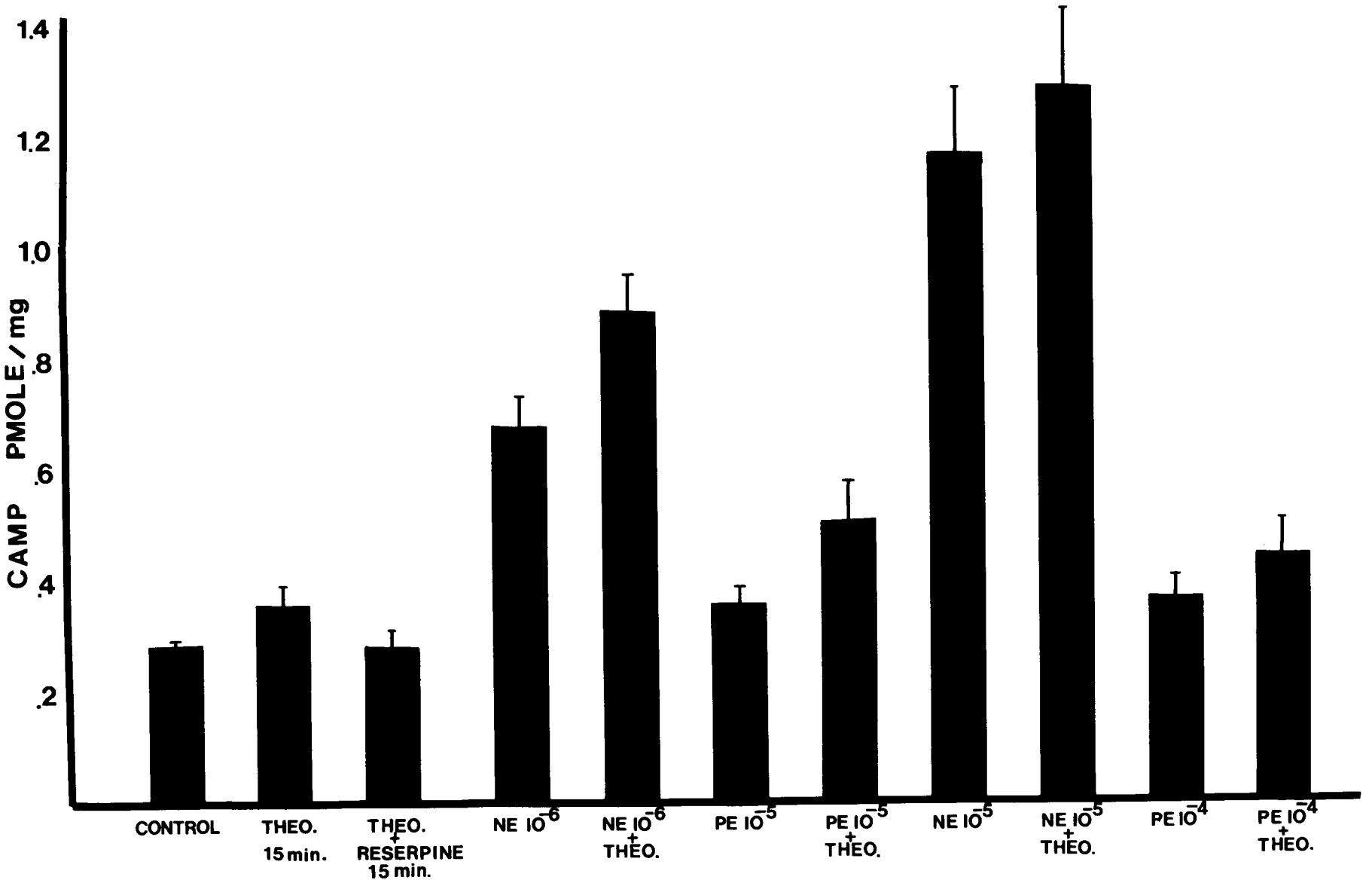

CAMP PMOLE / mg
1.4
1.2
1.0
.8
.6
.4
.2
CONTROL
THEO. 3 min.
NE 10^{-6}
NE 10^{-6} + THEO. 3 min.
NE 10^{-5}
NE 10^{-5} + THEO. 3 min.

Figure 25. The effect of exposure to theophylline ($5x10^{-4}$ M) for fifteen minutes, on cardiac cyclic AMP in response to norepinephrine and phenylephrine in driven left atria. Cyclic AMP concentrations were determined 10 seconds after the addition of norepinephrine or phenylephrine. Theophylline alone significantly increased cyclic AMP at 15 minutes exposure ($P<0.05$). Reserpine pretreatment 3 mg/kg twenty-four hours before the experiment abolished the cyclic AMP increase seen with theophylline. Exposure to theophylline increased the cyclic AMP response to norepinephrine and phenylephrine in an additive manner. None of the increases were significant when the effect of theophylline alone was subtracted. Each bar represents the mean of five to eight atria. One S.E.M. is shown.


CAMP PMOLE / mg
1.4
1.2
1.0
.8
.6
.4
.2
CONTROL
THEO. 15 min.
THEO. + RESERPINE 15 min.
NE 10^{-6}
NE 10^{-6} + THEO.
PE 10^{-5}
PE 10^{-5} + THEO.
NE 10^{-5}
NE 10^{-5} + THEO.
PE 10^{-4}
PE 10^{-4} + THEO.

