Regulation of Calcium Sensitivity in Perforated Mammalian Cardiac Cells

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ABSTRACT Sarcolemmal perforations can be produced in bundles of rat right ventricular cells by either perfusion of the heart or soaking of the bundles with a solution containing 10 mM EGTA. All cells are affected and lose ~40% of the surface membrane. In these cells it is possible to show cAMP regulation of contractility (maximum Ca-activated force) without cAMP regulation of Ca sensitivity (pCa for 50% of maximum Ca-activated force). Therefore, the target molecule for cAMP is different for the two regulatory systems. Both regulatory systems can be slowly washed out of the cell by 10 mM EGTA solution but not by relaxing or contraction solutions. A model for regulation of Ca sensitivity is proposed.

INTRODUCTION
To study the regulation of the contractile proteins in cardiac muscle, a preparation of rat ventricular cells in which the sarcolemma is highly permeable to small ions and molecules was developed (McClellan and Winegrad, 1978). This modification of the sarcolemma was produced by soaking the cells in a solution containing 10 mM EGTA, and the resulting cells were called hyperpermeable. The high permeability of the sarcolemma allows the contractile proteins to be activated directly by a Ca EGTA buffer system added to the bathing solution, eliminating the influence of the action potential or electromechanical coupling system on the amount of force generated. The change in permeability of the sarcolemma does not, however, destroy many of its metabolic functions. All three major components of the β-adrenergic system, that is, the receptor, the GTP binding protein, and adenylate cyclase, continue to function (McClellan and Winegrad, 1980). Muscarinic receptors are also present and active (Horowits and Winegrad, 1981). With this preparation it has been possible to show the existence of β-adrenergically mediated systems for regulating the concentration of Ca required for activating the contraction (McClellan and Winegrad, 1978; Mope et al., 1980) and the amount of force developed at an optimal concentration of Ca (McClellan and Winegrad, 1980).

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A modification of the hyperpermeable cardiac cell has been developed to allow additional types of study. Perforations are produced in the membrane so that large molecules like soluble proteins can be exchanged between the fluid in the immediate vicinity of the myofibrils and the bathing solution. These cells, called perforated cardiac cells, are described in the following report. The most important conclusion that has resulted so far from the use of the new preparation in studying the \(\beta\)-adrenergic control of the contractile proteins is that the target molecules for cAMP that actually effect the control of Ca sensitivity (concentration of Ca necessary for 50% of maximal activation of force) and contractility (maximum Ca-activated force) are different, thereby permitting the cell to regulate each property separately.

METHODS

Preparation of Tissues

Natural bundles of hyperpermeable cells from the right ventricle of the rat were prepared by an overnight soak in a solution containing 140 mM K propionate, 10 mM EGTA, 5 mM ATP, and 2 mM Mg acetate buffered to pH 7.2 with imidazole (10 mM EGTA solution) as already described (McClellan and Winegrad, 1978). They were mounted in a continuous perfusion chamber with continuous recording of tension and bathed with a solution consisting of 140 mM KCl, 7 mM MgCl\(_2\), 5 mM ATP, 3 mM EGTA, 15 mM creatine phosphate, 0.1 mg/ml creatine kinase, and 25 mM imidazole at pH 7.2. CaCl\(_2\) was added according to the desired concentration of Ca\(^{++}\). Concentrations of ionic Ca were calculated according to the stability constants used by Fabiato and Fabiato (1979).

Perforated cardiac cells were produced by two different protocols that resulted in cells with important differences. In the first method the ventricular bundle was treated in the same way as in the production of standard hyperpermeable cells except for the exclusion of ATP from the 10 mM EGTA solution that produced the hyperpermeable state. In the second method hearts were removed from rats, washed clean of blood, and then mounted for retrograde perfusion through the aorta as in a Langendorff preparation. The aorta was connected to a peristaltic pump and modified Krebs solution (140 mM NaCl, 4 mM KCl, and 2.5 mM MgCl\(_2\) buffered with 10 mM imidazole at pH 7.2 and oxygenated) perfused through the heart for 20–30 min at 1.5–2.0 ml/min. Then the heart and pump were placed in an insulated chamber cooled with ice to \(~4^\circ\text{C}\) and the perfusion medium was switched to the 10 mM EGTA solution with or without ATP. Perfusion was stopped at different times in different experiments, the heart was removed, and bundles were dissected from the endocardial surface of the right ventricle. The bundles were mounted in the continuous perfusion chamber, attached to a force transducer, and treated in the same way as hyperpermeable bundles.

