

Cyclic Nucleotide Regulation of the Contractile Proteins in Mammalian Cardiac Muscle

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ABSTRACT The contractile system of rat cardiac muscle that has been made hyperpermeable by soaking the tissue in EGTA (McClellan and Winegrad, 1978. *J. Gen. Physiol.* **72**:737-764) can be probed directly with Ca buffer from the bathing solution without significant interference from either sarcoplasmic reticulum or mitochondria on the Ca concentration. Changes in Ca-activated force are due therefore to changes in the properties of the contractile system itself and not to regulation of Ca concentration. The addition of cAMP, cGMP, and GTP, guanylyl imidodiphosphate (GMP-PNP), or epinephrine to the bath does not alter maximum Ca-activated force, but when these drugs are added with 1% nonionic detergent to the bath, contractility increases by as much as 180%. An inhibitor of phosphodiesterase must be present for the inotropic effect of cAMP but not cGMP, GTP, GMP-PNP, or epinephrine. The inotropic response to cAMP is independent of the Ca sensitivity of the contractile system, but guanine nucleotides enhance contractility only when Ca sensitivity is not high. The inotropic effect of epinephrine is inhibited to a large extent by cGMP but not by GMP-PNP. These data can be explained by a model in which contractility is enhanced by a cAMP-regulated phosphorylation that can be controlled through the β -receptor adenylate cyclase complex in the sarcolemma. The regulation involves two reactions, one a phosphorylation and a second that occurs in the presence of detergent. Phosphorylation of neither the myosin light chain nor the inhibitory subunit of troponin appears to be involved in this mechanism for regulating contractility.

INTRODUCTION

The mechanisms for the regulation of contractility of the cardiac cell are poorly understood. Most of the attention has been focused on a variation of the amount of Ca used to trigger the contraction in response to changes in the action potential, Ca conductance of the excited membrane (New and Trautwein, 1972; Reuter and Scholz, 1977), or rate of reaccumulation of Ca by the sarcoplasmic reticulum (Kirchenberger et al., 1974). Direct evidence for physiological regulation of the contractile proteins¹ themselves is sparse

¹ In this paper "contractile proteins" is used to include the regulatory proteins in the myofibril, as well as actin and myosin.

(Tsien and Weingart, 1976; Tsien 1977). Phosphorylation of the inhibitory subunit of troponin (TNI) can occur and is associated with the increased contractility produced by catecholamines (England, 1976; Solaro et al., 1976), but phosphorylation of TNI only increases the concentrations of Ca required for activation of the ATPase in isolated myofibrils without enhancing ATPase activity (Ray and England, 1976). Analogous regulation of the Ca sensitivity without change in contractility exists in the hyperpermeable rat heart (McClellan and Winegrad, 1978). Under certain circumstances a close relation between contractility and the ratio of cAMP to cGMP can be demonstrated in the intact frog heart (Singh et al., 1978), but it is not clear that this is due to a change in the contractile proteins.

In the studies described in this paper, regulation of the inotropic state of the contractile system itself has been studied in rat ventricles that have been made hyperpermeable by treatment with EGTA (McClellan and Winegrad, 1978). In this preparation, it is possible to bypass the normal electromechanical coupling steps and probe the properties of the contractile proteins directly with Ca buffers without mechanically removing the sarcolemma. Inasmuch as hyperpermeable cells retain soluble intracellular proteins as well as many membrane proteins, regulatory systems are more likely to remain intact in hyperpermeable cells than in mechanically skinned or glycerol-extracted tissues. Rather than attempting to alter the isolated protein system by known reactions to produce a state of enhanced contractility—an unsuccessful approach thus far—this work is directed towards producing a state of enhanced contractility and then trying to identify the regulatory reactions. Three specific questions will be addressed:

- a) Is the contractile state of the myofibrils, including both the contractile and the regulatory proteins, regulated directly?
- b) What are the roles of catecholamines and cyclic nucleotides in the regulation?
- c) What is the contribution of phosphorylation reactions involving myofibrillar proteins such as troponin and the light chains of myosin to the regulation (Cole et al., 1978)?

