

# The Regulation of the Calcium Sensitivity of the Contractile System in Mammalian Cardiac Muscle

GEORGE B. McCLELLAN and SAUL WINEGRAD

From the Department of Physiology G4, School of Medicine, University of Pennsylvania, Philadelphia, Pennsylvania 19104

**ABSTRACT** Treatment of rat ventricular cells with 10 mM EGTA makes the sarcolemma highly permeable to small ions and molecules without removing its restriction of the diffusion of larger molecules or inactivating all of its enzymatic functions. These hyperpermeable cardiac cells have been used to study the regulation of the range of concentration of Ca over which activation of the contractile proteins occurs (Ca sensitivity). The Ca sensitivity can be varied from three- to sixfold without any significant alteration in the general shape of the relation between force and Ca concentrations. Although cyclic nucleotides in concentrations of  $10^{-9}$  to  $10^{-5}$  M do not influence Ca sensitivity, in the presence of a phosphodiesterase inhibitor, cGMP increases and cAMP decreases Ca sensitivity. Treatment of the hyperpermeable cells with a nonionic detergent raises Ca sensitivity as does removal of the phosphate donor by complete substitution of CTP for ATP. These data indicate that Ca sensitivity is probably modulated by a cAMP-dependent phosphorylation that decreases Ca sensitivity. The sarcolemma is required for this reaction to take place. The effect of this reaction is antagonized by a cGMP-dependent reaction occurring inside the cell. Studies involving the perfusion of the heart with and without epinephrine before the exposure to EGTA indicate that epinephrine can regulate this system of control of Ca sensitivity. The functional considerations of this regulatory system are discussed.

## INTRODUCTION

Because every myocardial cell normally contracts in every cardiac cycle, the modulation of contractility must occur at the cellular rather than the organ level. Four general mechanisms have been proposed for the regulation of cellular contractility: (a) the organization of the contractile filaments (49); (b) the accumulation and release of Ca from the sarcoplasmic reticulum (10, 13, 23, 45); (c) Ca influx during the action potential (34, 39, 56); and (d) alteration in the contractile proteins themselves (37, 42). It is now apparent that the amount of filament overlap is not critical in the physiological regulation of force generation (24, 41, 55). Modification of the contractile state of the heart from changes in inward Ca current or Ca uptake by the reticulum can be produced by alteration of the action potential or by phosphorylation of a reticular protein, respectively (23, 40, 45), and may be physiologically important.

Attention has been focused on the contractile proteins themselves since the

demonstration of several phosphorylation sites including some which are dependent on cAMP (12, 35, 42, 47, 50). The phosphorylation of troponin I (TNI) may produce a change in the concentration of Ca required for activation of the cardiac myofibrillar ATPase (37, 42) and modifications in contractility have been observed in perfused hearts in association with altered cAMP and cGMP concentrations (12, 18, 47); however, no consistent relation between cyclic nucleotide concentration and contractile state has been demonstrated as yet. One limitation inherent in the study of intact cells is that the contractile properties of the protein cannot be directly assayed inasmuch as any regulation that may exist in excitation-contraction coupling cannot be bypassed. Consequently, the inotropic state of the tissue cannot be directly correlated with the degree of phosphorylation of the contractile proteins.

In the studies reported here, a preparation in which the surface membrane has been made very permeable to small ions and molecules without the loss of the diffusion barrier to large intracellular molecules has been used to probe the changes in the contractile proteins that can be produced by the remaining sarcolemma and other cellular components. It is a hyperpermeable rather than a skinned fiber preparation because the membrane remains and still retains some of its normal biochemical functions.

#### METHODS

After male rats weighing between 50 and 150 g were killed by decapitation, their hearts were rapidly removed and transferred to oxygenated, modified Krebs' solution at 4°C. The right ventricle was opened, and ties of raw silk were placed around the ends of the central and lateral papillary muscles and any ventricular trabeculae that were free for at least 2–3 mm of their length. The free ends of the silk were tied together to form loops for holding the tissue. The papillary muscles and trabeculae were freed from their attachments to the wall of the ventricle and transferred to a small chamber containing "disruption solution" (see below), where the isolated tissues were pinned at approximately their length in the opened but unstretched heart. The tissues were exposed to the disruption solution at 0°C for at least 12–72 h. Their mechanical response to Ca ions under standard experimental conditions described below was stable throughout this period. This "disruption" procedure is a modification of the one originally described in 1971 which used EDTA for 15 min at room temperature (54). The new procedure prevents any reversal of chemical skinning in concentrations of Ca up to 0.2 mM, the highest tested. It also produces a more stable preparation that responds reproducibly to a given concentration of Ca<sup>++</sup> over many hours.

The EGTA-treated tissue was transferred to a Lucite chamber with a volume of 0.15 ml. One end of the tissue was attached by the loop of silk to an Endevco semiconductor force transducer (model 8107, Endevco, San Juan Capistrano, Calif.) and the other to an arm mounted on a micrometer screw assembly which was used to stretch the tissue to the length it had in the disruption solution. The chamber was continuously perfused at a rate of 0.3 ml per min with a syringe pump, and the effluent was aspirated by a vacuum system. Changes in solution were made by means of a sliding valve. The total dead space of the system was <0.2 ml.

#### *Solutions*

The electrolyte solution used during dissection of the fresh muscle contained 140 mM NaCl, 4 mM NaCl, 4 mM KCl, 2.5 mM MgCl<sub>2</sub>, 2.5 mM CaCl<sub>2</sub> and was buffered to pH 7.2

with 10 mM imidazole. The disruption solution used for making the tissue hyperpermeable contained 140 mM K propionate, 2 mM Mg acetate, 10 mM EGTA, 5 mM Na ATP, and was buffered to pH 7.2 with 5 mM imidazole. Although imidazole is a phosphodiesterase inhibitor, its presence does not significantly affect the experimental results because the same phenomena were seen when equimolar trismaleate buffer was used in place of imidazole. The basic relaxing solution contained 140 mM KCl, 7 mM MgCl<sub>2</sub>, 3 mM EGTA, 5 mM ATP, 15 mM creatine phosphate, 0.1 mg/ml creatine phosphokinase (Sigma Chemical Co., St. Louis, Mo.), and was buffered to pH 7.0 with 25 mM imidazole. The relaxing solution had an ionic strength equivalent to 180 mM KCl. Contracture solutions were made by adding CaCl<sub>2</sub> to the basic relaxing solution. The free Ca<sup>+2</sup> concentration was estimated by solving the set of dissociation equations using the dissociation constants of Fabiato and Fabiato (14). The concentration of free Ca<sup>+2</sup> is given as the pCa (negative log of the Ca<sup>+2</sup> concentrations). Addition of the CaCl<sub>2</sub> to the contracture solutions increased the ionic strength of the basic relaxing solution by a maximum of 3%, a negligible amount with respect to force production. In experiments where the concentration of ATP was varied, the concentration of MgCl<sub>2</sub> was reduced in parallel to maintain a 2 mM excess of Mg over the ATP, and the ionic strength was maintained constant by adding the appropriate amount of KCl to the solution. When the ionic strength was increased to 210 mM KCl in a few experiments, the total force developed by the hyperpermeable muscle was reduced significantly, but the relationship between the relative tension and pCa remained essentially constant. Reduction of the ionic strength to the equivalent of 150 mM KCl, as was used by Kerrick and Donaldson (21), produced no significant change in the relative tension-pCa relation. The high concentration of pH buffer, and the use of steady-state tensions in the analysis of the data minimizes any possible influence of changes in pH inside the preparation (32). This conclusion is supported by the similar results with two buffers with different pKs.

#### *Glycerol Extraction*

Papillary muscles and trabeculae from rat right ventricles were isolated and extracted in 50% glycerol according to the procedure of Nayler (33).

#### *Analytical Measures*

The total Ca content of all solutions was routinely measured with an atomic absorption spectrometer (Perkin-Elmer Co., Norwalk, Conn., model 290). The activity of glutamate oxalacetate transaminase (GOT) was measured by a modified Reitman-Frankel method (38) available in kit form from Dade Diagnostics, Inc., Miami, Fla.

#### *Electron Microscopy*

Tissues were prepared for examination with the electron microscope by securing the ends of the tissue and fixing overnight in 6% glutaraldehyde in cacodylate buffer at pH 7.0 at 4°C. After a rinse in buffer and 25 min in 1% osmium, the tissues were then dehydrated with an ethanol series, embedded in Spurr and sectioned with a Sorvall MT-2B ultramicrotome (Dupont Instruments, Wilmington, Del.). Gold sections were examined with a Zeiss EM9 electron microscope (Carl Zeiss, Inc., New York). Two sets of tissues were examined: (a) rat ventricular trabeculae that had been dissected from isolated hearts and allowed to recover in Krebs solution at room temperature and (b) trabeculae that had been soaked in 10 mM EGTA overnight after removal from the chilled isolated heart.