Electron Microscopy

Tissues were fixed for electron microscopy in the following way. Isolated bundles were soaked for 60–75 min in 5% glutaraldehyde in a relaxing solution consisting of 5 mM ATP, 15 mM creatine phosphate, 3 mM EGTA, 7 mM MgCl\(_2\), and 140 mM KCl buffered to pH 7.2 with imidazole. In experiments where the effect of elimination of ATP from the solution was being studied, ATP and creatine phosphate were omitted. The tissue was then cut into small pieces and washed twice for 15 min each time in
0.1 M sodium cacodylate buffer. This was followed by post-fixation in 1% osmium in 0.1 M sodium Ca cacodylate for 1 h at 4°C followed by two more 15-min washes in cacodylate buffer at 4°C. The tissues were dehydrated in an ethanol series, embedded in epon, sectioned with a Sorvall MT-2B ultramicrotome, and viewed with a Zeiss 109 electron microscope (Carl Zeiss Inc., New York) after staining with uranyl acetate and lead citrate. Some hearts were fixed by perfusion with the glutaraldehyde solution retrograde through the aorta, cut into small pieces, and then treated as described above.

In the morphometrical studies it was important to select sections and areas for study without bias. Most muscles that were selected for morphometrical study had also been studied physiologically first, and their physiological performance was representative of the entire group of similarly treated preparations. One or two complete transverse sections and one or two complete longitudinal sections were carefully examined for each preparation. Generally, more than one tissue section was required because of the obstruction of grid bars. A low-power montage of each transverse or longitudinal section was prepared and divided into 16 regions of equal area. Each region was numbered, and numbers were chosen blindly to establish an order of study. Every bit of visible cell surface in each region was examined at high power using the tilt stage to provide the best angle for visualizing the unit membrane structure. In cells where the percentage of absent membrane was estimated, the entire cell surface was photographed at optimal tilt. When only the percentage of cells with defects was measured, all regions in which defects were suspected were photographed.

Phosphorylation Studies

Phosphorylation of proteins in the cardiac tissue was studied using the procedure already described (Mope et al., 1980). 8-10 trabeculae were removed from the same region of the endocardial surface of the right ventricle. Since trabeculae from the same region of the heart produce very similar pCa tension relations and exhibit very similar responses to drugs (McClellan and Winegrad, 1980), all but two of the bundles were used for radioactive studies and the remaining two were used for physiological studies of the mechanical responses of the tissue. The responses of the two bundles, which were always very similar, were used as a continuous check on the homogeneity of each group of trabeculae. All studies were performed at room temperature.

For the $^{32}$PO$_4$ labeling studies, the muscle bundles were pinned at in vivo length (the same relative length that was used in the mechanical studies for the other trabeculae) in a Lucite chamber and continuously superfused by syringe pumps. The muscles were first exposed to normal relaxing solution (140 mM KCl, 7 mM MgCl$_2$, 5 mM ATP, 15 mM creatine phosphate [CP], 3 mM EGTA, and 25 mM imidazole at pH 7.2) for 10-15 min. In one set of experiments the solution was then switched to $[^{32}$P$_4$]ATP relaxing solution that contained no CP in order to prevent dilution of the specific activity of the ATP. The specific activity varied among the experiments from 100 to 150 Ci/ml of solution. The solutions were then changed according to the following protocol: relaxing solution with $[^{32}$P$_4$]ATP for 10 min, relaxing solution with $[^{32}$P$_4$]ATP and 5 mM theophylline for 10 min, and then relaxing solution with $[^{32}$P$_4$]ATP, 5 mM theophylline, and 1 μM cAMP for 10 min. Experiments were terminated by bathing the tissue for 10 min in either a relaxing solution containing 20 mM NaF or 50 mM KH$_2$PO$_4$ and 70 mM NaF to inhibit dephosphorylation (Holroyde et al., 1979).