CHAPTER IV

DISCUSSION

The Mechanism of Action of Norepinephrine and Phenylephrine on Cardiac Contractility

The present series of experiments was designed to determine the time course of cyclic AMP and contractility changes in response to norepinephrine and phenylephrine. It was also intended to separate the direct and indirect effects of phenylephrine on cyclic AMP, and to determine the importance of alpha adrenergic receptors in the inotropic effects of sympathomimetic agents.

The results indicate that while norepinephrine and phenylephrine do cause an increase in cyclic AMP levels prior to contractile force (figures 5 and 6), both amines have the ability to produce an increase in cardiac contractility in which cyclic AMP is not involved (figure 6,7, and 9). The adrenergic receptors of cardiac muscle have been generally classified as beta-receptors. A number of investigators (Benfey and Varma, 1967; Berger and Mokler, 1969; Govier, 1968; Nakashima et al., 1971; Wagner, Endoh, and Reinhart, 1974; Wenzel and Su, 1966), however, have reported evidence supporting the existence of alpha adrenergic receptors in the heart which were capable of mediating a positive inotropic

effect in response to sympathomimetic agents. It seems reasonable, therefore, to attribute the increases in contractile force in response to norepinephrine and phenylephrine, which do not involve cyclic AMP, to alpha receptors.

Ahlquist, 1948, first formulated the concept of alpha and beta adrenergic receptors based primarily on the different quantitative responses of end organs to catecholamines. The receptors most sensitive to isoproterenol were called beta-adrenergic receptors and those most sensitive to norepinephrine were considered alpha-adrenergic receptors. The order of effectiveness for myocardial contractility in rabbit, cat, and dog was reported by Ahlquist, 1948, as isoproterenol > epinephrine > norepinephrine. The relative order reported by Govier, 1968, on guinea-pig atria, and by Wenzel and Su, 1966, on rat ventricle was isoproterenol > norepinephrine > phenylephrine. Phenylephrine has been considered to exert a direct effect on alpha-adrenergic receptors only (Ahlquist and Levy, 1959), although it has also been shown to have an indirect effect on releasing norepinephrine stores (Govier, 1968). Wenzel and Su, 1966, working on rat ventricles, reported that phentolamine potentiated the positive inotropic response to norepinephrine and epinephrine, blocked the phenylephrine response, and exerted no blockade of the isoproterenol-induced response. Govier, 1968, working on guinea-pig atria, reported that phentolamine antagonized the first component of a two-component phenylephrine-induced positive inotropic response, but exerted no effect on the

responses to norepinephrine, epinephrine, or isoproterenol. it was also reported that pronethalol did not block the phenylephrine positive inotropic response. Govier, 1968, suggested that norepinephrine and epinephrine possess alpha-adrenergic receptor stimulating activity but that it is not possible to demonstrate blockade of epinephrine and norepinephrine by alpha-adrenergic antagonists alone because the response to beta-adrenergic receptor stimulation is so great that the alpha-receptor component is statistically insignificant in comparison. In their experiments reduction of the beta-adrenergic receptor response with pronethalol made the alpha-adrenergic response a greater proportion of the total inotropic response and consequently alpha-receptor blockade resulted in a significant reduction in the positive inotropic response. On the basis of these experiments it was postulated that guinea-pig atria and rat ventricle myocardium contain alpha-adrenergic receptors (Wenzel and Su, 1966; Govier, 1968).

The failure of pronethalol to block the positive inotropic effect of low concentrations of phenylephrine suggested to Govier, 1968, that this effect is not mediated through the cyclic AMP system. He was led to the conclusion, therefore, that the positive inotropic effects of sympathomimetic agents resulting from alpha and beta adrenergic receptor stimulation are produced through different basic mechanisms. The data in the present series of experiments support this conclusion. The first evidence that two different basic mechanisms might be involved in the inotropic response came from the obser-

vation that the magnitude and time course of cyclic AMP changes induced by norepinephrine and phenylephrine were remarkably different while the magnitude of the contractile responses were not (figures 5,6, and 7). Pretreatment with reserpine established that the cyclic AMP response of phenylephrine is an indirect effect associated with the release of stored catecholamines. The contractile effect of phenylephrine could thus be dissociated from an increase in cyclic AMP, whereas with norepinephrine the two effects were still associated. It is confirmed that phenylephrine exerts a direct effect on alpha receptors only.

Norepinephrine is known to possess both beta- and alpha-adrenergic receptor stimulating activity (Wenzel and Su, 1966). Govier, 1968, was able to separate the alpha- and beta-receptor components of phenylephrine by first diminishing the beta receptor response with pronethalol so that the alpha-adrenergic response became apparent. In the present series of experiments concentrations of norepinephrine (10^{-5} M) and propranolol (10^{-6} M) were chosen such that the blockade of the inotropic response produced by propranolol had just been overcome by the higher concentration of norepinephrine. It was reasoned that at this point the greater proportion of the total inotropic response would be mediated by alpha-adrenergic receptors. Under these conditions the magnitude and time course of the contractile response resembled the phenylephrine induced inotropic effect, while there was no increase in the level of cyclic AMP (figure 9). The alpha-adrenergic antagonist phentolamine was inef-

fective in altering either the time-course or magnitude of the contractile response induced by norepinephrine. The cyclic AMP increase was slightly less in the presence of phentolamine than after norepinephrine alone. This could possibly be related to a nonspecific interference of phentolamine with the beta receptor. Rabinowitz, Parmley, and Katz, 1972, however, have reported that neither alpha-stimulation nor blockade affected the adenylate cyclase from cat heart muscle.