### *Electrophysiology*

Transmembrane potentials of hyperpermeable cells were measured with glass microelectrodes filled with 3 M KCl. The microelectrodes had resistances of 8–10 M $\Omega$  and tip potentials of no more than 5 mV. The transmembrane potential of six to eight surface cells was measured first in the standard relaxing solution containing 100 mM KCl and  $10^{-9}$  M Ca and then in a relaxing solution with NaCl replacing KCl to eliminate any voltage clamping effect of the 100 mM K in the bath. After subsequent reequilibration with the standard KCl relaxing solution, the bundle was exposed to a contracture solution containing pCa 4.5, and the membrane potential of another six to eight cells was measured in the contracture solution over a period of 30 min in the high Ca solution.

## RESULTS

### *Properties of the Preparation*

The purpose of this study is to determine whether the sarcolemma regulates the concentration of Ca required for activating the contractile proteins and how it might do it. These experiments require a preparation that both retains its control mechanisms and allows the contractile proteins to be directly probed. The cardiac fiber that has been made hyperpermeable by EGTA fulfills these requirements inasmuch as it has the following experimentally demonstrable properties: (a) the surface membrane is a minimal diffusion barrier to small ions and molecules; (b) the cells retain a high level of activity of soluble enzymes indicating that the surface membrane remains a diffusion barrier to large molecules; (c) the effect of intracellular Ca sinks, especially the sarcoplasmic reticulum, on the sarcoplasmic concentration of Ca is small because 3 mM EGTA in the bathing solution is an effective Ca buffer; (d) at least some sarcolemmal enzymes are still functional in spite of the altered state of the membrane; and (e) the preparation is sufficiently stable to allow meaningful comparisons of performance within a single experiment. Although a precise description of the size of molecules that will pass through the membrane requires an extensive study involving labeled compounds of different molecular sizes, the exact description is not crucial for interpreting the studies presented below.

The addition of Ca ions at an adequate concentration to activate the contractile system produces an increase in force, and the reduction of the concentration produces a relaxation (Fig. 1). The half-times of the changes in force are short,  $\sim 1$  min, and the responses prompt considering the 100–300- $\mu$ m diameter of the preparations. A similarly rapid mechanical response to changes in the concentrations of ATP, creatine phosphate (Fig. 2), and phosphoenol pyruvate indicates that small ions and molecules move very quickly from the bath through the membrane to the contractile system. The cells retain soluble proteins like creatine phosphokinase and GOT. Creatine phosphokinase is found primarily in the sarcoplasm of intact cells (2), although a small amount may be bound to the contractile proteins at the M line (44), and the ease with which the enzyme leaks through damaged membranes has been used as a clinical tool for detecting early damage to myocardial cells (46). After a hyperpermeable preparation has been put into rigor by replacing ATP with ADP in the absence of creatine phosphate, the rigor state can be rapidly

reversed by adding creatine phosphate without either ATP or exogenous creatine phosphokinase (Fig. 2). The relaxation does not occur in the absence of ADP. These results indicate that the cells retain a high level of activity of creatine phosphokinase, and analogous experiments indicate the presence of considerable pyruvate kinase.

The retention of soluble sarcoplasmic enzymes by the hyperpermeable cardiac fiber can be demonstrated in another way. After ventricular trabeculae had been soaked in the disruption solution at 0°C for 50 h, the activity of GOT was measured in the surrounding solution, the undamaged central third of the fiber bundle and the ends, which contained all of the damaged tissue as well as some

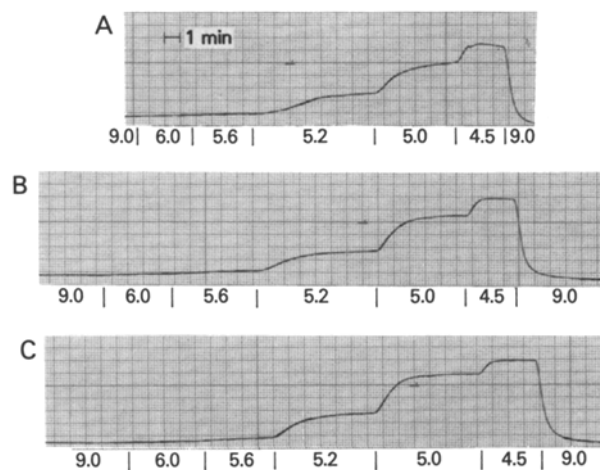


FIGURE 1. A record of force produced by direct Ca activation of the contractile system of a hyperpermeable rat ventricular bundle. The three records are from three consecutive runs on the same preparation over a period of ~100 min and show the stability of the hyperpermeable cardiac bundle. Numbers below indicate pCa.

undamaged tissue. The activity of the enzyme was over 1,000 times greater in the undamaged tissue than in the bathing solution and 2-3 times greater per unit wet weight than in the damaged ends. Approximately 50% of the ends appeared to be composed of damaged tissue by gross inspection under the microscope. If one assumes that the remaining undamaged tissue in the ends contained the same enzymatic activity as the undamaged center, then most, if not all, of the transaminase activity in the bathing solution had come from damaged tissue, and the undamaged cells did not leak this enzyme. This conclusion is further supported by the fact that practically all of the enzymatic activity in the bath appeared within the first 2 h.

The sarcoplasmic Ca concentration is controlled adequately by 3 mM EGTA in the bathing solution. With this Ca buffering capacity, very little tension develops below a free Ca concentration of 1  $\mu$ M. If, however, the concentration of EGTA is reduced to 30  $\mu$ M, spontaneous contractions appear at a pCa of ~6.6. As Fabiato and Fabiato (13) have shown, these contractions are due to Ca-

induced releases of Ca from the sarcoplasmic reticulum that transiently raise the concentration of ion in the sarcoplasm to activating levels (Fig. 3). Elevation of EGTA back to the 3 mM immediately terminates the spontaneous activity. The addition of 10 mM caffeine to a bathing solution containing 30  $\mu$ M EGTA produces a large contracture as a result of the release of Ca from the sarcoplasmic reticulum. With 3 mM EGTA present for adequate buffering, no increase in force is produced by the same concentration of caffeine. The constant relation between tension and pCa at concentrations of the buffer above 3 mM is additional evidence for the adequacy of Ca buffering by 3 mM EGTA.

The same relation between force and Ca concentration can be produced several times during a single experiment with only a trivial change in the maximum developed tension (Fig. 1). The preparation has even been stored overnight in disruption solution at 0°C at the end of an experiment and still produced the same tension-pCa curve on the following morning. Because of the

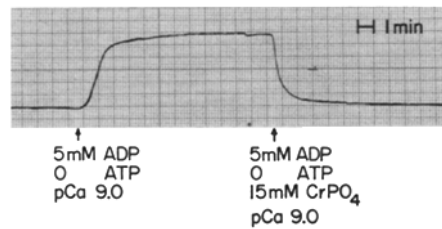


FIGURE 2. The removal of ATP produces sustained elevated force, even in the presence of 5 mM ADP, but the addition of creatine phosphate causes an immediate, complete relaxation indicating the rapid formation of ATP from ADP and creatine phosphate. Inasmuch as no creatine phosphokinase was added there must be a high level of activity of endogenous creatine phosphokinase.

stability of the preparation, changes in this relation during an experiment are functionally significant and cannot be attributed to deterioration of the tissue.

Evidence for the presence of functional adenylate cyclase in the sarcolemma of the hyperpermeable cardiac fiber will be presented below.

The trabeculae and papillary muscles used in these studies are natural bundles of cardiac cells and do not have either uniform shape or cross sectional area along their length. Any estimate of force in terms of cross-sectional area is therefore unreliable and useless for quantitative considerations. To evaluate the effect of producing the hyperpermeable state on the force producing capacity of the contractile system, the tension generated in response to electrical stimulation in Krebs' solution before treatment with EGTA was compared with the maximal Ca activated tension after EGTA treatment. Massive electrodes were employed to insure activation of all fibers, and the optimal frequency of stimulation for contractile force was used. The EGTA-treated rat heart behaved as the EDTA-treated frog and rabbit hearts (53) in producing at least the same force with Ca activation as with electrical stimulation. The influence of damaged tissue at the ends of the bundle on the development of tension by the hyperpermeable tissue was probably nominal as measurements were made from

steady-state forces. The compliance introduced by the damaged tissue would primarily influence the kinetics rather than steady-state force. In early experiments the effect of the resting length on the changes observed with increasing Ca was examined and found to be independent of resting length as would be expected if the series compliance was not important. In addition, the stability of the response of the tissue to  $\text{Ca}^{++}$  argues against any major influence of damaged tissue on the basic phenomena that were observed.