Experiments were also conducted by using $^{32}$PO$_4$ in the presence of succinate, ADP, creatine, and O$_2$ and creatine to allow the mitochondria to synthesize radioactive
ATP. The results of these experiments were not different from those with [\(^{32}\)Py]ATP. In a few studies where the experiments were terminated by bathing the tissues in 15% (wt/vol) trichloroacetic acid to inhibit phosphatase activity (Westwood and Perry, 1981), there was no significant difference.

At the conclusion of the experiment, the tissue was homogenized at room temperature with a micro Potter-type homogenizer in a solution of 3 mM Tris-Cl buffer and 20 mM NaF at pH 8.4. Then sodium dodecyl sulfate (SDS) and mercaptoethanol were added to a final concentration of 1% each and the homogenate was brought to 100°C for 7 min. The samples, which contained all of the protein of six to eight trabeculae, were divided into two approximately equal aliquots, and each was loaded on a different lane of an electrophoresis box that already contained a 10% polyacrylamide gel in Tris-glycine buffer at pH 8.9 with 0.1% SDS and 0.1% mercaptoethanol. Markers were added to the other lanes of the slab gel, the gel was streaked with bromphenol blue tracking dye, and then electrophoresis was carried out at 7-10°C according to the method of Weber and Osborne (1969). After completion of the electrophoresis, the gel was removed, stained overnight in Coomassie Brilliant Blue, and then destained in 7-10% glacial acetic acid. Protein content in the bands in the gels was measured with a spectrophotometer connected to a Hewlett-Packard Reporting Integrator 3390A (Hewlett-Packard Co., Palo Alto, CA). The gels were dried with a Bio-Rad gel slab dryer (Bio-Rad Laboratories, Richmond, CA) and then stored for 3-14 d with x-ray film to produce autoradiographs for localization of \(^{32}\)P. The film was developed with Kodak liquid x-ray developer (Eastman Kodak Co., Rochester, NY).

RESULTS

Morphology of the Perforated Cell

Holes in the sarcolemma of cardiac cells result from soaking the heart for \(\geq 12\) h in 10 mM EGTA solution containing no ATP (Fig. 1). This is clearly different from the structurally intact membranes of hyperpermeable cells produced when ATP was present. Quantitative morphometry was carried out on cells that had been soaked in 10 mM EGTA solution without ATP for 24 h to determine the extent of the defects in the sarcolemma. In each of four bundles, every one of the 25 cells chosen at random for examination had gaps in the membrane. The amount of surface membrane that was missing from a complete transverse section of 10 cells from each of the four bundles, as estimated by planimetry, was 40 \(\pm 12\)% (SEM) of the total cell surface. Therefore, after 24 h of soaking in 10 mM EGTA containing no ATP, every cell developed large enough holes in the sarcolemma for soluble protein molecules to leak and for bidirectional exchange of large molecules or ions to occur. In view of the uniform presence of gaps in the sarcolemma, these cells are called “perforated cells.”

The production of the perforations occurs relatively slowly. After 6 h in the EGTA solution without ATP, the cells have no sarcolemmal perforations (Fig. 1A). They are hyperpermeable, have a low Ca sensitivity (pCa required for 50% of maximum Ca-activated force) that will respond to cAMP and cGMP, and will undergo an increase in contractility (defined by the maximum Ca-activated force) in response to detergent with catecholamines, guanine triphosphate, benzyl alcohol, or the combination of cAMP and theophylline
Figure 1. Electron micrographs of cells that have been soaked for 6, 12, and 24 h in 10 mM EGTA solution without ATP. Note the normal appearance of the sarcolemma after 6 h (A), and the gaps (arrows) in the membrane after 12 h (B) and 24 h (C). The contractile filaments have a normal appearance in the micrographs.

