Calcium Sensitivity of the Contractile System and Phosphorylation of Troponin in Hyperpermeable Cardiac Cells

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ABSTRACT Bundles of cells from rat right ventricular myocardium were made "hyperpermeable" by an overnight soak in 10 mM EGTA (McClellan and Winegrad. 1978. J. Gen. Physiol. 72:737-764). In this preparation the cytoplasmic concentration of Ca++ and ATP could be controlled while sarcolemmal receptors and enzymes were retained. The Ca sensitivity of the tissues (as indicated by the pCa for 50% maximum activation) was altered to different extents in the presence of [32P]ATP by treatment with cyclic nucleotides, catecholamines, or a low concentration of nonionic detergent. The proteins of the tissue were then isolated by SDS-polyacrylamide gel electrophoresis, and the identity of 32P-labeled proteins was determined. The Ca sensitivity is inversely related to the relative amount of 32P incorporated into the inhibitory subunit of troponin (TNI). Extrapolation of the relation to the lowest Ca sensitivity observed gives a stoichiometry of about 0.8 mol PO_4 per mol TNI. These results support the hypothesis that Ca sensitivity of cardiac myofibrils is regulated by a phosphorylation of TNI that is stimulated by cyclic AMP (cAMP) and inhibited by cGMP.

Recent work using bundles of rat ventricular muscle in which the membranes had been made permeable to Ca, EGTA, ATP, and other small molecules and ions has shown that calcium sensitivity (the range of concentrations of Ca that is necessary for activation of the contractile system) can be varied over a fivefold range by a cellular mechanism (10). Indirect evidence indicates that the sensitivity is decreased by a cyclic AMP (cAMP) sensitive phosphorylation that can be regulated by catecholamines interacting with the sarcolemma. Calcium sensitivity is increased by a cGMP-regulated reaction that opposes the cAMP-regulated phosphorylation. In keeping with these results, the concentration of Ca required for activating the ATPase of isolated cardiac myofibrils is increased by a cAMP-regulated phosphorylation of the inhibitory subunit of troponin (TNI) (2, 4, 12, 13). The studies described in this paper were performed to determine whether phosphorylation of TNI was also responsible for regulating Ca sensitivity in the "hyperpermeable" rat ventricular fibers (10).
METHODS

Hyperpermeable bundles of rat ventricular fibers were produced as already described (10) by soaking trabeculae dissected from the endocardial surface of the rat right ventricle overnight at 2°C in a solution containing 140 mM K propionate, 2 mM Mg acetate, 5 mM Na₂ ATP, 5 mM imidazole, and 10 mM EGTA. Stability constants used for calculating free calcium were the same as those used by Fabiato and Fabiato (5). Eight or nine trabeculae were removed from the same region of the right ventricle. All eight bundles from a given region produced very similar tension-pCa relations and responses to drugs when their mechanical properties were studied on several occasions. Therefore, all but two of the bundles were used for radioactive studies and SDS-gel electrophoresis, and the remainder were used for parallel mechanical studies (10) in order to have an accurate knowledge of the mechanical properties of the tissues studied by electrophoresis. Originally one trabecula was used for the electrophoresis, but the amount of tissue (~ 0.2 mg) was too small to give well-defined bands for all myofibrillar proteins. The two bundles always had essentially identical mechanical responses both to different Ca concentrations and to the drugs used in the electrophoretic studies, thus supporting their use for indicating the mechanical properties of the bundles studied electrophoretically. All studies were performed at room temperature (23–24°C).

For electrophoretic studies the muscle bundles were pinned at approximately in vivo length in a Lucite chamber (DuPont, Wilmington, Del.) and continuously superfused by syringe pumps. The muscles were first exposed to normal relaxing solution (140 mM KCl, 7 mM MgCl₂, 5 mM ATP, 15 mM creatine phosphate (CP), 3 mM EGTA, and 25 mM imidazole at pH 7.0) for 10–15 min and then treated according to one of several protocols described in Results. Throughout all mechanical and biochemical experiments, the bundles were kept at their lengths in the opened but unstretched heart so that the relative lengths of the tissues used for the electrophoretic and physiological studies were the same. The solutions, the flow rates, and the durations of exposure to each solution were identical in the two types of experiments.

No creatine phosphate was added to the solutions containing [³²Pγ]ATP in order to prevent dilution of the specific activity of the radioactive adenosine triphosphate [³²Pγ]ATP. Since the volume of the superfusion solution was approximately 2,000 times the volume of the tissue, any dilution of specific activity of the radioactivity of the solution by phosphate in the tissue would be trivial. All radioactive superfusion solutions during a given experiment were labeled with [³²Pγ]ATP to have the same specific activity. Specific activity varied among all of the experiments from 100 to 150 μCi/ml of solution. Experiments were terminated by bathing the tissue for 10 min in relaxing solution containing either 20 mM NaF or 50 mM KH₂PO₄ and 70 mM NaF to inhibit phosphatase. Omission of F⁻, an activator of adenylate cyclase, did not change the results, but the F⁻ was retained as a precaution. For the electrophoresis, 10% acrylamide slab gels containing 0.1% sodium dodecyl sulfate (SDS), 0.1% mercaptoethanol, and 20 mM NaF were used.