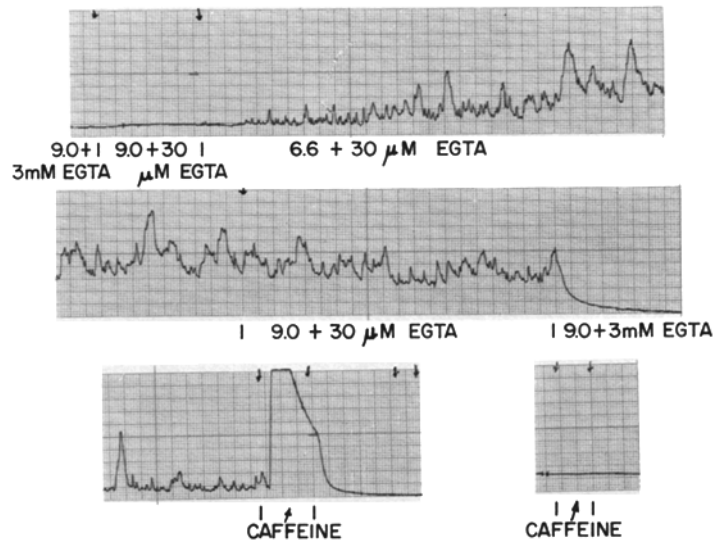
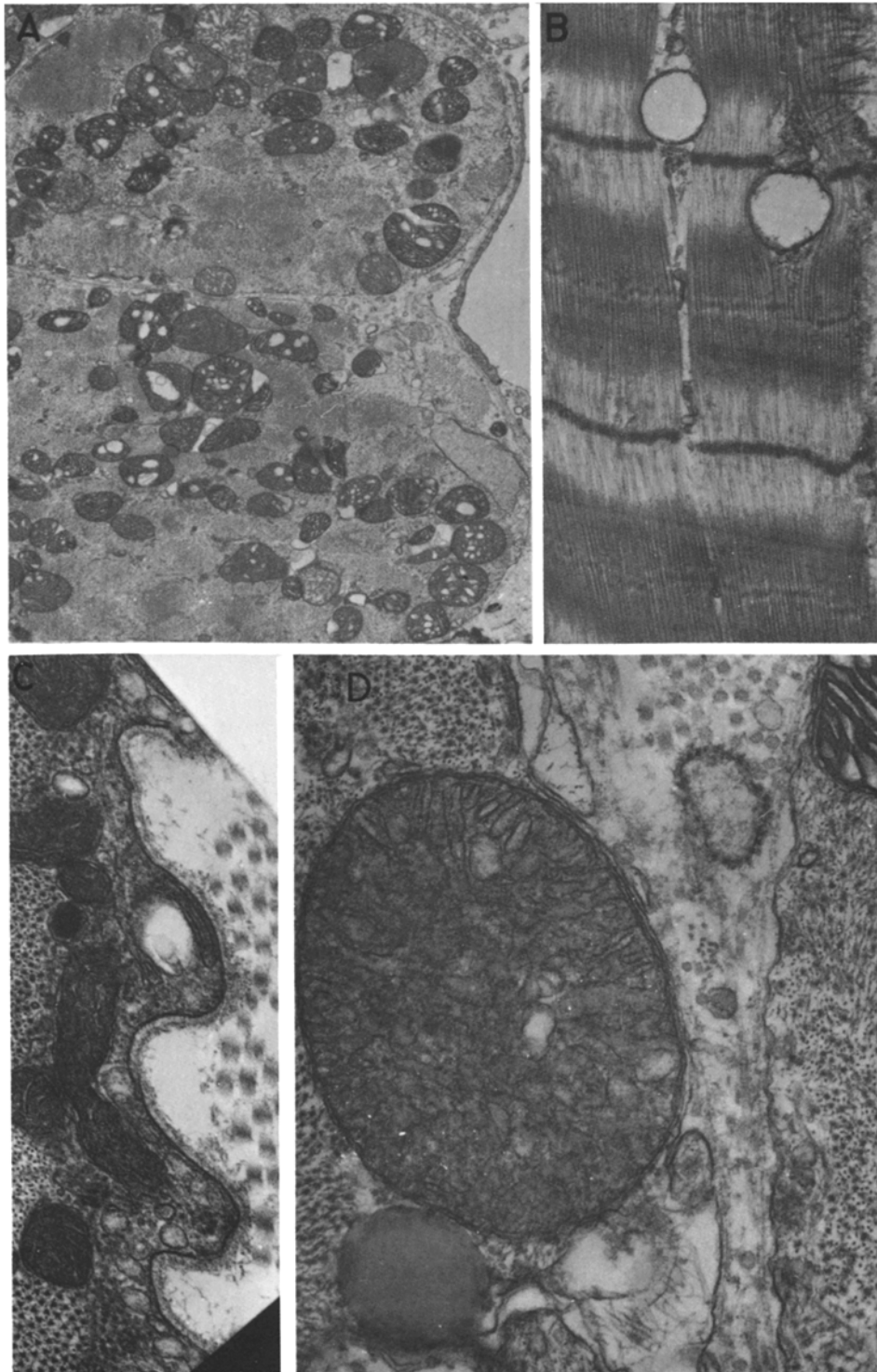


FIGURE 3. In the presence of 3 mM EGTA, no force is developed until pCa is lowered to 6 or less, but in the presence of only 30  $\mu\text{M}$  EGTA, spontaneous contractions are initiated at a pCa of 6.6. The spontaneous activity is presumably due to Ca-induced Ca release and quickly disappears when the concentration of EGTA is returned to 3 mM. Caffeine produces a large contracture in the presence of 30  $\mu\text{M}$  EGTA (lower left), but not in the presence of 3 mM EGTA (lower right). This indicates that 3 mM EGTA is an adequate buffer for sarcoplasmic Ca.

The EGTA-treated fibers retain a more normal appearance than mechanically skinned fibers (Fig. 4). Although the mitochondria swell considerably as in the mechanically skinned cells, there is much less swelling of the sarcoplasmic reticulum, and in a majority of the regions examined the reticulum appeared relatively normal. The myofibrils remain well organized and retain the hexagonal array of filaments. The sarcolemma, however, is considerably altered in appearance. Instead of the well- and uniformly stained membrane that is seen in the untreated tissue, the EGTA-treated membrane is poorly stained in some places. The major change produced by EGTA, however, is in the basement membrane, which becomes less dense and more fibrous in appearance, and frequently is separated from the sarcolemma. Changes in the appearance of EGTA-treated skeletal fibers have been recently reported (9). A more detailed study of the change in morphology of the sarcolemma from treatment with EGTA will soon be reported by Robinson and Winegrad.





In the standard relaxation solution the difference in potential across the membranes of hyperpermeable cells averaged  $-7.5 \pm 0.8$  mV. When the KCl was replaced by NaCl, the average membrane potential was unchanged at  $-8.6 \pm 1.5$  mV, and the addition of  $3 \times 10^{-5}$  M Ca (pCa 4.5) increased the potential slightly to  $-5.5 \pm 0.3$  mV. These values are not very different from the  $-10$  to  $-20$  mV measured in hyperpermeable frog heart cells by Winegrad (54). The potential measured by the microelectrode is presumably some kind of diffusion potential associated with the fixed charge on the proteins rather than a true transmembrane potential.