(McClellan and Winegrad, 1980). After 9 h of soaking, some perforations of the cell membrane begin to appear, but they are not uniformly distributed among all of the cells present. At this time the Ca sensitivity has increased so that a pCa of $\sim 5.2-5.3$ is necessary for 50% activation of maximum Ca-
activated contraction. Contractility will still respond to the combination of detergent and the substances listed above. After 12 h of soaking in the EGTA solution (Fig. 1B), ~85% of the cells that were examined had at least one large hole in the sarcolemma, and after 18 h every cell had large holes in its sarcolemma. The contractile filaments and myofibrils retain their normal appearance throughout, but the mitochondria are distorted and the sarcoplasmic reticula are swollen.
The maximum Ca-activated force that was produced by optimal Ca concentration in perforated cells did not differ significantly from the values found in hyperpermeable cells, and the rate at which tension developed, as defined by the half-time for the rise of tension at comparable levels of force, was the same for the two preparations. The Ca sensitivity of perforated cells was uniformly
high, however (Fig. 2, Table I). After 12 h of soaking in 10 mM EGTA solution without ATP, pCa required for 50% of maximum Ca activation was 5.2, and it could be decreased with cAMP or increased with either cGMP or detergent to ~5.4. Further exposure to the EGTA solution without ATP increased the Ca sensitivity and decreased the ability of cyclic nucleotide or detergent to alter Ca sensitivity.

![Figure 2](image)

**Figure 2.** Tension tracing of a trabecula that had been soaked in 10 mM EGTA without ATP for 48 h. The contraction solution with pCa values is indicated below the tracing. Note that >50% of maximum tension is produced in pCa 5.6. In the presence of completely or almost completely phosphorylated TNI, a pCa of <5.0 is required (Mope et al., 1980).

**Table 1**

| Duration (d) | Ca sensitivity | Percent increase in contractility |
|-------------|----------------|----------------------------------|
| Without cGMP | With cGMP | Δ | cAMP, theophylline, and detergent | Benzyl alcohol and detergent |
| 12 h | 5.20 | 5.40 | 0.20±0.02 | 174±12 |
| 2 d | 5.60 | 5.60 | 0 | 172±14 |
| 4 d | 5.70 | 5.70 | 0 | 203±21 |
| 1.5 h | 5.20 | 5.40 | 0.20±0.02 | 221±29 |
| 3 d | 5.50 | 5.60 | 0.10±0.01 | 57±19 |
| 2 d | 5.80 | 5.80 | 0 | 18±7 |
| 5 d | 5.70 | 5.70 | 0 | 21±6 |
| 2-5 d | 5.70 | 5.70 | 0 | 7±8 |
| 2-5 d | 5.70 | 5.70 | 0 | 2±6 |

Standard errors are not given for the Ca sensitivities but only for changes in Ca sensitivity, as these are the more relevant values.

After 2 d of exposure to the 10 mM EGTA solution without ATP, Ca sensitivity was maximal or almost maximal (Fig. 2), and was unresponsive to cAMP, cGMP, or detergent. Calcium sensitivity of the cells very closely resembled that of hyperpermeable cells in which all phosphorylation of troponin (TNI) has been inhibited by substituting cytosine triphosphate for
ATP (McClellan and Winegrad, 1978). The contractile system was still calcium regulated, but its Ca sensitivity was very high. At this time very little increase in contractility could be produced by epinephrine, GTP, or benzyl alcohol followed by detergent, but the combination of cAMP, theophylline, and detergent increased contractility by 172 ± 14%, the same increase in contractility as in the hyperpermeable fibers. In other words, agents that enhance contractility in hyperpermeable fibers by increasing activity of endogenous adenylate cyclase in the membrane were ineffective in perforated cells, but cAMP increased contractility equally in both hyperpermeable and perforated fibers. Therefore, in bundles consisting entirely of perforated cells, cAMP-induced increase in contractility can occur, but a cAMP-induced decrease in Ca sensitivity cannot occur.