Shanfeld, Frazer, and Hess, 1969, have shown that the effect of a small dose (30 ng) of norepinephrine on cyclic AMP formation was somewhat more inhibited in the presence of isopropylmethoxamine (IMA) than was the positive inotropic effect. The evidence for a dissociation between the two effects did not appear to be conclusive, especially in view of the small total changes produced by this small dose of norepinephrine, since the inotropic response to norepinephrine was also reduced in the presence of IMA by about 50% in these experiments, whereas cyclic AMP was elevated by about 25% (which was not significant). In contrast, the present experiment clearly separates the beta- and alpha-adrenergic receptor stimulating activity of norepinephrine on contractility and cyclic AMP.

Several investigatiors (Lee and Yoo, 1964, 1970; in isolated rabbit atria; Kabela, et. al., 1969, in isolated dog heart), have reported that beta adrenergic antagonists inhibit the inotropic effect of phenylephrine in spontaneously beating cardiac tissue and that alpha adrenergic antagonists were ineffective. Other investigators, however, (Starke, 1972) in

perfused rabbit heart, and Osnes and Oye, 1975, in perfused rat heart) have found that the positive inotropic effects of phenylephrine were blocked by phentolamine but not by propranolol. A possible explanation for the divergent and contradictory experimental results may be found in a recent publication by Endoh and Schumann, 1975b. They have reported that the positive inotropic effects of the alpha-agonists methoxamine, maphazoline, and oxymetazoline decreased with increasing frequency of stimulation (0.5-1-1.5 Hz). They considered on the basis of previous experiments (Endoh, Wagner, and Schumann, 1975) that the stimulation of alpha-adrenergic receptors is capable of increasing the intracellular calcium to a higher level, either by increasing the influx of calcium per contraction or by decreasing the efflux. Winegrad and Shanes, 1962, had shown that the frequency of stimulation strongly effects the flux of calcium through myocardial cell membrane. Endoh and Schuman, therefore reasoned that when intracellular calcium has already been raised by an increase of the frequency of stimulation, alpha-adrenergic receptor stimulation is not able to induce a further increase of intracellular calcium.

In the present series of experiments the cyclic AMP response to phenylephrine (10^{-4} M) (which has been shown to be due to the release of stored catecholamines) and norepinephrine (10^{-5} M) were similar in the rate controlled (1 Hz.) and the spontaneously beating preparations (figures 5,6, and 7). In addition increasing the rate of stimulation from one to two Hz. had no effect on the cyclic AMP response to norepinephrine

(figure 11). The time course of the phenylephrine contractile response, however, was remarkably different between the rate controlled and the spontaneously beating preparation (figures 6 and 7). The time required to reach peak contractile response was changed from 100 seconds in the driven preparation to 40 seconds in the spontaneously beating tissue. The difference in the time course of the phenylephrine-induced contractile response in the slow-paced verses the spontaneously beating preparation is most likely due to the ability of the chronotropic response to affect the flux of calcium through the cell membrane (Winegrad and Shanes, 1962). The chronotropic response of phenylephrine has been shown by a number of investigators to be blocked by beta-adrenergic antagonists (Leong and Benfey, 1968; Krell and Patil, 1969; Starke, 1972; Wagner, Endoh, and Reinhardt, 1974). The use of spontaneously beating preparations may, thus, introduce other variables such as rate which can greatly affect the contractile response. Such variables may at least partially account for the divergent experimental results reported in the literature.

The rate controlled preparation may offer a better system for attempting to correlate the physiological and biochemical effects. For example, the time course of the norepinephrine-induced contractile response in slow paced atria is greatly different in the presence and absence of propranolol (180 seconds to reach peak tension in the presence of propranolol verses 60 seconds to reach peak tension in the absence) (figure 9). This difference may be related to the lack of cyclic AMP

elevation in the former situation. This observation would have been obscured by the presence of a chronotropic response.

It should always be kept in mind when pacing a tissue electrically that excessive depolarization per se may elevate cyclic AMP contents (Wollenberger, Babskii, Krause, Genz, Blohm, and Bogdanova, 1973) and may also liberate endogenous catecholamines. In the present series of experiments different voltage stimuli from four to twenty volts had no effect on cardiac cyclic AMP levels indicating that neither of the above possibilities had taken place (figure 10).

In conclusion, norepinephrine and phenylephrine do cause an increase in cyclic AMP levels prior to contractile force; however, both amines have the ability to produce an increase in cardiac contractility in which cyclic AMP is not involved. The phenylephrine-induced cyclic AMP response is an indirect effect associated with the release of stored catecholamines. It seems reasonable to attribute the increases in contractile force in response to norepinephrine and phenylephrine which do not involve cyclic AMP to alpha receptors. This does not, however, imply a dissociation between beta receptor function, cyclic AMP, and the inotropic response.