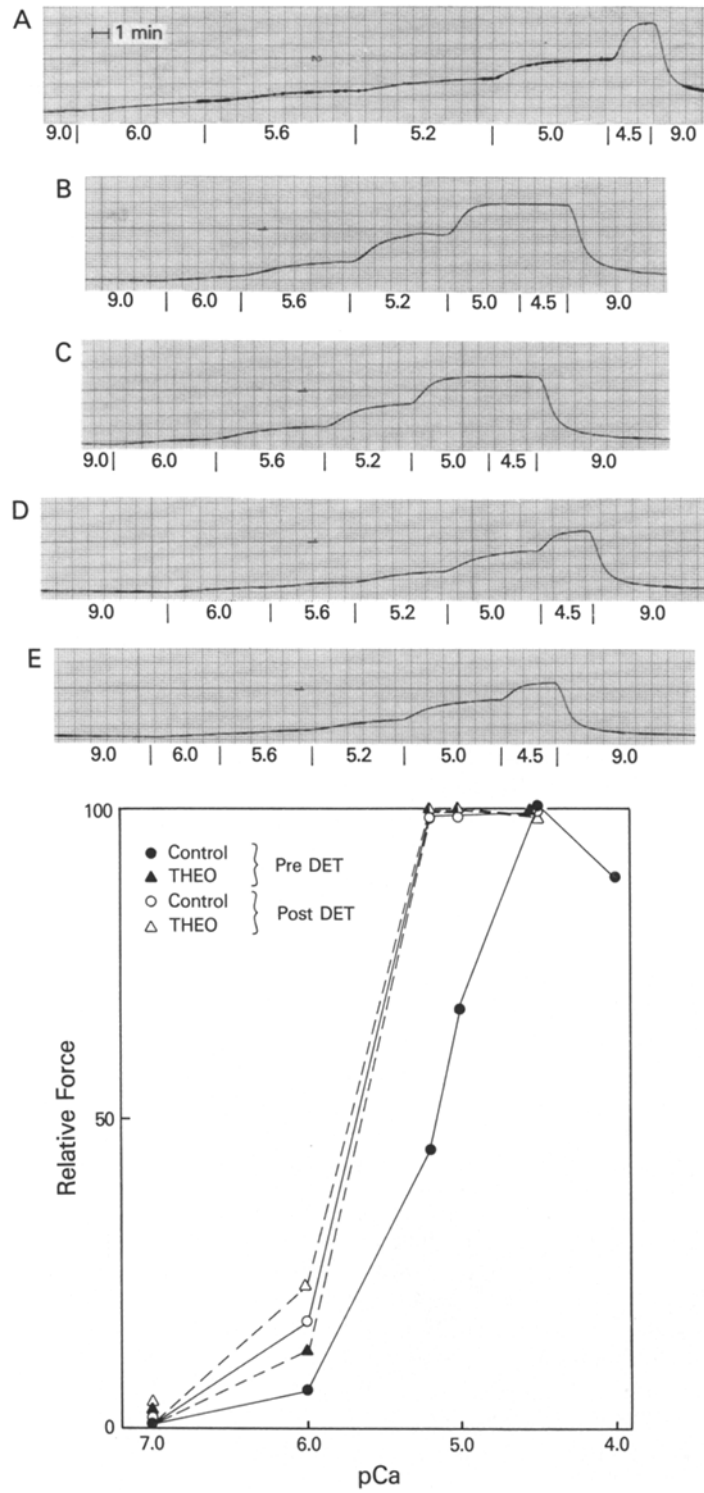
#### *Regulation of Ca Sensitivity*

**CHANGE IN THE RELATION BETWEEN CALCIUM AND DEVELOPED FORCE** In hyperpermeable ventricular trabeculae or papillary muscles from young rats (50–100 g), most of the activation of the contractile system is produced between pCa 5.6 and 4.5 (Figs. 1 and 5). Although there is some small variation in the range where the slope of the relation is very steep, maximum force quite uniformly is produced by a pCa of 4.5, and reducing the pCa further causes a small decline in developed tension. Very little force is generated at pCa of 6.0 or greater as long as the MgATP concentration is well maintained.

When 5 mM theophylline, a potent inhibitor of phosphodiesterase, is added to the bathing solution, the concentration of Ca necessary for the activation of force is decreased by a factor of 3–6. Maximum force is generally produced in pCa of 5.2 and significant tension occurs in pCa of 6.0. An essentially identical effect can be produced by using another phosphodiesterase inhibitor (Squibb Drug Co., no. 20009) instead of theophylline, indicating that the shift in the tension-pCa curve is probably due to a change in the concentration of one or both the cyclic nucleotides, cAMP and cGMP (1). The addition of either of these cyclic nucleotides to the bath without a phosphodiesterase inhibitor does not generally (see below) produce any alteration of the tension-pCa curve. This is true for concentrations of the cyclic nucleotides from  $10^{-9}$  to  $10^{-5}$  M. The effectiveness of a phosphodiesterase inhibitor suggests that the contractile proteins are sensitive to changes in the concentration of cyclic nucleotides, but the lack of response to cyclic nucleotides in the absence of the inhibitor shows that the preparation contains sufficient phosphodiesterase activity to prevent an adequate increase in the concentration of cyclic nucleotides in the immediate vicinity of the appropriate protein kinase. Removal of the theophylline restores the tension-pCa curve to its original position.

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**FIGURE 4.** Electron micrographs of rat ventricular trabecula (A), (B), (D) after treatment with EGTA and (C) before treatment with EGTA. (A) A lower power view of part of two cells showing essentially normal appearance of myofibrils, mild swelling of sarcoplasmic reticulum, and considerable swelling of the mitochondria. (B) Longitudinal section showing normal structure of sarcomeres. (C) Well-stained, uniform appearance of sarcolemma in untreated cell; basement membrane is dense and lies along sarcolemma. (D) Less well-stained and nonuniform sarcolemma with loss of material from basement membrane and separation of basement membrane from sarcolemma.



The treatment of the hyperpermeable fibers with a 1% solution (in relaxing solution) of the nonionic detergents Triton X-100, Lubrol WX, or Brij 58 for 30 min produces the same change in the tension-pCa relation as theophylline, and the addition of theophylline to the solution bathing a preparation that has been treated with detergent does not change the tension-pCa relation (Fig. 5). There is no significant change in either the maximum Ca-activated force or the rate of rise of tension as a result of the treatment with detergent. In 21 experiments, post-detergent force rises by  $8 \pm 8\%$ , and the respective half-times for the rise of tension before and after detergent are  $1.03 \pm 0.04$  and  $1.01 \pm 0.05$  min. These observations and the fact that the steady-state forces that are produced before treatment with detergent are not influenced by increasing the concentration of the EGTA-buffering system to 10 mM indicate that the increased Ca sensitivity is not the result of removal of a major diffusion barrier to the entry of Ca into the cells. The change in Ca sensitivity produced by the detergent could be due to alterations in the activity of either the phosphodiesterase or the nucleotide cyclases. The latter enzymes appear to be the more likely target in view of the smaller effect of added cAMP after treatment of the tissue with detergent than before treatment (see below). In changing the Ca sensitivity, the detergent must be acting on the sarcolemma rather than the sarcoplasmic reticulum because Brij treatment of a mechanically skinned rat ventricular fiber, which has a functional sarcoplasmic reticulum, produces no alteration in the tension-pCa relation (13). The tension-pCa relation of the mechanically skinned fiber is essentially the same as that of the detergent-treated hyperpermeable fiber.

**ROLE OF cGMP** The addition of  $10^{-7}$  or  $10^{-6}$ M cGMP alone produced either no effect or a very small increase in the calcium sensitivity of the hyperpermeable fiber in most preparations. In four preparations that initially had very low Ca sensitivities, the addition of  $10^{-6}$  cGMP did produce a significant increase in Ca sensitivity (Fig. 6, Table I). In the nine experiments in which  $10^{-6}$  M cGMP was added before treatment of the preparation with detergent, including the four experiments with the relatively Ca insensitive tissues, the pCa required for 50% of maximal activation was increased by an average of  $0.14 \pm 0.04$ , which is equivalent to a reduction of 28% in the Ca requirement. When added with theophylline, cGMP enhanced the sensitivity of the fiber to a greater degree than the nucleotide or the phosphodiesterase inhibitor alone (Fig. 7).

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FIGURE 5. Record A-E: a record of force produced by a hyperpermeable cardiac bundle before (A), during (B, C), and after (D, E) the addition of 5 mM theophylline (THEO) to the bath to block phosphodiesterase activity. Note the completely reversible change in the pCa required for activation that is produced by theophylline. The reduction in maximum force produced by the drug is reproducible and is the subject of a paper in preparation. Lower panel: the relation between developed tension and pCa is shifted to higher pCa (lower Ca concentrations) by addition of 5 mM theophylline or treatment with a nonionic detergent (DET). There is no significant difference between the tension-pCa relation in theophylline or after 1% detergent in relaxation solution. Curves are normalized to the value for maximum force. Each point is the mean of results from at least five different experiments.

The Ca sensitivity of the fiber in the presence of theophylline and  $10^{-7}$  or  $10^{-6}$  M cGMP is the same before and after treatment with detergent (Fig. 8). Apparently, the cell membrane is not a major factor in determining Ca sensitivity when cGMP concentration is controlled by the contents of the bathing solution, but the membrane is very important if cyclic nucleotides and a phosphodiesterase inhibitor have not been added to the bathing solution.

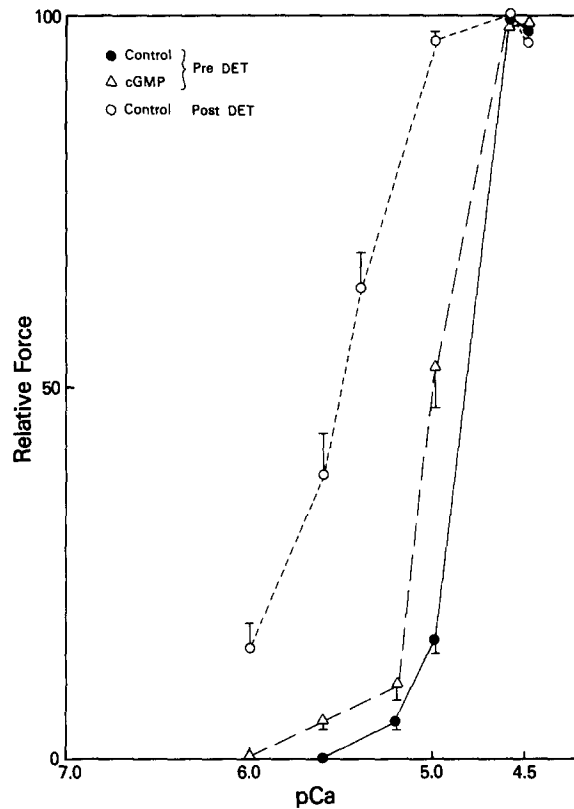


FIGURE 6. The relation between force and pCa in four hyperpermeable bundles that were initially particularly insensitive to Ca. Addition of  $10^{-6}$  M cGMP causes a significant increase in Ca sensitivity even without the presence of a phosphodiesterase inhibitor. Treatment with detergent (DET) produces a much more marked increase in Ca sensitivity. Bars indicate 1 SEM.