The difference in the ability of the perforated and hyperpermeable cells to modify Ca sensitivity through a cAMP-regulated phosphorylation was studied more directly by measuring the amount of phosphorylation of the inhibitory subunit of TNI that can be produced in each preparation. Hyperpermeable and perforated cells were incubated with cAMP and theophylline in the presence of [32P]ATP, and then the tissues were homogenized, boiled in SDS, and electrophoresed on polyacrylamide gels. No evidence of loss of any of the contractile or regulatory proteins from the prolonged soak in EGTA without ATP could be detected from quantitative densitometry of the SDS gels (Fig. 3). In autoradiographs of the gels from hyperpermeable cells, there is considerable 32P labeling of a protein band that co-migrates with TNI, whereas in gels from perforated cells prepared by a 2-d soak in 10 mM EGTA without ATP, there was no detectable 32P present in the same band (Fig. 3). In perforated cells that had been soaked in 10 mM EGTA without ATP for only 12 h, where Ca sensitivity was less than maximal but still sensitive to cyclic nucleotides, some 32P was present in the TNI band on the gel.

Other functional properties of the perforated cell preparation were examined. The ability of the cell mitochondria to carry out oxidative phosphorylation was measured by the degree to which rigor tension produced in the absence of ATP could be reversed by the addition of ADP, inorganic PO₄, O₂, and succinate, a regimen that quickly and completely reverses rigor tension in hyperpermeable fibers. In perforated fibers, rigor tension was only slowly and incompletely reversed by this treatment.

The ability of the sarcoplasmic reticulum to accumulate and to release Ca was assayed by examining the response of tension to the addition of either 5 mM caffeine or Ca in subthreshold concentrations to relaxing solution with only 30 μM EGTA (Fabiato and Fabiato, 1975). In hyperpermeable fibers in 30 μM EGTA, caffeine caused a large contracture, and a subthreshold concentration of Ca produced cyclic increases in force. Both mechanical responses were inhibited by 3 mM EGTA. In perforated cells, only a weak caffeine response and weak cyclic contractions were produced after several minutes of Ca loading in 30 μM EGTA, and these responses were prevented by exposing the cells to 3 mM EGTA after a period of Ca loading in 30 μM EGTA. These results have been interpreted as indicating the presence of Ca release in response to caffeine or Ca-induced release signals to the sarcoplasmic
reticulum, but also a large degree of leakiness in the reticulum impairing its ability to retain calcium already taken up.

**Perforated Cells Prepared by Perfusion**

After perfusion of the heart through its coronary circulation with 10 mM EGTA solution with or without ATP, there were large holes in the sarcolemma that had the same appearance as those described in cells that had been soaked in 10 mM EGTA without ATP for ≥9 h (Fig. 4). Holes were present in a majority of cells after only 30 min of perfusion with 10 mM EGTA, the shortest perfusion time that was studied. Quantitative morphometry was carried out on cells that had been perfused with 10 mM EGTA overnight to

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**Figure 3.** SDS slab gel electrophoresis of hyperpermeable trabecula (c) and perforated trabeculae (d) (48 h soak without ATP) exposed to cAMP and theophylline in the presence of [32Pγ] ATP. Lanes a and b are the autoradiographs of c and d. The hyperpermeable trabeculae contain a considerable amount of 32P in the 150,000- and 28,000-dalton bands, but the perforated cells do not. Lanes e, f, and g contain, respectively, carbonic anhydrase, myosin (which had undergone some proteolysis during storage), and actin (courtesy of Dr. Annemarie Weber) for markers.
determine the extent of the defects in the sarcolemma, and the results were not significantly different from those from bundles soaked in 10 mM EGTA solution without ATP for 24 h. In every one of 100 cells randomly chosen from four bundles, there were gaps in the sarcolemma. An average of only 55 ± 11% (SEM) of the cell surface was covered with unit membrane. The contractile filaments and myofilaments appeared normal.