At the conclusion of the experiment the muscle tissue was homogenized at room temperature in a micro Potter-type homogenizer using 3 M Tris-Cl buffer, pH 8.9, and 20 mM NaF. Then SDS and mercaptoethanol were added to a final concentration of 1% each and the homogenate placed in boiling water for 7 min. The sample was loaded into the appropriate slot of the electrophoresis box, which already held the gel in a Tris-glycine buffer at pH 8.9 containing 0.1% SDS and 0.1% mercaptoethanol. Markers were added, the gel was streaked with bromphenol blue tracking dye, and then the electrophoresis was carried out at 7–10° C according to the method of Weber.
and Osborne (17). After the electrophoresis was complete, the gel was removed and was stained overnight in Coomassie Brilliant Blue. 7–10% glacial acetic acid was used with horizontal electrophoresis for destaining. The gels were sliced by hand so as to include all of a given band in a single section. The thickness of individual sections therefore varied from ~ 3 to 8 mm. Each section was ground with scintillation fluid and counted in a liquid scintillation counter (Packard Instrument Co., Inc., Downers Grove, Ill.). Aliquots of the superfusion solutions were diluted with and without the addition of a representative slice of both stained and unstained nonradioactive gel to evaluate any quenching, which turned out to be insignificant.

In two experiments with [α²P³]ATP, myofibrils were isolated from the rest of the cellular material and their proteins were separated on polyacrylamide gels by electrophoresis. In these experiments at the end of the incubation in [α²P³]ATP, all eight trabeculae were washed out with relaxing solution containing 50 mM KH₂PO₄ and 70 mM NaF and then with a solution consisting of 0.2 mM dithiothreitol, 80 mM KCl, 20 mM NaF, and 5 mM imidazole. The tissues were homogenized in a micro Potter-type homogenizer and after centrifugation at 8,000 rpm in a GSA rotor for 10 min, the pellet was resuspended in the same solution. The pellet was homogenized, centrifuged, and resuspended for four additional cycles. It was then centrifuged at 7,000 rpm for 5 min, and the pellet was resuspended in 3 M Tris-Cl buffer, pH 8.9, containing 20 mM NaF. The pellet, which contained the myofibrillar material, was then treated with SDS and mercaptoethanol, and electrophoresis was carried out as above.

RESULTS

Previous work (10) has shown that the Ca sensitivity of hyperpermeable rat ventricular bundles can be changed to different extents by several procedures: (a) addition of the phosphodiesterase inhibitor theophylline to the bathing medium; (b) addition of cAMP to the medium; (c) addition of cGMP to the medium; (d) treatment of the tissues with 1% nonionic detergent; and (e) treatment of the tissue with 1% nonionic detergent containing cAMP and a phosphodiesterase inhibitor. An example of the change in Ca sensitivity, in this case produced when 5 mM theophylline is added to the bath, is shown in Fig. 1. The change in Ca sensitivity produced by either exogenous or endogenous cyclic nucleotides from the bath or phosphodiesterase inhibition is completely reversible whereas the effect of detergent on Ca sensitivity is not (10). Since the general shape of the relation between force and Ca concentration is unchanged when the Ca sensitivity is altered, the concentration of Ca at which force is 50% of maximum can be used as a reasonable measure of the Ca sensitivity.

The relation of the phosphorylation of the contractile proteins to changes in Ca sensitivity was studied by examining the labeling of contractile proteins from [α²P³]ATP added to relaxing solution with or without theophylline and cAMP and after treatment with 1% Triton X-100 (Rohm & Haas Co., Philadelphia) in radioactive relaxing solution containing either no drug, cGMP with theophylline, cAMP with theophylline, or epinephrine. After exposure to the radioactive solutions, the muscles were homogenized and the proteins were separated by SDS-polyacrylamide gel electrophoresis (Fig. 2). Isolated myofibrils from rat ventricle and skeletal
muscle and specific markers such as myoglobin, lactoglobulin, lysozyme, alcohol dehydrogenase, lactic dehydrogenase, carbonic anhydrase, actin, and phosphorylase A were included in the electrophoresis to facilitate the identification of bands produced by the hyperpermeable fibers. Tropomyosin, the inhibitory subunit of troponin (TNI), and the two light chains of myosin (LCI and LCII) produced clear and distinct bands whereas actin and the tropomyosin-binding subunit of troponin (TNT), which differ in molecular weight by only 1,000, ran together in 10–11% polyacrylamide gels although they were separated with gels containing a lower concentration of polyacrylamide. The band for the Ca-binding subunit of troponin (TNC) is visible but less distinct, running ahead of the 19,000 dalton light chain of myosin.