The time-course of the contractile response induced by adrenergic amines may be remarkably influenced by the chronotropic response in spontaneously beating preparations while the cyclic AMP response is not greatly affected. This indicates that events beyond the receptor level, possibly involving the effect of rate changes on calcium fluxes, are taking place. It is

therefore suggested that rate controlled preparations may offer a better system for attempting to correlate physiological and biochemical events.

The Effect of Temperature on Cardiac Inotropic and Adenylate Cyclase Activating Adrenoceptors

The second series of experiments was designed to be a through investigation of the biochemical and physiological changes induced by lower temperature in mammalian cardiac muscle. The purpose was to determine whether alpha- and beta-adrenergic receptors represent allosteric configurations of the same receptor macromolecule and whether there was indeed a dissociation of cardiac inotropic and adenylate cyclase activating adrenoceptors at low temperature. The results indicate that there is no interconversion of alpha- and beta-adrenergic receptors mediated by temperature.

It has been confirmed repeatedly that the strength of contraction of isolated heart muscle from homeothermic animals increased when its temperature is lowered below normal (Clark, 1920; Feigen, et. al., 1952; Fuchgott, and Gubareff,1958; Garb, and Chenoweth, 1949; Hollander, and Webb, 1955; Kelly, and Hoffman, 1960; Saunders, and Sanyal, 1958). The inotropic effects of temperature changes and of pharmacological agents are not necessarily additive. Lowering the temperature may so increase the strength of contraction as to leave little room within the limits of contractility for a drug to exert a positive inotropic effect. The positive inotropic effects of cardiac glycosides (Brown and Cotten,

1954; Meyer and Kukovetz, 1952; Saunders and Sanyal, 1958) and of epinephrine and norepinephrine (Barlow, and Sollman, 1926; Booker, 1960; Brown, and Cotten, 1954; Cotten, and Cooper, 1962) have been found to be decreased by cooling, and this has been attributed at least in part to the influence of limits on contractility (Blinks and Koch-Weser, 1963).

In the present series of experiments the inotropic effect of temperature was again confirmed (see figure 16). The increase in contractile force mediated by cooling to 17° C was so great that the positive inotropic effects of all the amines were greatly reduced (figure 13). This decrease in responsiveness appeared to be due to an increase in the baseline and not to a decrease in efficacy. The fact that the order of potency is altered by the decrease in temperature (figure 13 verses figure 12) has been interpreted by some investigators to indicate that an interconversion of receptors from beta to alpha has taken place (Amer and Byne, 1975). However phenylephrine, the most specific alpha agonist, was the least potent. In fact phenylephrine produced a negative inotropic effect which would tend to rule out a receptor change. The decrease in potency of isoproterenol is most likely due to the inotropic effects of temperature changes masking a portion of its dose-response curve.

The inotropic effects of the adrenergic amines at 17° C were too small to be useful in evaluating the activity of alpha and beta adrenergic antagonists. It was therefore decided

to compare the activity of these agents at 37° C and 22° C. It was found, however, that at 22° C phentolamine caused an increase in the positive inotropic effect of temperature change, while propranolol caused a decrease in the strength of contraction (figure 16).

In order to understand how the adrenergic blocking agents may influence the inotropic effect of temperature on myocardial tissue it is necessary to review the effects of temperature changes on the duration of the active state. Over a wide range of temperature, the direction of temperature-dependent changes in the strength of contraction seems to be determined by changes in the duration of the active state (Blinks and Koch-Weser, 1963). It is well known that the duration of the contractions of heart muscle increases with decreasing temperature. This has been observed in muscle from cold-blooded (Hajdu, and Szent-Gyorgyi, 1952; Wiegmann, et al., 1957; Heintzen et al., 1956; Eckstein, 1920) and warm-blooded animals (Trautwein and Dudel, 1954; Schmidt and Chang, 1961; Garb and Chenoweth, 1949; Hegnauer et al., 1950; Hirvonen, and Lybeck, 1956). The increased duration of the active state with cooling is associated with an increase in the duration of the action potential (Brooks et al., 1955; Cranefield, and Hoffman, 1958; Heintzen et al, 1956; Hollander and Webb, 1955; Kelly and Hoffman, 1960). During contraction the calcium ion concentration remains elevated as a result of the slow inward current carried by calcium during the plateau (sustained depolarization) phase of the action potential. Thus the action potential, by regulating the

calcium ion concentration, not only triggers the contraction but influences its magnitude and duration as well. The positive inotropic effect of temperatue change may be a direct result of this increase in the slow calcium inward current during the action potential.

Phentolamine has been found to prolong the action potential duration in guinea-pig papillary muscle (Quadbeck and Reiter, 1975); guinea-pig atria (Pappano, 1971); Purkinje fibers of sheep (Giotti et al., 1973); and dog (Rosen et al, 1971). Phentolamine could conceiveably mediate this effect without interacting with any adrenoceptors. It is also possible that the effect of phentolamine on the action potential may be related to its direct myocardial stimulant component (Goodman and Gilman, 1970; Ahlquist et al., 1947; Lum and Nickerson, 1946) or to its ability to cause a secondary release of endogenous catecholamines (Goodman and Gilman, 1970).