The Ca sensitivity and contractility of cardiac cells perforated by perfusion
was different from those of either hyperpermeable cells or cells perforated slowly by soaking 10 mM EGTA without ATP (Table II). In cells from hearts that had been perfused for 1 h, the pCa required for 50% of maximum activation was 5.05, and it was not altered by either cAMP, cGMP, cGMP and theophylline, or detergent. Contractility was high—maximum Ca-activated force equaled 6 g/mm²—and treatment with the combination of cAMP, theophylline, and detergent produced only a 46 ± 6% increase in contractility. When trabeculae isolated from these hearts were soaked overnight in 10 mM EGTA solution without ATP, calcium sensitivity rose so that a pCa of 5.38 was required for 50% of maximum activation, but Ca sensitivity still did not respond to cyclic nucleotides or detergent. Contractility was increased by 68 ± 11% after treatment with combination of cAMP, theophylline, and detergent.

Trabeculae that were perfused overnight with 10 mM EGTA solution with or without ATP had near-maximum Ca sensitivity; the pCa for 50% of maximum activation was 5.45. Ca sensitivity could not be changed by cyclic nucleotides or detergent. Contractility remained high without significant change from the value after only 1 h of soak. Unlike the cells that had been perfused for only 1 h, however, treatment with cAMP, theophylline, and detergent produced no change in contractility at all (4 ± 9%). When the volume of the 10 mM EGTA solution that perfused the heart overnight was restricted by recirculating the fluid without changing the perfusion rate, contractility and calcium sensitivity were different; the smaller the volume of the perfusion medium was, the lower the Ca sensitivity was. This was true for solution with and without ATP, so the difference could not have been breakdown of the nucleotide. For a 50-ml recirculated perfusion volume, pCa for 50% of maximum activation was 5.20 (Table II), and with volumes between 10 and 20 ml, the necessary pCa was 4.98. Ca sensitivity in these cells, in contrast to cells from hearts that were perfused without recirculation, did respond to cyclic nucleotides and detergents. Cyclic GMP or detergent increased Ca sensitivity by ~0.2 pCa units, and cAMP opposed this effect.

| Duration of perfusion | Volume of perfusion | Ca sensitivity | Percent increase in contractility in cAMP, theophylline, and detergent |
|-----------------------|---------------------|----------------|---------------------------------------------------------------|
| h                     |                     | Without cGMP   | With cGMP Δ                                                  |
| 1                     | No recirculation    | 5.05           | 5.05 0                                                      | 46±6                                               |
| 15                    | No recirculation    | 5.45           | 5.45 0                                                      | 4±9                                                |
| 15                    | 50 ml               | 5.20           | 5.40 0.20±0.01                                              | 3±6                                                |
| 15                    | 10-20 ml            | 4.98           | 5.20 0.22±0.02                                              | 63±9                                               |
The effect of cGMP was completely reversible (Fig. 5), which indicates that dephosphorylation as well as phosphorylation of TNI could occur.

Although Ca sensitivity increased with time of perfusion with 10 mM EGTA solution, it did not change over 4 h of soaking in relaxing and contraction solutions. Since there were large gaps in the membrane, the protein kinase and phosphatase that were involved in the reactions could not be freely diffusible, but on the contrary should have been rather tightly bound. Perforated cells were bathed in relaxing solution without ATP solution for 20 min to see if the kinase could be washed away when the reaction was blocked by the absence of one of its substrates. In three of four experiments, there was no change in the regulation of Ca sensitivity after the period of zero ATP, and in the fourth experiment only a small increase in Ca sensitivity
occurred. This suggests that even during a period when the enzyme was not active, it was tightly bound.

Contractility of cells from hearts that had been perfused with a limited volume also responded to the combination of cAMP, theophylline, and detergent. The smaller the volume of the recirculated perfusion solution was, the larger was the increment in contractility (Table II). With the smallest recirculating volume of 10 ml, contractility increased by 80%.