**INFLUENCE OF AGE OF ANIMAL** The results discussed thus far have come from experiments with rats weighing 30–100 g. The initial tension-pCa relations of hyperpermeable cardiac strands from older rats (over 150 g) frequently differ from similarly treated tissue from younger rats. They closely resemble the relation of ventricular tissue from younger rats that have been treated with either theophylline or a detergent, and they undergo a much smaller change in calcium sensitivity after treatment with theophylline or

detergents. The transition from the less sensitive tension-pCa relation of the young rat to the more sensitive relation of the older rat appears to occur gradually as the animal grows to its adult weight, but this developmental change requires more detailed study.

**ROLE OF cAMP** The Ca sensitivity is not changed by the addition of cAMP to the bathing solution in the absence of a phosphodiesterase inhibitor, but with an inhibitor present a decrease in Ca sensitivity occurs. The extent of the decrease depends on the original sensitivity. The effect of cAMP on shifting

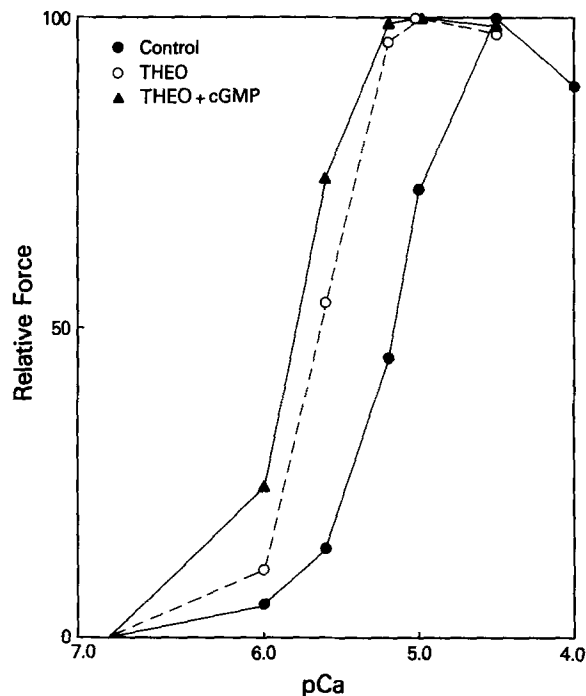


FIGURE 7. The addition of  $10^{-7}$  M cGMP shifts the tension-pCa relation to even higher pCa than exists with just 5 mM theophylline (THEO). Curves have been normalized.

the Ca sensitivity was most prominent in hyperpermeable strands from some older rats that initially showed a high sensitivity to calcium (Fig. 9), but it is qualitatively similar although quantitatively smaller with tissues from younger rats and less sensitive older rats. In the latter, the addition of cAMP reduces the amount of increased Ca sensitivity that occurs with theophylline alone.

The same general shift in Ca sensitivity produced by cAMP and theophylline occurs regardless of whether the preparation has been treated with detergents, but the decrease in sensitivity produced by a given concentration of cAMP is always greater before treatment with detergent than afterward (Fig. 10) (Table I). This consistent difference in the response to cAMP was independent of the age of the animal from which the tissue had been taken. There are at least four

general kinds of explanations: (a) in the presence of a functioning, though hyperpermeable, membrane the production of cAMP by the membrane results in a higher background concentration of the nucleotide in the cell enhancing the effect of the added nucleotide; (b) the membrane inhibits the effect of cGMP in increasing Ca sensitivity, and the release of this inhibition with detergent treatment makes cAMP-induced decrease in Ca sensitivity less noticeable; (c) there is a detergent-sensitive reaction occurring in the membrane that

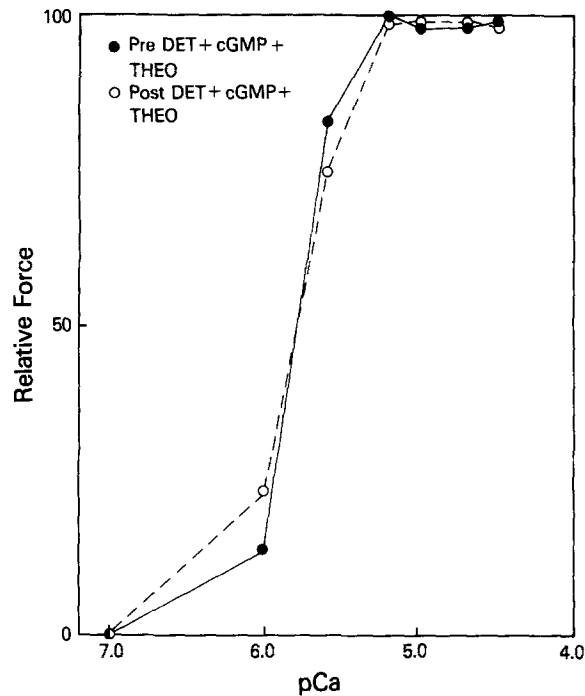


FIGURE 8. The tension-pCa relation in  $10^{-7}$  M cGMP with 5 mM theophylline (THEO) is the same regardless of whether the tissue has been treated with detergent (DET) first. Curves have been normalized.

enhances the effect of cAMP; and (d) the hyperpermeable preparation loses some of its cAMP-dependent protein kinase after treatment with detergent as a result of the increased membrane permeability. The experiment examining the role of membrane lipids presented below argues against the fourth explanation. The second explanation, a membrane inhibition of cGMP, seems unlikely in view of the same response of Ca sensitivity to a given concentration of cGMP before and after treatment with detergent. The possibility that a simple concentration effect was in operation was examined by increasing the concentration of cAMP used after the detergent 10-fold over the  $10^{-6}$ M in the bath before the detergent treatment. The elevated cAMP concentration did increase the Ca sensitivity after detergent treatment a small amount, but the 10-fold larger concentration did not produce the response that was observed before detergent

treatment. These results do not rule out a concentration effect, but they do suggest that the possibility of another membrane reaction capable of influencing the cAMP response should be seriously considered. Modulators of cyclic nucleotide action on protein kinase have been described (28, 29, 35).

**ROLE OF MEMBRANE LIPIDS** There are considerable data in the literature describing the influence of membrane lipids on the activity of membrane proteins (25, 27, 28, 30). Of particular interest, in view of the role of cyclic nucleotides in the regulation of Ca sensitivity, is the report that detergents

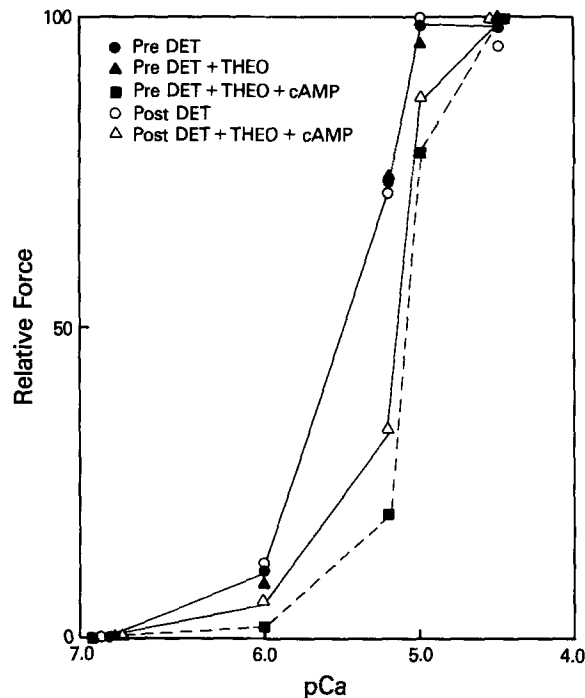


FIGURE 9. In hyperpermeable cardiac strands from older rats, where the initial Ca sensitivity is high, the addition of theophylline (THEO) plus  $10^{-6}$  M cAMP decreases the Ca sensitivity both before and after treatment with detergent (DET). Curves have been normalized.

inactivate the activator-nucleotide cyclase complex by uncoupling them and specific phospholipids reverse this uncoupling (20, 22). Studies were carried out to determine whether the alteration of the calcium sensitivity by detergents could be reversed with phospholipids.