Propranolol, on the other hand, has been found to shorten the action potential of guinea-pig papillary muscle (Quadbeck and Reiter, 1975). The actions of propranolol on the heart could result from at least two different mechanisms of action. The first is attributable to the beta-blocking action of propranolol which inhibits both positive inotropic and arrhythmic actions of catecholamines (Lucchesi et al., 1966). The second is a nonspecific 'local anaesthetic' action which appears to result from a direct action of propranolol that is not related to its beta-blocking activity. The direct effect on myocardial excitability predominates in the prevention of digitalis-induced

arrhythmias arising from myocardial ischemia (Barrett and Cullum, 1968). In the present experiment it is unlikely that beta-adrenergic blockade is involved in the antagonism of the inotropic effect of temperature by propranolol as the control levels of cyclic AMP were not elevated by decreased temperature alone (figure 5, 17, and 18). This would indicate that the beta receptor was not active in mediating the positive inotropic effect of temperature. It therefore seems illogical that the effect of propranolol on the inotropic effect of temperature may be due to blockade of increased calcium fluxes through the cell membrane. It is therefore possible that propranolol may antagonize the inotropic effect of temperature by acting as a local anesthetic.

Before considering the effects of the alpha- and beta-adrenergic antagonists on the log dose-respones (LDR) curves of the adrenergic amines, it may be appropriate to review a few important points concerning the analysis of antagonism in the framework of the LDR curve. The position of a LDR curve on the x-axis reflects the affinity of the drug for its receptor. A typical LDR curve is symmetrical about the point at which 50 per cent of the maximum response is obtained, and its maximum slope and point of inflection occur at this midpoint. The lower the ED50 (the dose for half-maximal response) the more potent the drug. In the presence of a competitive antagonist, the curve for an agonist will be shifted to the right, but neither the slope nor the maximum response would be expected to change. The antagonist simply

alters the effective affinity of agonist drug for receptor. The effect of a noncompetitive antagonsit upon the LDR curve will be quite different. The agonist curve may or may not be shifted to the right, however, the slope will be reduced and the maximum response will diminish, in relation to the degree of noncompetitive blockade established. A through coverage of the different categories of these two basic types of antagonism is covered by Webb, 1963.

Since the studies which have indicated there is an interconversion of cardiac inotropic receptors from beta to alpha mediated by temperature have consistently reported their results in dose ratios (Kunos and Szentivanyi, 1968; Kunos et al., 1973; Benfey et al., 1974) instead of complete dose or time response curves, it is important to understand the advantages and limitations of the dose ratio method of expressing antagonism. The dose ratio is the ratio of the two agonist concentrations producing the same response, usually a half-maximal response, in the presence and absence of antagonist. When the LDR curves are parallel, this ratio is constant. However, when the curves are not parallel the dose-ratio varies. It is not the best method of expressing an antagonist effect under these conditions. Furthermore, it is impossible to tell anything about the nature of the antagonism from a simple presentation of the dose ratios.

It is possible to illustrate mathmatically the difference between competitive and noncompetitive antagonism as they pertain to dose ratios. The equation for competitive inhibition of a receptor may be reduced to the form

$$\frac{(A)_I}{(A)_0} = \frac{(I)}{K_I}$$

where $(A)_0$ is the concentration of agonist to produce an arbitrarily chosen response, $(A)_I$ is the concentration needed to produce the same response in the presence of concentration (I) of the antagonist, $1/K_I$ is the affinity of the antagonist for the receptor, and $(A)_I/(A)_0$ is the dose ratio (Webb, 1963; Goldstein, 1974). The equation for noncompetitive antagonism will not reduce to the above form. Thus the chief advantage of the dose ratio in competitive inhibition, that it is a convenient measure of the affinity of the antagonist for the receptor, is lost in noncompetitive inhibition.

The effects of phentolamine and propranolol on the LDR curves of the adrenergic amines at 37° C is shown in figure 14. As would be expected, phentolamine had no effect on the isoproterenol dose-response curve. Phentolamine did, however, cause a small noncompetitive antagonism of the norepinephrine curve and a small competitive antagonism of the phenylephrine curve. A similar attenuation of the positive inotropic effects of norepinephrine by phentolamine has been reported by Rabinowitz et al., 1974, in the isolated cat papillary muscle. The shift of norepinephrine and phenylephrine curves to the right is probably mediated by a competitive antagonism of alpha adrenergic receptors. The mechanism of the phentolamine mediated supression of the norepinephrine maximal response remains obscure. It may be

related to a nonspecific interaction, possibly involving the sympathomimetic activity of phentolamine or a supression of beta receptor function. It may also reflect a specific antagonism of alpha receptors. Propranolol caused the isoproterenol LDR curve to shift to the right. In addition, the efficacy of isoproterenol was increased in the presence of propranolol which may be due to separation of the beta-blocking and local anesthetic effects. If isoproterenol were able to overcome the beta-blockade of propranolol while cardiac microsomal and sarcoplasmic reticulum uptake and binding of calcium were still inhibited, the resultant increase in free intracellular calcium could then account for the elevation of maximal contractile force. It would be possible to test this hypothesis by repeating this experiment using sotalol or practolol which are almost devoid of local anesthetic potency instead of propranolol. Propranolol competitively antagonized the norepinephrine LDR curve. Propranolol did not cause a true noncompetitive antagonism of the phenylephrine LDR curve, although the slope was reduced and the maximum response was diminished. The fact that it was not possible to further suppress the phenylephrine-induced positive inotropic response by increasing the concentration of antagonist suggests that propranolol was able to antagonize only the second component of phenylephrine activity which is due to catecholamine release (Govier, 1968). These data support the view that phenylephrine exerts its major positive inotropic effect through alpha receptors.