These results can be explained by the loss of the systems for regulating Ca sensitivity and contractility as the sarcolemma is perforated. The level of contractility remains unchanged after the loss of the regulatory system. Ca sensitivity, on the other hand, slowly increases with perfusion as, presumably, TNI is dephosphorylated. When the 10 mM EGTA perfusion solution is recirculated, the intracellular contents continue to be bathed by the regulatory systems in low concentration, and the regulatory systems are retained to an extent that depends on their concentrations in the bathing solution. The regulatory systems remain bound to insoluble cellular components in relaxing and contraction solutions but not in 10 mM EGTA solution.

DISCUSSION

The first important conclusion that can be drawn from results of similar experiments with hyperpermeable and perforated cardiac cells is that the target molecule for the cAMP regulation of contractility of the contractile proteins is different from the membrane-bound protein kinase that regulates TNI phosphorylation and Ca sensitivity. In spite of the total loss of regulation of calcium sensitivity in cells perforated by a long soak in 10 mM EDTA solution without ATP, treatment with cAMP, theophylline, and detergent produces a large increase in contractility. Even though cAMP is important in both regulatory systems, the cell can modify Ca sensitivity and contractility of the contractile proteins separately. The target molecule for cAMP regulation of contractility must be bound inside the cell since it is retained in the perforated cells. An alternative explanation for the inability of the perforated cells to phosphorylate TNI is that the prolonged exposure to a high concentration of EGTA in the absence of ATP modifies the molecule’s ability to undergo phosphoryl transfer reactions. There are several arguments against this possibility. No change in the molecule could be detected after electrophoresis on SDS gels. Second, several proteins that were phosphorylated in the hyperpermeable fibers are not phosphorylated in the perforated fiber, which suggests that the kinase rather than the substrate is at fault. Third, the results of the perfusion with 10 mM EGTA without ATP strongly support the notion that something is lost from the perforated cell to inhibit phosphorylation of TNI because after 15 h of perfusion by recirculation, phosphorylation of TNI remains (Table II), and the gels indicate that none of the regulatory proteins has been lost or even reduced in concentration.

The existence of functionally different pools of cAMP-regulated protein kinases in cardiac cells can account for the separation of cAMP-sensitive regulation of Ca sensitivity from contractility. Although studies in rabbit hearts using photo-affinity labeling indicate that only the 49,000- and 54,000-
dalton regulatory subunits of the two types of protein kinase significantly bind cAMP (Brunton et al., 1981), elevation of cAMP in cardiac cells by different agents does not always produce the same responses (Corbin et al., 1977; Honeyman et al., 1979; Keely, 1979). Cyclic AMP and contractility are both increased by isoproterenol, but PGE raises only cAMP. Isoproterenol increases the transfer of protein kinase activity from the particulate to the soluble fraction, and PGE does not (Brunton et al., 1981; Corbin et al., 1977).

Modification of Ca sensitivity of the contractile proteins of cardiac muscle by cyclic nucleotide-regulated reactions has been shown in hyperpermeable fibers (McClellan and Winegrad, 1978; Mope et al., 1980), mechanically skinned fibers (Fabiato, 1981), detergent-treated fibers (Herzig et al., 1981), isolated myofibrils (Ray and England, 1976), and isolated contractile proteins (Holroyde et al., 1979a), but in only the hyperpermeable fibers has the regulation both been reversible and involved a relatively large change in the concentrations of Ca required for contraction. A change of >0.7 pCa unit in Ca sensitivity can be reversibly produced in hyperpermeable fibers, but in detergent-treated fibers the change is irreversible, and in both detergent-treated and mechanically skinned cells as well as isolated proteins, the change is only ~0.2 pCa units. The more limited regulation of Ca sensitivity in these preparations is similar to what has been observed after a large fraction of the system regulating Ca sensitivity has apparently been lost with the perforation of the cell membrane. The importance of the integrity of the sarcolemma for maximal control of Ca sensitivity (McClellan and Winegrad, 1978) is not surprising since evidence indicates that the protein kinase for phosphorylation of TNI is located in the surface membrane until it is released by cAMP (Brunton et al., 1981; Corbin et al., 1977).