METHODS

The general method has already been reported by McClellan and Winegrad (1978). Natural bundles of tissues were removed from the endocardial surface of rat right ventricles and soaked overnight in a solution containing 10 mM EGTA at 0°C. This treatment produces a preparation in which the membrane is permeable to ions and small molecules. The tissues were mounted in a chamber for continuous superfusion and measurement of tension. The solutions used before and after the exposure to detergent had identical compositions. Reagents were obtained from Sigma Chemical Co. (St. Louis, Mo.). All nucleotides were tested for purity by one- or two-dimensional thin layer chromatography, and only a small breakdown of nucleotide triphosphate to nucleotide diphosphate was detected. This change should have been reversed in the superfusion solutions because of the presence of the phosphocreatine-creatine phosphokinase system.

RESULTS

The hyperpermeable rat ventricular strip has characteristics that make it suitable for the study of functional properties of the contractile proteins (McClellan and Winegrad, 1978). Because it is very stable, even relatively small changes in the mechanical response to a given concentration of Ca^{+} ions are significant. The membrane is freely permeable to a Ca-EGTA buffer system that prevents the influence of the mitochondria or sarcoplasmic reticulum on sarcoplasmic Ca concentration. Therefore, changes in the response to a given concentration of Ca are the result of alterations of the contractile proteins rather than other organelles (McClellan and Winegrad, 1978).

Contractility of hyperpermeable cells, defined as the maximum Ca-acti-

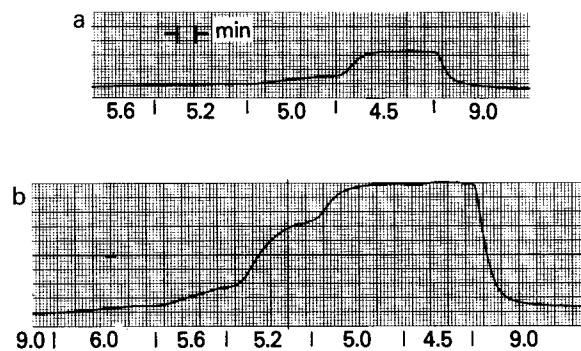


FIGURE 1. Tension record of a hyperpermeable bundle of rat ventricular fibers. Numbers below record indicate pCa in contraction solution. Between *a* and *b* tissue was exposed to relaxing solution with 5 mM theophylline for 5 min; relaxing solution with 5 mM theophylline and 10^{-6} M cAMP for 10 min; relaxing solution with 5 mM theophylline, 10^{-6} M cAMP, and 1% Triton X-100 for 30 min; and finally relaxing solution for 30 min. Note large increase in Ca-activated force in *b*.

ated force, is not altered by adding from 10^{-9} to 10^{-5} M cAMP or cGMP to the bathing solution either before or with the activating Ca. The presence of the phosphodiesterase inhibitor theophylline with the cyclic nucleotides does not change the result. Epinephrine and GTP are similarly without effect on the maximum Ca-activated force.

If a nonionic detergent such as Triton X-100, Brij 58, or Lubrol WX is present with the cyclic nucleotides or catecholamine, marked changes in Ca-activated force result (Fig. 1; Table I). Inasmuch as Ca-activated force is reduced in the presence of detergent, although the inhibitory effect is completely reversible and disappears after removal of the detergent (Table I), the contractility was compared before and after detergent and drug treatment. Hyperpermeable fibers were exposed to relaxing solution with the drug for 5 min and then to relaxing solution containing both the drug and 1% detergent

for up to 30 min. After the detergent and the drug had been washed out with relaxing solution for another 30 min, the response to Ca was tested and compared with the response before exposure to the combination of drug and detergent. The increased contractile state induced in this way by nucleotides or catecholamines is very stable and unresponsive to further exposure to cyclic nucleotides or catecholamines in either relaxing or contraction solution. Detergent alone in relaxing solution does not lead to enhanced contractility. The enhanced contractility cannot be either (a) reversed by relaxing media with detergent or (b) increased any further by a second exposure to the combination of drug and detergent. The change in contractility produced by the drug in detergent can be blocked by first exposing the hyperpermeable

TABLE I
MAXIMUM Ca-ACTIVATED FORCE

Procedure	Experiment <i>n</i>	Relative force
Control		100
Detergent	21	110±6
Theophylline + detergent	7	99±7
cAMP + detergent	6	106±11
Theophylline + cAMP + detergent	6	158±16*
Theophylline, theophylline + cAMP + detergent	18	259±10‡
Epinephrine + detergent	14	273±37‡§
Epinephrine + cGMP + detergent	11	158±20*§
Guanine nucleotide + detergent	25	201±14‡
Epinephrine + GMP-PNP + detergent	9	238±25‡
5'-GMP	4	102±6
Dibutyryl cAMP + detergent	4	102±9

Relative force refers to the maximum Ca-activated force produced after each tissue was treated as indicated in the procedure compared with the initial values in Ca solution without drugs.