The effects of phentolamine and propranolol on the LDR curves of the adrenergic amines at 22° C is shown in figure 15. Phentol-

amine reduced the slope and diminished the maximum response for all the amines. The affinities of the drugs for the receptors, however, were not altered by phentolamine as can be seen by the concentrations of the agonists causing 50% of the maximum effect. The nonspecific, noncompetitive antagonism observed in the presence of phentolamine is probably due to the ability of phentolamine to increase the strength of contraction (raise the baseline) so as to leave little room within the limits of contractility for a drug to exert a positive inotropic effect (figure 16). Propranolol competitively antagonized the isoproterenol and the norepinephrine curves, causing a shift of the LDR curves to the right in both cases. Propranolol did not antagonize the inotropic effect of phenylephrine. The apparent increase in efficacy of all the amines in the presence of propranolol was probably due to the ability of propranolol at 22° C (figure 16) to decrease the inotropic effect of temperature changes and thus leave more room within the limits of contractility for the drugs to exert their positive inotropic effects. It should be noted that the use of dose ratios to characterize the changes in the LDR curves at 22° C is inappropriate due to the appearance of nonspecific, noncompetitive effects which may complicate the interpretation.

Lowering the temperature from 37° C to 22° C and 17° C decreased both the rate and maximum levels of cyclic AMP production in response to norepinephrine (figures 5, 17,and 18). It was not possible to detect an increase in cyclic AMP mediated by phenylephrine at 22° C or 17° C in contrast to the results obtained at 37° C (figures 6, 17, and 18). This probably

reflects the overall decrease in adenylate cyclase activity at lower temperature. In agreement with Benfey et al., 1974, the stimulation of cyclic AMP production in response to norepinephrine remained an entirely beta-adrenoceptor response at all temperatures tested (figure 19).

Kunos and Szentivanyi, 1968, used phentolamine and propranolol as adrenergic antagonists in the experiments where they first suggested that alpha and beta adrenergic receptors may represent allosteric configuration of the same receptor macromolecule. The present series of experiments have shown that the results upon which this conclusion was based were due to nonspecific effects. Subsequently, Kunos et al., 1973, reported similar results using phenoxybenzamine and propranolol as the antagonists. Phenoxybenzamine may act in a manner similar to phentolamine in the present experiment to increase the inotropic effect of temperature. An alternate explanation has also been suggested. Benfey, 1975, found the blocking effects observed with phenoxybenzamine at low temperature to be nonspecific in frog ventricles. He suggested that the changes in phenoxybenzamine effects with temperature are related to differences in the rate of formation and stability of the intermediate aziridium ion and its rate of alkylation of nucleophilic centers. It is difficult, however, to see how such an explanation could account for the observed nonspecific antagonism. Whatever the true explanation for the phenoxybenzamine activity at low temperature, it is clear that it is a nonspecific effect.

Kunos et al., 1973, have also reported that the heart binds twice as much phenoxybenzamine at 14° C than at 24° C. This was interpreted as proof of additional binding to receptors which had been converted from their beta to their alpha configuration. There is no reason to suppose, however, that the ability to bind phenoxybenzamine should be the same at 14° C and 24° C. The great majority of the binding in any case will be nonspecific, and differences encountered between the two temperatures may be related to such a phenomenon as phase changes in membrane lipids which have been shown to alter the permeability of membranes (DeGier et al., 1968). It is also possible that the different binding characteristics may be related to the rate of formation and stability of the intermediate aziridium ion as has been suggested by Benfey, 1975. The fact that the temperature of incubation did not influence the effect of phenoxybenzamine on either the contractile or the cyclic AMP response of norepinephrine (figure 20) would indicate that there is no specific binding of phenoxybenzamine to the beta receptor at either temperature.

In conclusion, the results indicate that there is no interconversion of alpha- and beta-adrenergic receptors mediated by temperature. The apparent blockade of the adrenergic amine-induced inotropic response by phentolamine and the apparent lack of blockade by propranolol has been found to be related to the ability of the blocking agents to modify the inotropic effect of low temperatures. Although adrenergic amines have the ability to cause increases in contractile force

which are mediated by alpha receptors and which do not involve cyclic AMP (Benfey and Carolin, 1971; Osnes and Oye, 1975; Martinez and McNeill, 1975), no dissociation has yet been found between the beta receptor, cyclic AMP, and the inotropic response, It therefore seems logical, in view of the close association of these parameters, that cyclic AMP may be one of the second messengers mediating the inotropic response in cardiac tissue. These data support the role of cyclic AMP as a mediator of beta-adrenergic receptor function.