Dephosphorylation, which is regulated by a cGMP-dependent reaction (Mope et al., 1980), is equally important in the overall regulation of Ca sensitivity. Unlike the enhanced inotropic state and the phosphorylation of phosphorylase kinase, TNI phosphorylation that has been stimulated by catecholamines is not rapidly reversed by washing out the catecholamine (England, 1976; McCullough and Walsh, 1979; Onorato and Rudolph, 1981) or even by addition of the beta blocker propranolol. Phosphorylation can be reversed by cholinergic muscarinic agents in hyperpermeable fibers (Horowits and Winegrad, 1981) and decreased by acetylcholine in perfused hearts that have been exposed to catecholamines (England, 1975, 1976).

Regulation of Ca sensitivity may depend not only on cAMP-induced release of the catalytic subunit from protein kinase, but also on a beta-adrenergically controlled increase in the affinity of the myofibril for the catalytic subunit inasmuch as perforated cells can, under appropriate conditions, retain the ability to regulate Ca sensitivity for several hours in relaxing or contraction solutions in spite of large holes in the sarcolemma. This effect would explain residual regulation of sensitivity in cardiac cells after brief treatment with detergent (Herzig et al., 1981) or mechanical skinning (Fabiato, 1981). The change in affinity may also account for the fact that Ca sensitivity is modified by a lower concentration of catecholamine than contractility (Allen and Kurihara, 1980).
From these and previous results (McClellan and Winegrad, 1978; Mope et al., 1980), a general mechanism for the regulation of TNI phosphorylation can be formulated. Important observations in forming this model are (a) in standard hyperpermeable fibers, cAMP stimulates TNI phosphorylation; (b) after detergent treatment, phosphorylation of TNI is markedly but not completely reduced; (c) addition of mixed phospholipids after treatment with detergent produces some but not complete restoration of cAMP-stimulated phosphorylation of TNI; (d) the catalytic subunit of protein kinase that produces TNI-P\textsubscript{04} is sufficiently tightly bound to the myofibril that it is not easily washed out; (e) a cGMP-dependent reaction controls dephosphorylation; (f) cholinergic drugs increase cell cGMP and produce dephosphorylation of TNI.

In the proposed model the relevant protein kinase is bound to the sarcolemma. Cyclic AMP releases its catalytic subunit, which diffuses to the myofibril and phosphorylates TNI. \(\beta\)-adrenergic stimulation may enhance the affinity of the myofibril for the catalytic subunit. A decline in \(\beta\)-adrenergic activity causes an increase in affinity of the regulatory subunit for the catalytic subunit, leading to a recombination in the sarcolemma. The protein kinase in some way interacts with the cytoskeleton at the membrane, but requires a certain phospholipid milieu for its activity. Dephosphorylation results from the action of a cGMP-regulated phosphatase, possibly under cholinergic control.

The retention of the cAMP-dependent system for regulating contractility by the slowly perforated cells, but the loss to the bathing solution from cells rapidly perforated by perfusion may be an indication of a change in its interaction with catecholamine stimulation. The perfused cell is perforated while the \(\beta\)-adrenergic system is still coupled and active. On the other hand, the membrane of cells soaked in 10 mM EGTA without ATP is perforated only after the \(\beta\)-adrenergic system has been relatively uncoupled by the loss of GTP through the hyperpermeable membrane. Further work is necessary to evaluate this possibility.

It is not clear from the experiments reported why perfusion of the coronary circulation with 10 mM EGTA solution with or without ATP produces sarcolemma perforations, whereas soaking only in the solution without ATP makes holes. It may be related to the rate at which the concentration of EGTA in the immediate vicinity of the sarcolemma rises.

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