* Significant difference from control at <0.05.

‡ Significant difference from control at <0.01.

§ Significant difference from presence of cGMP at <0.05.

|| No significant difference between the two values.

cells to detergent in relaxing solution. The detergent seems to exert a permissive effect on reactions that alter inotropy and at the same time slowly remove the reactants required for the production of the altered state (see Discussion).

Regulation of Contractility by cAMP

The addition of 10^{-6} M cAMP and 1% detergent to the bathing medium for as long as 30 min produces no observable change in the maximum Ca-activated force. If 5 mM theophylline is included with the cAMP, the maximum Ca-activated force is increased by a significant but variable amount (Table I). The hyperpermeable cells apparently have an active phosphodiesterase which almost totally eliminates the effects of exogenous cAMP, and the addition of theophylline is not always successful in producing a significant block of the same enzyme. The extent of the increase in contractility is not correlated with the Ca sensitivity of the contractile system (defined as the pCa

necessary to produce 50% of maximum activation), indicating that the extent of phosphorylation of the inhibitory subunit of troponin (TNI) is probably not important in this regulatory mechanism (Table I).

When 5 mM theophylline is added to the bathing solution a few minutes before the cAMP, there is a large and consistent increase in contractility (Fig. 1; Table 1). The procedure of theophylline followed by theophylline and cAMP and then the combination cAMP, theophylline, and detergent produced an average increase in contractility of 159%. The phosphodiesterase inhibition is more complete when the inhibitor is added before the cyclic nucleotide. Theophylline itself with or without detergent did not change contractility.

The change in contractility from cAMP and theophylline must occur rapidly because a very brief exposure to the drugs produces essentially the same result as a 30-min exposure. The lipid soluble form of the nucleotide, dibutyl cAMP, is equally effective, but it too requires the presence of a detergent and a phosphodiesterase inhibitor to produce a change in contractility.

Exposure to cAMP and theophylline in the absence of detergent does change the latent properties of the hyperpermeable cells even though the effect is not immediately apparent on Ca-activated force. Bathing the preparation with relaxing solution and detergent 5–10 min after a 10-min exposure to cAMP and theophylline produces a $79 \pm 19\%$ increase in maximum Ca-activated force. Apparently cAMP can induce changes in the preparation in the absence of detergent, although these changes are gradually reversed in relaxing solution. These data indicate that there may be more than one reaction involved in the production of the higher contractility state, one sensitive to cAMP, and second that takes place in the detergent and can not be merely the inhibition of reactions reversing the effect of cAMP. These conclusions are supported further by the results of experiments with ATP γ S and benzyl alcohol.

Studies with ATP γ S

ATP γ S is an analog of ATP which provides a phosphate for phosphorylation reactions that is removed only very slowly by phosphatases. This ability to support phosphorylation but inhibit dephosphorylation makes the compound a useful probe for studying the physiological effects of phosphorylation. When all of the ATP in the solution bathing hyperpermeable fibers is replaced by ATP γ S, the maximum Ca-activated force declines. This reduction in force production, which is completely reversed when ATP is restored, results from the fact that ATP γ S is a relatively poor substitute for ATP in the reaction between actin and myosin. Although the maximum force is the same after a 5-min exposure to ATP γ S in either relaxing solution or a contraction solution, the properties of the hyperpermeable cells have been changed in two observable ways. There is a small but definite increase in Ca sensitivity (Fig. 2) that is irreversible. In all eight experiments where ATP γ S was used, the Ca concentration necessary for 50% activation of contraction decreased from 20 to 40%, a smaller shift in Ca sensitivity than that produced by theophylline or

detergent. In addition, the hyperpermeable cells treated with ATP γ S respond to 1% nonionic detergent in relaxing solution with an increase in the maximum Ca-activated force even without exposure to cyclic nucleotides or catecholamines. The extent of the increase in contractility, $180 \pm 26\%$, is the same as that produced by detergent with cAMP and theophylline in hyperpermeable fibers not exposed to ATP γ S. Both the overt effect on Ca sensitivity and the latent effect on contractility of ATP γ S are undiminished even 60 min after ATP γ S has been removed.