The Effect of Theophylline on Amine-Induced Cardiac Cyclic AMP and Cardiac Contractility

The third series of experiments was designed to investigate the effect of theophylline on adrenergic amine-induced cardiac cyclic AMP and cardiac contractility. The positive inotropic effects of the methylated xanthine derivatives caffeine, theophylline, theobromine, and papaverine are well known, however, the mechanism responsible for this action remains unclear. Two recent developments: 1.) the demonstration that caffeine causes the release of calcium from and decreases the net uptake of calcium in microsomes (Weber, 1968; Johnson and Inesi,,1969; Fuchs, 1969; Ogawa, 1970), and 2.) the discovery that the methylxanthines are inhibitors of phosphodiesterase (Sutherland and Rall, 1958; Butcher and Sutherland, 1962), the enzyme responsible for the breakdown of cyclic AMP in muscle, have stimulated interest in these compounds. The fact that catecholamines stimulate the production of cardiac cyclic AMP has led to the suggestion that catechol-

amines and the methylxanthines may both exert their inotropic effects through increased concentrations of cyclic AMP. The present results indicate the inotropic effects of the methylxanthines are not mediated through cyclic AMP but are more readily explained by the effects of these agents on calcium metabolism.

The methylxanthine phosphodiesterase inhibitors are known to release catecholamines from heart tissue (Westfall and Fleming, 1968). Several investigators have, therefore, proposed that theophylline may influence cardiac muscle through the secondary release of endogenous catecholamines (Nuzher et al., 1967: Westfall and Fleming, 1968). In the present series of experiments, however, theophylline was found to exert a direct contractile effect in reserpine pretreated tissue which was not significantly different from the control response (figure 21). In contrast, reserpine pretreatment abolished the cyclic AMP increase seen with theophylline (figure 25). Thus, the theophylline-induced cyclic AMP response is an indirect effect associated with the release of endogenous amines while the greater part of the contractile reasponse is not.

A further dissociation between the contractile and the cyclic AMP responses can be seen in figure 22. Theophylline ($5x10^{-4}$ M) significantly ($P<0.05$) enhanced the contractile response to norepinephrine after 15 minutes. The contractile response to norepinephrine after 3 minutes exposure, however, was attenuated ($P<0.01$). There was no difference in the cyclic AMP concentrations at either of these times (figure 24

and 25). After 60 minutes exposure, the tissue was completely unresponsive to norepinephrine. It is difficult to see how these results can be resolved on the basis of phosphodiesterase inhibition and cyclic AMP elevation, however, they may be readily explained on the basis of calcium metabolism. The methylxanthines are known to alter intracellular calcium concentrations by decreasing the rate of calcium sequestration by the sarcoplasmic reticulum (Weber, 1968), and mitochondrial accumulation of calcium (Nayler, 1967). Transport of calcium by the cell membrane either to increase influx (Scholz, 1971) or to decrease efflux (Shine and Langer, 1971) or both, may also be affected. more calcium is thus available for excitation-contraction coupling and a positive inotropic effect results. In the present series of experiments, the attenuation of the norepinephrine contractile response observed after 3 minutes exposure to theophylline may result from excessive release of calcium ions. De Gubareff and Sleator, 1965, have demonstrated that guinea-pig atria respond in a positive fashion to concentrations of caffeine up to 1.5 mM in the presence of a normal amount of calcium in the bath. Higher concentrations of caffeine produced a negateve inotropic effect unless the calcium concentration in the bath was lowered. In the presence of calcium , caffeine produced only a negative inotropic effect.

These authors suggested that increasing intracellular calcium beyond this optimal level (by increasing the dose of caffeine or raising the concentration of calcium in the bath) would result in a decrease in contractility. It seems reasonable that the rate of theophylline-induced calcium release would be greatest when the contractile response induced by theophylline first reached its peak. At this time release would be maximally stimulated and the calcium reserves would not yet be seriously depleted. Norepinephrine might then increase the intracellular level of ionized calcium beyond the optimal level for contraction and produce a negative inotropic effect (figure 22). After 15 minutes exposure to the same dose of theophylline, the calcium reserves might be sufficiently depleted that the combined effect of theophylline and norepinephrine on calcium mobilization would then be in the optimal range for contraction, and an enhancement of the inotropic response would result. After 60 minutes exposure, the intracellular calcium reserves may be so much further depleted that no inotropic response to norepinephrine would be possible (figure 22). It has previously been shown that the methylxanthines can both enhance and depress the contractile effect of norepinephrine depending on the dose of both drugs and on the amount of calcium present in the physiological solution (McNeill et al., 1969, 1973b). In the present study the time of exposure to theophylline has also been shown to affect the results.

A number of investigators (Hamakawa et al., 1972; Endoh

and Schumann, 1974; Kalsner, 1971) have reported that theophylline in a sufficient concentration to enhance the contractile effect of isoproterenol, epinephrine, or norepinephrine, did not alter the positive inotropic effect of phenylephrine. This observation is again confirmed in the present series of experiments (figure 23). Since all increases in cardiac contractility were plotted against the original baseline before the addition of theophylline the increase in contractile force seen with low doses of phenylephrine are due to the inotropic response of theophylline alone. It can clearly be seen that there was no increase in the maximum contractile effect of phenylephrine in contrast to the results obtained with norepinephrine (figure 22). Endoch and Schumann, 1975, have interpreted these results to be inconsistent with the ability of theophylline to act through a calcium-dependent mechanism as proposed by Blinks et al., 1972, and by McNeill et al., 1969. However, one would not expect the maximum response mediated mostly by alpha-adrenergic receptors (phenylephrine stimulated) to be enhanced by the methylxanthines if the same sources of calcium were utilized by both groups of compounds. Only if an additional factor (such as a high intracellular level of cyclic AMP) which was able to mobilize additional resources of calcium, was introduced would an increase in efficacy be apparent. Endoch et al., 1975, have recently demonstrated differences in the calcium fluxes mobilized by alpha and beta-adrenergic receptors. Similarly, one would not expect the methylxanthines to affect calcium dose-response