The addition of a chloroform-methanol extract of brain tissue (Sigma Chemical Co.), which consisted of 10–20% phosphatidyl inositol, 50–60% phosphatidyl serine, and 5–10% cerebrosides, to the bath at a concentration of 0.3 mg/ml caused a reversible decrease in force (which is the subject of a paper in preparation) but no change in the tension-pCa curve of hyperpermeable strands with low Ca sensitivities. The same concentration of the brain extract, however,

completely reversed the increase in calcium sensitivity that had been produced by detergent and restored the original ability of theophylline to increase calcium sensitivity (Fig. 11). Presumably, by displacing the essential lipids from the vicinity of the reactive site, the detergent reversibly inhibits a membrane reaction that maintains a lower calcium sensitivity. The inhibitory reactions can be reactivated by the appropriate phospholipids. A chloroform-methanol extract of rat heart was equally effective but lysolecithin alone was not.

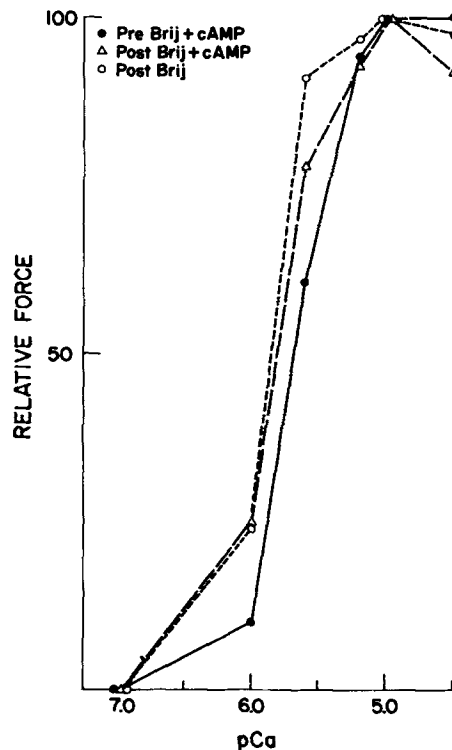


FIGURE 10. The ability of  $10^{-6}$  cAMP plus 5 mM theophylline to decrease the Ca sensitivity is greater before than after detergent treatment. 5 mM theophylline is present in both cases with the cAMP. Curves have been normalized.

**EFFECT OF CYTOSINE TRIPHOSPHATE** Although cytosine triphosphate (CTP) will substitute for ATP in the force-generating reactions of the contractile system (19), it does not serve as a good phosphate donor in either cAMP-dependent or -independent protein phosphorylating reactions (20, 52). In the presence of an active phosphatase, replacing ATP with CTP should lead to a dephosphorylation of any phosphorylated contractile proteins; therefore, the CTP substitution should indicate the Ca sensitivity of the unphosphorylated contractile system. Substitution of CTP for ATP results in a shift of the relation between force and Ca concentrations to lower Ca concentrations (Fig. 12) without changing maximum force. The restoration of ATP reverses the change



in Ca sensitivity. The shape and the position of the relation between Ca and force of muscle in CTP bathing solution closely resembles those of muscles exposed to theophylline or treated with detergent. In CTP, the calcium sensitivity did not respond to theophylline.

The absence of either a rise in the base-line tension in  $10^{-9}$ M Ca or an increase in the peak tension indicates that the fibers in CTP are not deficient in high energy phosphate and that the  $\text{CrPO}_4$ -CPK system is an adequate regenerating system for CTP.

TABLE I  
EFFECT OF CHANGES IN BATHING SOLUTION ON CONCENTRATION OF Ca  
TO PRODUCE 50% MAXIMUM ACTIVATION

Solution change	No. expts.	Shift in pCa	$\frac{[\text{Ca}]_{\text{after}}}{[\text{Ca}]_{\text{before}}}$
Detergent treatment	18	$+0.52 \pm 0.03$	0.30
Addition of cAMP to theophylline before detergent in preparation with initially high Ca sensitivity	6	$-0.41 \pm 0.05$	2.57
cAMP and theophylline after detergent compared with cAMP and theophylline before detergent	14	$+0.15 \pm 0.01$	0.71
Addition of cGMP before detergent	9	$+0.14 \pm 0.04$	0.72
Addition of cGMP to theophylline before detergent	8	$+0.16 \pm 0.02$	0.69
cGMP and theophylline after detergent compared with cGMP and theophylline before detergent	11	$-0.006 \pm 0.02$	1.01
CTP for ATP	6	$+0.79 \pm 0.04$	0.16

**TENSION-pCa RELATION OF THE GLYCEROL-EXTRACTED FIBER** Trabeculae and papillary muscles that had been extracted in glycerol to destroy the membrane regulatory mechanisms had relations between force and pCa similar to those seen with hyperpermeable bundles in theophylline, in CTP, or after treatment with detergent (Fig. 13). The glycerol-treated fibers were insensitive to theophylline. Inasmuch as the Ca-tension relation of these fibers was not well maintained on successive runs, the preparation was not used for further study.

**INFLUENCE OF MAGNESIUM-ATP ON CALCIUM SENSITIVITY** The tension pCa relation can also be changed by lowering the concentration of MgATP in the bathing solution (3, 14). This change is analogous to the increased Ca sensitivity of myofibrils in the presence of low MgATP, and is presumably due to an increased affinity of troponin for calcium (4, 17). To determine whether the membrane mechanism for regulating calcium sensitivity is the same as that involved in the response to low MgATP, hyperpermeable cardiac strands from young rats were put into a state of high Ca sensitivity by treatment with detergent, and their response to Ca was examined in the presence of 0.2 mM ATP. A further increase in Ca sensitivity was produced by the lower MgATP so that 0.1  $\mu$ M Ca, which does not raise force in 5 mM MgATP (Fig. 14), produced ~15% of maximum tension in the presence of 0.2 mM MgATP. These results

indicate that the alteration in Ca sensitivity with low ATP and cyclic nucleotides involves different mechanisms.

**EFFECT OF PERFUSION OF HEART** The condition of the heart that has been isolated from an animal is influenced to a considerable degree by the manner in which the animal has been handled before sacrifice (8, 11, 16). Decapitation causes a marked increase in the plasma levels of norepinephrine

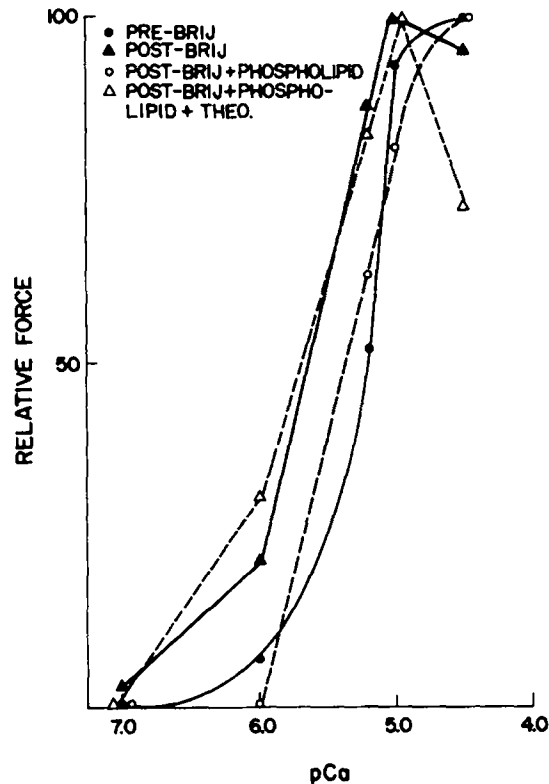


FIGURE 11. The increase in Ca sensitivity which is produced by treatment with detergent is reversed by adding mixed phospholipids to the bathing solution. The addition of the phospholipids also restores the ability of theophylline (THEO) to increase Ca sensitivity. Curves have been normalized.

(8) and a decrease in the phosphate content of cardiac myosin (47). If the animal is anesthetized before sacrifice the plasma norepinephrine level is much lower, and the phosphate content of cardiac myosin and TN1 is increased and decreased, respectively. Perfusion of the isolated heart after decapitation has approximately the same effect as anesthesia before removal of the heart. These studies indicate that the heart removed immediately after decapitation or stunning is under strong influence of catecholamines, and that this is reversed by *in vitro* perfusion.

Because the effects of cyclic nucleotides on Ca sensitivity suggest that epinephrine might be important in the regulation of this property, the relation

between Ca and force was examined in hyperpermeable bundles from hearts treated in three different ways: (a) hearts isolated after decapitation were immediately cooled to 4°C, bundles were dissected and then incubated in EGTA solution; (b) hearts were perfused for 5–10 min with modified Krebs' at 25°C before cooling and dissection; (c) hearts were perfused for 5 min with modified Krebs' at 25°C and then for 7–10 min with Krebs' that contained 2  $\mu$ M epinephrine.