These results indicate that the same phosphorylation that appears to result from cAMP and theophylline has occurred during the exposure to ATP γ S,

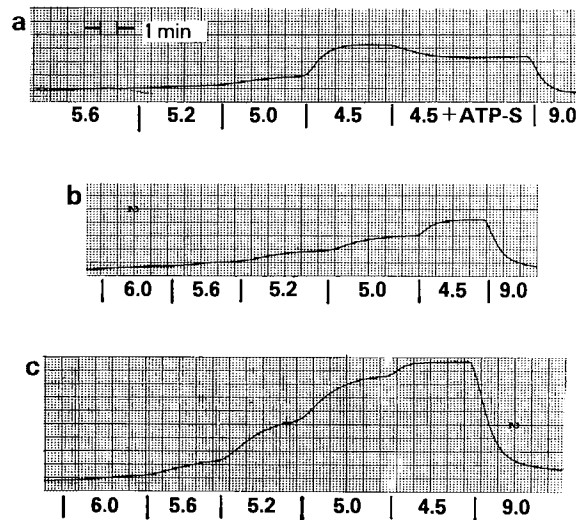


FIGURE 2. Effect of ATP γ S on hyperpermeable fibers. Between exposures to Ca-containing contraction solutions in *a* and *b*, tissue was exposed for 10 min to relaxing solution in which all of the ATP had been replaced by ATP γ S. Note the increase in Ca sensitivity but the same maximum Ca-activated force. Between *b* and *c* the tissue was exposed for 30 min to relaxing solution with 1% Triton X-100 and then to relaxing solution for an additional 30 min. Note increase in maximum Ca-activated force.

but it is insufficient to enhance contractility. The change in contractility apparently requires two separate reactions: one a phosphorylation and the second, still undefined but induced by detergent. The irreversible increase in Ca sensitivity shows that some turnover of phosphate on TNI occurs even in the absence of cyclic nucleotides and phosphodiesterase inhibitors with the cGMP-regulated reaction that increases Ca sensitivity predominating (McClellan and Winegrad, 1978; Mope et al., 1980).

Additional data support the notion that there is a two-step mechanism for regulation of contractility. Benzyl alcohol has been shown to activate adenylate cyclase in the membranes of liver cells, apparently by changing the properties of the membrane lipids (Houslay et al., 1976; Dipple and Houslay 1978). Treatment of hyperpermeable cardiac cells with 0.1% benzyl alcohol in

relaxing solution produces the same changes in the cells as ATP γ S substitution for ATP, that is, a modest increase in Ca sensitivity and a large increase in maximum Ca-activated force after treatment with detergent in relaxing solution (Fig. 3). The changes are similarly irreversible, remaining for at least 60 min after the exposure to benzyl alcohol in relaxing solution. During the exposure to the benzyl alcohol Ca-activated force may be reduced by up to 10%, but this completely reverses when the alcohol has been removed. Higher concentrations produce larger but still reversible decreases in Ca-activated force.

Effect of Epinephrine

The maximum Ca-activated force is also increased by epinephrine in concentrations greater than 10^{-8} M (Table I). The largest response, almost a tripling

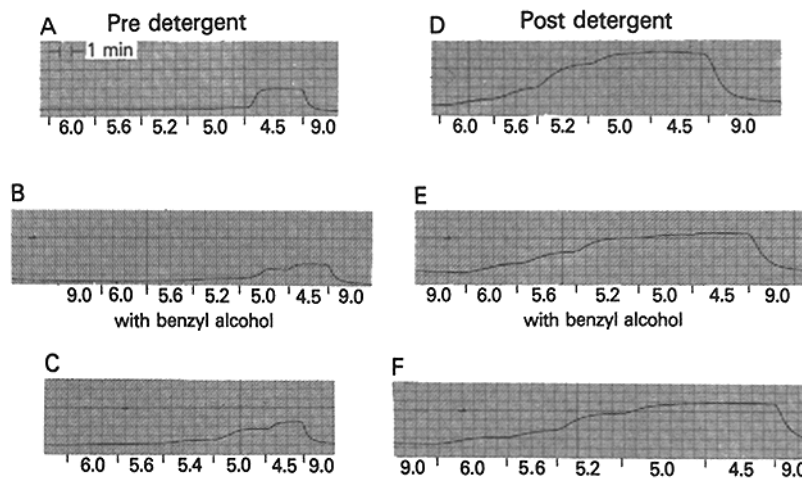


FIGURE 3. Effect of 0.1% benzyl alcohol on hyperpermeable cells. A,C,D,F without benzyl alcohol. Note increase in Ca sensitivity after benzyl alcohol and increase in force after detergent.