curves (Endoch and Schumann, 1975a; Skelton et al., 1971). The amount of calcium released from internal stores by theophylline might be too small to significantly affect calcium dose-response curves which occur between 10^{-4} and 10^{-2} molar (Endoch and Shumann, 1975a). In any case, it should be noted that merely elevating extracellular calcium may not be sufficient to demonstrate an intracellular calcium effect. Several investigators (Massingham and Nasmyth, 1972; Endoch and Schumann, 1975a)have demonstrated a positive interaction between electrical stimulation and theophylline in cardiac muscle. Such an effect would not likely be mediated through cyclic AMP and is more likely explained by an increase in intracelluar calcium. Alternate explanations are also possible. Kalsner, 1971, has demonstrated that the methylxanthines inhibit the extraneuronal inactivation of catecholamines by inhibiting catechol-o-methyl-transferase in vascular smooth muscle. A similar mechanism might operate in cardiac muscle and would thus specifically enhance the norepinephrine but not the phenylephrine response. Further studies are needed before any definite conclusions can be reached on the interpretation of this data.

Theophylline alone caused a small increase in cyclic AMP which was found to be due to the secondary release of endogenous catecholamines (figures 24 and 25). In all cases the effect of theophylline on cyclic AMP appeared to be additive and not potentiative with the adrenergic amine response. This would further tend to rule out phosphodiesterase inhibition as a factor

In the effect of theophylline on the amine-induced cardiac contractility. Many other discrepancies between phosphodiesterase inhibition and pharmacological effects have been noted in other tissues and have been reviewed by Blinks et al., 1972; and McNeill et al., 1969, 1974.

In conclusion our data support the work of McNeill et al., 1974, who reported a lack of interaction between norepinephrine and theophylline on cardiac cyclic AMP. The theophylline-induced cyclic AMP response is an indirect effect associated with the release of stored catecholamines. Futhermore, theophylline exerts a direct contractile effect which is unrelated to cyclic AMP. The ability of theophylline to enhance the norepinephrine and not the phenylephrine response may be due to mobilization of different sources of calcium by the alpha and beta receptors. The fact that the effect of theophylline on cyclic AMP appeared to be additive and not potentiative would tend to rule out phosphodiesterase inhibition as a factor in the effect of theophylline on the amine-induced cardiac contractility. In addition, there was no correlation between the contractile and the cyclic AMP effects at the different times tested. It therefore seems logical, in view of the lack of correlation observed, that the cardiac effects of the methylxanthines are not mediated through cyclic AMP. These results support the view that the methylxanthines exert their effects on cardiac tissue through calcium metabolism.

CHAPTER V

SUMMARY

1. Norepinephrine and phenylephrine cause an increase in cyclic AMP levels prior to contractile force, however, both amines have the ability to produce an increase in cardiac contractile force in which cyclic AMP is not involved.

2. The phenylephrine-induced cyclic AMP response is an indirect effect associated with the release of stored catecholamines.

3. It seems reasonable to attribute the increase in contractile force in response to norepinephrine and phenylephrine which do not involve cyclic AMP to alpha receptors.

4. The time course of the contractile response induced by adrenergic amines may be remarkably influenced by the chronotropic response in spontaniously beating preparations while the cyclic AMP response is not greatly affected.

5. It is therefore suggested that rate controlled preparations may offer a better system for attempting to correlate physiological and biochemical events.

6. There is no interconversion of alpha- and beta adrenergic receptors mediated by temperature.

7. The apparent blockade of the adrenergic amine-induced inotropic response by phentolamine and the apparent lack of blockade by propranolol have been found to be related to the ability of the blocking agents to modify the inotropic effect of low temperatures.

8. Although adrenergic amines have the ability to cause increases in contractile force which are mediated by alpha receptors and which do not involve cyclic AMP, no dissociation has yet been found between the beta receptor, cyclic AMP, and the inotropic response.

9. It therefore seems logical, in view of the close association of these parameters, that cyclic AMP may be one of the second messengers mediating the inotropic response in cardiac tissue.

10. The theophylline-induced cyclic AMP response is an indirect effect associated with the release of stored catecholamines.

11. Furthermore, theophylline exerts a direct contractile effect which is unrelated to cyclic AMP.

12. The ability of theophylline to enhance the norepinephrine and not phenylephrine response may be due to mobilization of different sources of calcium by the alpha and beta receptors.

13. The fact that the effect of theophylline on cyclic AMP appeared to be additive and not potentiative would tend to rule out phosphodiesterase inhibition as a factor in the effect of theophylline on the amine-induced cardiac contractility.

14. The effect of theophylline on amine-induced cardiac cyclic AMP and contractile force showed no correlation between the contractile and the cyclic AMP effects at the different times tested.

15. It therefore seems logical that the cardiac effects of theophylline are not mediated through cyclic AMP.

16. These results support the view that the methylxanthines exert their effects on cardiac tissue through calcium metabolism.

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