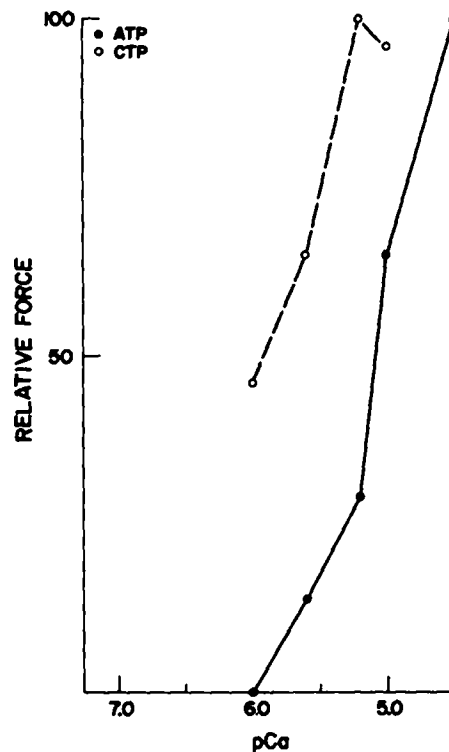


FIGURE 12. Replacement of ATP with 5 mM CTP produces a shift in the tension-pCa relation to higher pCa. The CTP can support the generation of force but it does not act as a good phosphate donor.

The tension-Ca relations in these muscles are shown in Fig. 15. Hyperpermeable bundles from hearts which had been perfused with Krebs' had greater sensitivity than those from hearts that had been cooled immediately after removal. Perfusion with epinephrine partially reversed the increase in Ca activity that had been produced by the perfusion with Krebs'. The Ca sensitivity of both the hearts that had been cooled immediately and those which had been perfused with Krebs' were the same, however, after inactivation of the cell membranes with 1% Triton X-100 solution. Epinephrine apparently can regulate the Ca sensitivity of the contractile system by an alteration in the state of the cell membrane, presumably by changing the rate of synthesis of cAMP.

## DISCUSSION

A preparation of mammalian ventricular muscle in which the surface membrane has been made very permeable to ions and small molecules was used to study the concentration of Ca ions required for activation of the contractile system. This model of a cardiac cell has the advantage of retaining not only sarcoplasmic proteins but also some membrane enzymes. It allows the assay of properties of

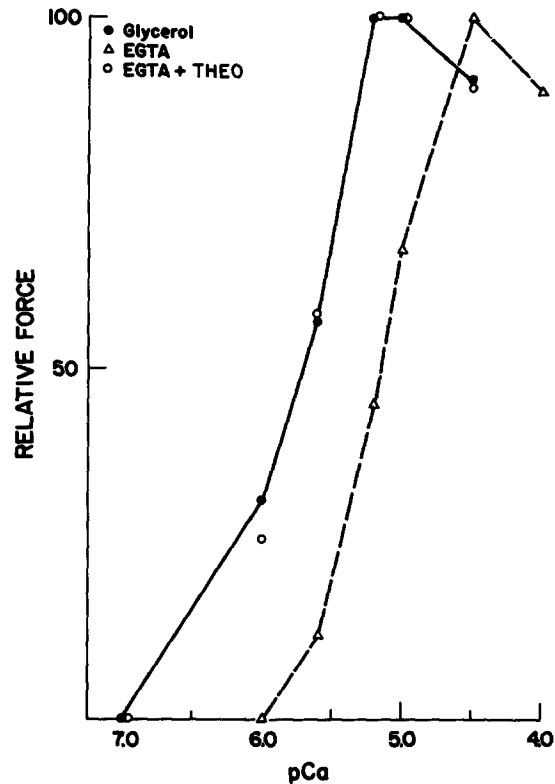


FIGURE 13. The tension-pCa relation of rat ventricular muscle which has been glycerol-extracted for several days is shifted to higher pCa and is the same as the relation in the hyperpermeable (EGTA) muscle which is bathed in a solution with 5 mM theophylline (THEO). Curves have been normalized.

the contractile system by direct activation with Ca in the presence of more of the constituents and functions of the intact cell than the mechanically skinned or glycerol-treated fiber preparation. A more rigorous biochemical description of the membrane than is possible from physiological experiments will require homogenization of the tissue and study of the isolated organelles, but this type of approach has the potential problem of inactivation of membrane constituents during the preparation.

In this model of a mammalian cardiac cell, the range of concentration of Ca ions that is required for the activation of the contractile system can be varied from three- to sixfold without a change in the general shape of the relation

between force and Ca concentration. The curve is merely shifted along the Ca concentration axis. The sarcolemma must be intimately involved in the regulation of Ca sensitivity because treatment with detergents produces a change in the Ca sensitivity. No change in Ca sensitivity of mechanically skinned rat ventricular cells, which have no surface membrane, results from treatment with detergent or cAMP (14, 15), although these skinned fibers have a functioning sarcoplasmic reticulum that is sensitive to detergents. The effect of the detergent on Ca sensitivity must be on the sarcolemma and not the intracellular mem-

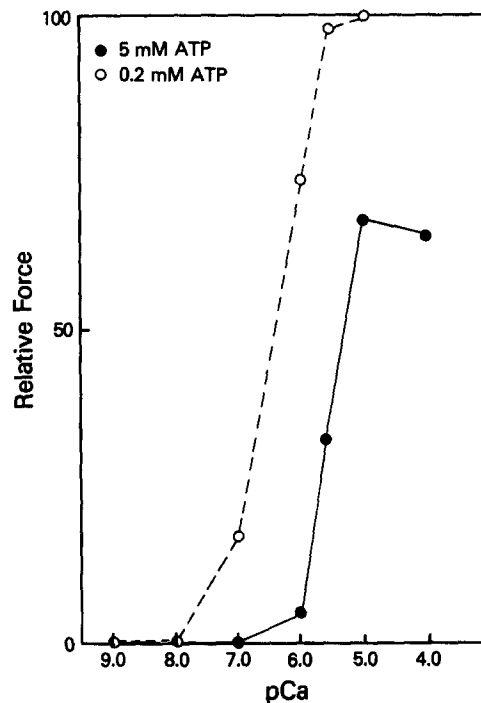


FIGURE 14. Decreasing the concentration of MgATP from 5 to 0.2 mM increases both Ca sensitivity and maximum force in hyperpermeable strands in which the Ca sensitivity has already been increased by treatment with detergent.

branes and, predictably, the Ca-tension relation of the skinned fiber is the same as that of the detergent-treated hyperpermeable fiber. The hyperpermeable cells seem to retain important regulatory functions in the membrane that are lost after detergent treatment.

The evidence for an important role of phosphorylation in the regulation of Ca sensitivity is considerable but neither direct nor conclusive. Ca sensitivity can be changed by treatment of the hyperpermeable fibers with cyclic nucleotides, with phosphodiesterase inhibitors, or by the removal of phosphate donors. The manner in which the cyclic nucleotides, phosphodiesterase inhibitors, and detergents influence Ca sensitivity of the hyperpermeable cardiac cell is very similar to the way they influence phosphorylation reactions in isolated systems of mammalian cardiac muscle. Nonionic detergents increase guanylate and

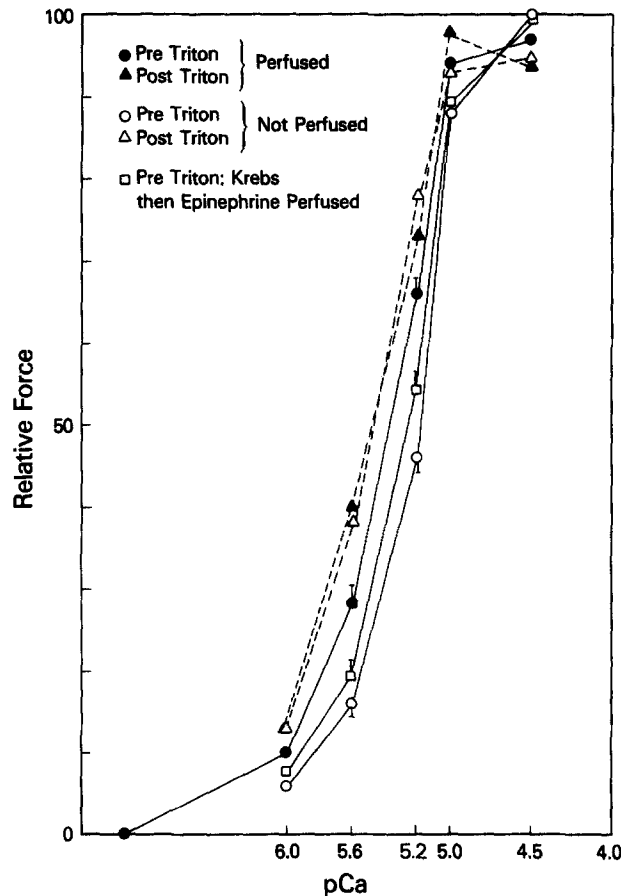


FIGURE 15. The effect of the state of the heart before exposure to EGTA on the Ca sensitivity of the hyperpermeable fibers. Perfusion of the isolated heart after acute sacrifice of the animal increases the Ca sensitivity, but addition of epinephrine to the perfusion fluid partially reverses this increase. The Ca sensitivity after treatment with detergent is independent of the state of the heart before exposure to EGTA. Each curve is the composite of at least three experiments on hearts removed from litter mates. Variation at each point is not shown because of the large number of data points, but all curves except the two postdetergent ones are significantly different from each other at  $<0.05$  level. (●) Pre-Triton, Krebs' perfused; (▲) post-Triton, Krebs' perfused; (○) pre-Triton, nonperfused; (△) post-Triton, nonperfused; (□) pre-Triton, Krebs' and the epinephrine perfused.