of maximum Ca-activated force, occurs with 10^{-7} M, and there is some suggestion that a concentration of 10^{-6} M or greater actually produces a smaller inotropic effect. In four experiments where paired bundles from the same hearts were studied, the increase in the Ca-activated force was always greater with 10^{-7} M than with 10^{-6} M epinephrine.

The inotropic response to epinephrine was reduced by about 70% when 10^{-7} M cGMP or 10^{-6} M GTP was added with epinephrine but the nonhydrolyzable guanine nucleotide guanylyl imidodiphosphate (GMP-PNP), which is believed to act primarily if not exclusively in coupling the β -receptor with adenylate cyclase, had no such effect. Isoproterenol produced the same results as epinephrine.

Effect of Guanine Nucleotides

In the presence of detergent, guanine nucleotides also increased contractility. 1 μ M GTP, 0.1–1.0 μ M cGMP, and 10 μ M CMP-PNP all produce very

similar effects on the maximum Ca-activated force with the largest increases reaching values of 225% (Fig. 4). Unlike the change in contractility produced by cAMP, the positive inotropic response to guanine nucleotides was dependent on the Ca sensitivity of the preparation (Fig. 4). Little or no modification of maximum Ca-activated force was seen when the Ca sensitivity was high, but in tissues with low values there was a large increase in maximum Ca-activated force. Although the exact correlation between the calcium sensitivity and the relative increase in force may not be the same for each guanine

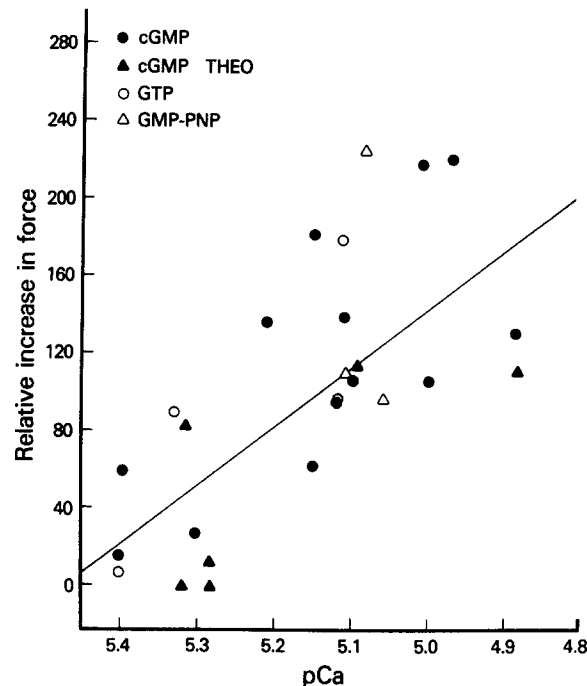


FIGURE 4. Relation of increase in contractility, produced by guanine nucleotides, to Ca sensitivity, indicated as the pCa for 50% of maximum Ca-activated force. Ordinate is the relative increase in maximum Ca-activated force.

nucleotide, there is a similarly positive correlation in each case. Neither addition of theophylline, variation of the concentration of GTP between 10^{-7} and 10^{-5} M, nor the addition of GTP 5 min before the detergent made any difference. 5'-GMP, the metabolic breakdown product of GTP, had no effect on contractility either in the presence or absence of detergent.

Studies with CTP

In view of the demonstration that the 19,000 dalton light chain of myosin can be phosphorylated in cardiac muscle by activation of light chain kinase with the combination of Ca and calmodulin (Frearson and Perry, 1978; Nairn and Perry, 1979), it was of interest to consider whether either of the two steps in the change in contractility involved phosphorylation of the light chain of

myosin. Light chain phosphorylation was unlikely during the first step inasmuch as the change in contractility could occur with cAMP, ATP γ S, or benzyl alcohol in the virtual absence of Ca (less than 10^{-8} M). Since CTP is an excellent substitute for ATP in the reaction between actin and myosin but a very poor phosphate donor in some phosphorylation reactions (Hasselbach, 1956; Walsh and Krebs, 1973), including that of light chains (Perry et al., 1975), CTP can be used as a probe to detect a requirement for phosphorylation of the light chain. When ATP was replaced with CTP there was a large increase in Ca sensitivity, which indicated inhibition of phosphorylation of TNI. The substitution of CTP for ATP in relaxing solution containing cAMP, theophylline, and detergent did not prevent the normal increase in contractility either when CTP was continued through the Ca-activating solutions, or when ATP was restored to the contraction solutions. The increase in contractility in the cAMP-theophylline-detergent solution, despite the absence of a good phosphate donor for myosin light chain, argues against light-chain phosphorylation as the critical second reaction in enhancing contractility.

DISCUSSION

The most important conclusion to be drawn from these experiments is that the force of contraction of cardiac cells can be regulated by changes in the properties of the contractile proteins. Any significant influence of the sarcoplasmic reticulum or the mitochondria on the steady-state, Ca-activated force of the hyperpermeable fibers was eliminated by the Ca buffer system present in all solutions (McClellan and Winegrad, 1978). Catecholamines, cAMP, and guanine nucleotides are involved in the regulation since each can influence the maximum Ca-activated force, but the mechanism appears to require two reactions, of which only one may be sensitive to cyclic nucleotides.

The need for detergent for the second reaction implicates a lipid phase of the cell and, in particular, cell membranes. The function of the detergent may be simply to overcome a lipid barrier to a larger molecule for diffusion between the bath and the cytoplasm or to facilitate either accessibility of reactants or release of products for reactions occurring within a membrane. Removal of Ca from the sarcolemma alters the properties of the membrane and apparently prevents some critical reaction among the molecules that are present (Ross and Gilman, 1977; Schramm et al., 1977; Ross et al., 1978). Membrane fluidity is altered and with it the reactivity of membrane proteins (Humphries and McConnell, 1975; Dipple and Houslay, 1978) and possibly interactions of membrane proteins with microfilaments and microtubules (Nicolson, 1976; Edelman, 1977). The ability of detergent to facilitate or inhibit reactions within membranes is well known (Levey, 1970; Johnson and Sutherland, 1973; Kimura and Murad, 1974; Horwood and Singhal, 1976; Houslay et al., 1976), and in referring to its effects on cholinergic receptors, Changeux (1974) has suggested that solubilization by detergents releases constraints created by either membrane lipids or proteins and stabilizes the receptor in a favorable conformation. On the other hand, prolonged exposure or exposure to high concentrations may inactivate the reaction by removing necessary lipid or protein molecules from the membrane.

Cyclic AMP produced an increase in contractility that bore no obvious relation to Ca sensitivity or TNI phosphorylation. Guanine nucleotides, however, produced increases in contractility that had a large positive dependence on TNI phosphorylation when the latter was estimated by the Ca sensitivity. Little or no increase in contractility occurred in cells with a high Ca sensitivity; large increases occurred when Ca sensitivity was low. Since TNI phosphorylation is controlled by epinephrine (Ray and England, 1976), the amount of phosphorylation should be an indication of the extent of epinephrine stimulation. In isolated membranes guanine nucleotides are necessary for coupling the epinephrine stimulated β -receptor to adenylate cyclase; the coupling determines the degree of activation of adenylate cyclase by catecholamines (Lefkowitz, 1975; Rodbell et al., 1975; Levitski, 1978). Guanosine triphosphate seems to be the physiological nucleotide in intact cells. In view of the relation to epinephrine, it is reasonable that guanine nucleotides increase contractility in hyperpermeable fibers by a similar coupling mechanism. (Pfeuffer, 1972; Sevilla and Levitski, 1977). These data do not explain why cGMP, which is not a good coupler with isolated membrane fragments (Lefkowitz, 1975), should be an effective inotropic agent. Its breakdown product, 5'-GMP, has very little inotropic activity, and although cGMP can produce GTP in the presence of pyrophosphate, pyrophosphate is normally broken down so rapidly that it never reaches an adequate concentration to support the reversal of the GTP-cGMP reaction (Greengard et al., 1969). The site of the inhibitory reaction of cGMP is not the β -receptor-adenylate cyclase coupling; cGMP can inhibit the positive inotropic response to epinephrine but GMP-PNP does not.