decrease adenylate cyclase activity (22, 53), as well as uncouple adenylate cyclase from the catecholamine-stimulated  $\beta$ -receptor (26). The uncoupling can be reversed by the addition of phospholipid (27). Treatment of the hyperpermeable fiber with detergent produces a cGMP-like effect and inhibits any cAMP-like action. Addition of phospholipids reverses these changes. Detergent does not alter the isolated protein kinase and, as indicated by the reversability of the detergent's effect on the hyperpermeable fibers, it does not denature the proteins involved in the regulation of Ca sensitivity.

Perfusion of the heart with epinephrine before treatment with EGTA changes Ca sensitivity in the same way as increased cAMP. The apparent durability of the epinephrine effect on the hyperpermeable fibers through prolonged exposure at 0°C to a solution without catecholamines is not surprising, in view of the maintained activation of rat cardiac protein kinase in spite of the removal of cAMP if the temperature is 0°C (6). Phosphorylation of TNI accompanies the perfusion (11, 12, 51), and in two laboratories (7, 37) phosphorylation of TNI by a cAMP-dependent protein kinase leads to about a three-fold increase in Ca sensitivity. This has not been a uniform finding (5, 42), and the difference in results has not yet been explained, but phosphorylation of TNI could be involved in the regulation of Ca sensitivity in the hyperpermeable fibers.

The observed changes in the properties of the hyperpermeable fibers must be due to modification of the contractile proteins themselves as the myofibrils are directly activated by a Ca buffer that is adequate to suppress the influence of other cell organelles on the cytoplasmic concentration of Ca ions. Clearly, however, proof of the relationship between Ca sensitivity and phosphorylation requires the study of both in the same tissue under the various conditions in which Ca sensitivity can be modified. Demonstration of changes in the concentration of cyclic nucleotides in the tissue by direct measurement is also critical for final evaluation of the proposed mechanism. Both kinds of experiments, which involve <sup>32</sup>P labeling, protein isolation, and sensitive assay of cyclic nucleotides, are currently being done in our laboratory. Dose-response curves relating epinephrine in the perfusion medium to the state of the contractile proteins are also important for judging the connection between the initial reaction on the membrane and the phosphorylation of the contractile protein.

Because the Ca sensitivity can be shifted back and forth by inhibitors of phosphodiesterase, the preparation must contain a system that can both phosphorylate and dephosphorylate the proteins. In the absence of a phosphate donor, as when CTP has replaced ATP, the phosphatase should reduce the proteins to a dephosphorylated state. Under these conditions maximal Ca sensitivity of the contractile system is observed.

The results of this study do not provide sufficient information for the formulation of a rigorous and complete model, but they do show that the sarcolemma can regulate the performance of the contractile proteins by a nonionic mechanism. A relatively coherent notion of the manner in which Ca sensitivity is regulated can be inferred from the following experimental facts (Fig. 16): (a) cGMP enhances Ca sensitivity; (b) cAMP depresses Ca sensitivity; (c) inhibition of phosphodiesterase increases Ca sensitivity; (d) inactivation of the surface membrane either increases Ca sensitivity or does nothing; (e) cGMP plus theophylline has the same effect with or without an active membrane; (f) cAMP plus theophylline is more effective before rather than after the membrane is inactivated; (g) absence of a phosphate donor produces a state of high Ca sensitivity. In the intact cell the concentration of Ca required for activation is the net result of cAMP-controlled phosphorylation, which increases the required concentration, and a cGMP-regulated antagonistic reaction. The former requires the sarcolemma whereas the latter does not. In the presence of a high concentration of catecholamines, the Ca sensitivity of the contractile proteins is

relatively low as a result of the predominance of cAMP over cGMP synthesis. When the hydrolysis of both cyclic nucleotides is inhibited by blocking phosphodiesterase activity, the cGMP-regulated reaction becomes predominant presumably because the  $K_m$  for cGMP-dependent reactions is  $\sim 0.1$  of that of cAMP-dependent reactions, and the action of cAMP is antagonized either directly or by a second phosphorylation that neutralizes the effects of the cAMP-dependent phosphorylation. With this type of regulation, Ca sensitivity would usually but not always be related to the cAMP concentration and the phosphate content of the protein (37, 47).

### CA SENSITIVITY REGULATION

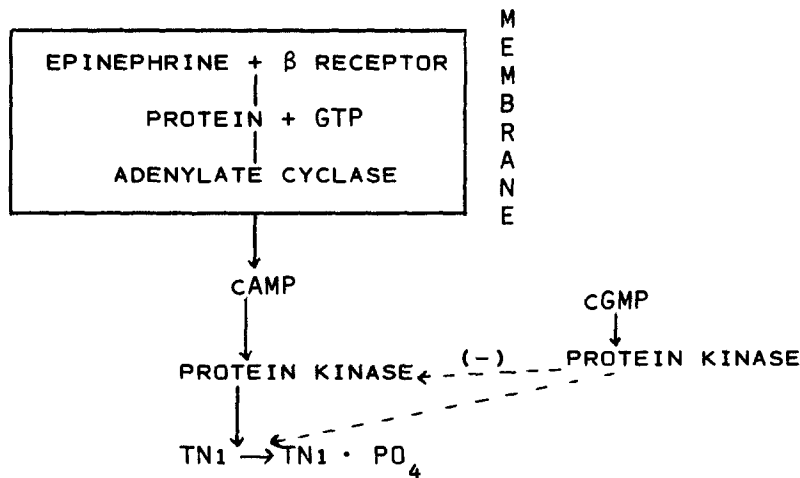


FIGURE 16. Proposed model for the regulation of Ca sensitivity by catecholamines. Interrupted lines are used for the antagonistic, cGMP-dependent reactions to indicate that the data do not allow a meaningful inference about the site of action.

Our results, which indicate that cGMP induces an increase and cAMP induces a decrease in Ca sensitivity, are consistent with the findings of Ray and England (37) and Cole et al. (7) that cAMP causes a decrease in sensitivity. The increased sensitivity reported by Rubio et al. (42) may have involved activation of the cGMP-regulated reaction.

Lipids are clearly involved in providing the appropriate molecular environment for the activity of the sarcolemmal regulatory proteins as well as for other membrane systems, such as the sarcoplasmic reticulum (30). This sensitivity of membrane proteins to lipids makes the lipids potential messengers from the organelles or sarcoplasm to the membrane. The membrane could operate as an integrator of both intracellular and extracellular information in addition to a regulator of the properties of the contractile proteins.

Regulating the range of Ca concentrations over which activation of the contractile proteins occurs is not a good mechanism for modulating contractility. The Ca necessary to raise the sarcoplasmic concentration constitutes only a small



fraction of the total Ca requirement, most of which is necessary for binding to troponin (10, 48). Increasing Ca sensitivity causes only a small decrease in the Ca requirement for activation, but the shift in the range of activating concentration may be useful to the cell in at least three ways. It would permit regulation of diastolic tone by varying the amount of activation of the so-called "resting muscle" at a given Ca concentration and, as a result, determine the number of links between actin and myosin that contribute to resting stiffness (31, 43, 55, 56). The degree to which the activation of the contractile system is coupled with other Ca-dependent cellular reactions could also be modulated by this mechanism. The ability to vary Ca sensitivity of the contractile system over a significant range also offers the cell a mechanism for activation of contraction with no change in Ca concentration. Phosphorylation could shift the range of Ca sensitivity into and out of the existing sarcoplasmic concentration range but, in view of the rate at which the cardiac contractile proteins are phosphorylated in solution, it is probably not an important mechanism in beat-to-beat regulation.

The insensitivity of the preparation to cyclic nucleotides in the absence of a phosphodiesterase inhibitor points out the potential importance of regulation of phosphorylation by this enzyme. It may be vital in controlling local cyclic nucleotide concentrations in strategically important regions of the cell.

The authors gratefully thank Dr. Thomas Robinson for the use of the electron micrographs in Fig. 4.

This work was supported by grants HL-16010 and HL-15835 from the U. S. Public Health Service.

*Received for publication 21 February 1978.*

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