The greater response to guanine nucleotides than to cAMP when there is no inhibition of phosphodiesterase is intriguing because both nucleotides are probably ultimately operating through the same protein kinase. The need of phosphodiesterase inhibition for any increase in contractility from cAMP, but not from guanine nucleotides, can be explained by a phosphodiesterase in the sarcolemma that protects the protein kinase from cAMP in the bath or, in the case of the intact cell, in the cytoplasm (Corbin et al., 1977). This protein kinase is normally available only to cAMP produced within the membrane by an ordered sequence of reactions involving epinephrine and a guanine nucleotide. As a result a functional compartmentalization would exist, and the cell could use cytoplasmic cAMP for the regulation of intracellular reactions without activating a protein kinase in the membrane. On the other hand activation of the membrane enzyme would depend on the combination of an extracellular transmitter, catecholamine, and an intracellular messenger, guanine nucleotide.

Although the data are insufficient to prove any detailed mechanism, it is useful to consider whether they can be synthesized with published material into a coherent and internally consistent model. Such a proposal is shown in Fig. 5 which depicts regulation of contractility by cAMP, GTP, and cGMP. Such a model is consistent with much existing physiological and biochemical data, and it indicates why the state of contractility cannot always be related

to cAMP or cGMP concentrations in the cell in a simple way. The two opposing reactions regulated by guanine nucleotides result in an interesting property of the system regulating the contractile proteins. The cell itself can either inhibit contractility or turn up the gain on the catecholamine-dependent positively inotropic system. According to this model, the cell and the organism negotiate the contractile state of the myocardial proteins through neurotransmitters like catecholamines and "intracellular messengers" like guanine nucleotides. The result is that the contractile state is a function of the organism's needs and the metabolic state of the cell.

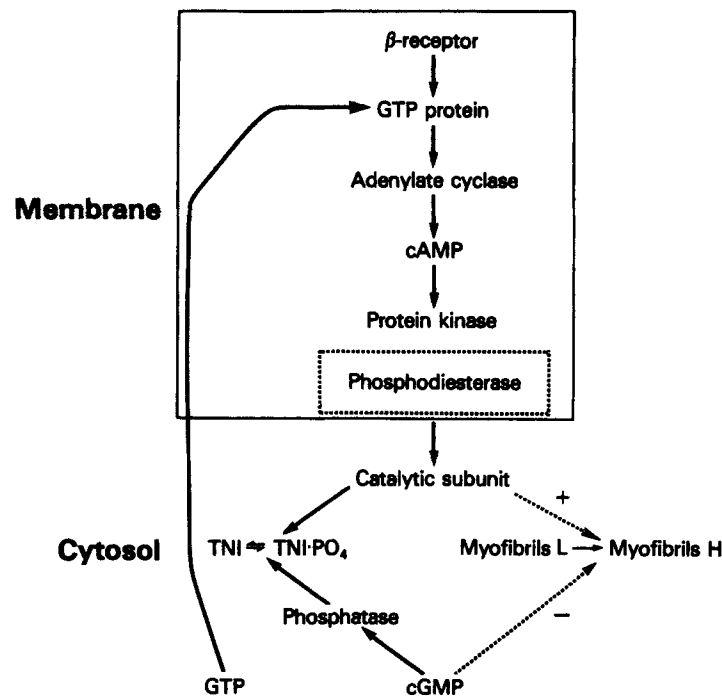


FIGURE 5. Model to explain regulation of contractility and Ca sensitivity by cyclic nucleotides and catecholamines. TNI phosphorylation produces lower Ca sensitivity and is blocked by cGMP (England, 1978; McClellan and Winegrad, 1978; Mope et al., 1980). See text for details. L and H after myofibrils indicate low and high inotropic state.

The actual reactions involved in altering contractility are not clear, but certain negative conclusions can be drawn. The physiological data indirectly indicate that the phosphorylation of TNI or the myosin light chain is not responsible for the cAMP-regulated increase in contractility of the myofibril.

These conclusions are supported by preliminary data involving SDS gel electrophoresis of hyperpermeable fibers in both the low and high states of contractility. The transition from low to high inotropy is not related to (a) TNI phosphorylation, although Ca sensitivity is (Mope et al., 1980), or (b) the phosphorylation of the 19,000 dalton myosin light chain.

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