The estimation of the relation of incorporated $^{32}$P to the amount of TNI cannot be rigorously made because of the presence of tissue with disrupted membranes at the cut ends of each hyperpermeable bundle. (In order to include as much normal tissue with relatively aligned cells as possible, the ends include a small portion of the ventricular wall.) Cardiac cells with disrupted membranes do not show regulation of Ca sensitivity by cyclic nucleotides (10), and the TNI present in these cells should not be included in the calculation of the extent of phosphorylation of TNI. This difficulty was avoided by estimating the volume of undamaged tissue in isolated bundles with a dissecting microscope and then calculating the TNI content of this tissue from the known concentration of TNI in cardiac muscle (9). The length and the diameter of the undamaged portion of bundles were measured without taking into account the nonuniformity in diameter of a bundle, but since each experiment included six or seven bundles which were randomly positioned, the relative error introduced between groups should be small. The average length and diameter were $1.9 \pm 0.1$ and $0.368 \pm 0.026$ mm, respectively, to

![Figure 1](image-url)

**Figure 1.** A tension record of a hyperpermeable bundle of rat ventricular cells exposed to different concentrations of Ca in the (a) absence and (b) presence of 5 mM theophylline. The numbers below the tracings indicate the pCa ($-\log (\text{Ca}^{++})$) in the bathing solution, and the vertical marks indicate the period of exposure at each pCa. Note the change in pCa required to produce force without any significant change in the maximum Ca-activated force.
give an average volume of $0.202 \pm 0.02 \text{ mm}^3$ and, using a specific gravity of 1.0, an average mass of $0.20 \pm 0.02 \text{ mg}$. Another independent estimate of the amount of undamaged tissue was made by measuring the dry weight of the undamaged portion of a group of bundles. The average was 55 $\mu$g, which, using a weight to dry weight ratio of 4, gives 0.22 mg per bundle in reasonably good agreement with the value calculated from the measured dimensions of the bundles. The radioactivity of the gel slice was converted to PO₄ from a direct measurement of the specific activity of the superfusion solutions and

\begin{figure}
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\includegraphics[width=\textwidth]{figure2.png}
\caption{Photograph of the electrophoretic pattern of homogenized hyperpermeable cardiac cells with markers run in parallel to facilitate identification of the bands. Horizontal lines indicate location of specific bands. (a) Myofibrils prepared from rat ventricle; (b) hyperpermeable cardiac fibers; (c) markers myoglobin (MYO) (17,200), lactoglobulin (LG) (18,400), carbonic anhydrase (CA) (29,000), actin (A); (d) markers lysozyme (LYS) (14,300), lactic dehydrogenase (LDH) (33,500), alcohol dehydrogenase (ADH) (37,000), phosphorylase A (PHO) (94,000). Carbonic anhydrase is a very good marker for TNI. Although amino acid sequencing gives a molecular weight of 23,500 for TNI (8), the molecular weight by SDS polyacrylamide gel electrophoresis is about 28,000 (7, 8, 15, 16).}
\end{figure}

the measured amount of ATP added to the solution, which was always 5 mM. This amount of PO₄ represents the addition and exchange that occurred in the $^{32}$P solutions and not the total phosphate content.

As an example of data from a typical experiment, the distribution of $^{32}$P bound by proteins from the $[^{32}P]_\text{ATP}$ added to the medium in an experiment in which six muscles had been exposed to 1% Triton in relaxing solution with 5 mM theophylline and $10^{-6}$ M cAMP is shown in Fig. 3. The radioactivity in slices of the gel is indicated and the band pattern of the tissue proteins is drawn above. There was very little $^{32}$P incorporation into any of the contractile proteins except TNI, which contained a relatively large amount of radioactivity.
Hyperpermeable muscles that have been incubated with relaxing solution containing $[^{32}\text{P}]\text{ATP}$ for 20 min show very little incorporation of $^{32}\text{P}$ into contractile proteins or any other bands. An additional 15 min in relaxing solution containing 5 mM theophylline produces a significant incorporation of $^{32}\text{P}$ into TNI that increases after switching the bathing medium back to radioactive relaxing solution. An even larger amount of $^{32}\text{P}$ is incorporated into TNI if the bundles are superfused with radioactive relaxing solution containing cAMP and theophylline. Apparently there is very little turnover of phosphate in TNI in the absence of a significant concentration of cyclic nucleotides, but when the cyclic nucleotide concentrations are presumably raised by inhibiting phosphodiesterase activity, the rates of both phosphorylation and dephosphorylation increase considerably. This means that in order to measure the change in the amount of phosphorylated TNI, the existing phosphate must first be labeled by bathing the tissue in radioactive relaxing solution with theophylline to inhibit phosphodiesterase activity. Therefore two general types of experiments were performed, one in which $^{32}\text{P}$-labeled tissues with high Ca sensitivity from soaking in radioactive relaxing solution with 5 mM theophylline were subjected to procedures that lower Ca sensitivity and a second in which $^{32}\text{P}$-labeled tissues with low Ca sensitivity from soaking in radioactive relaxing solution with $10^{-6}$ M cAMP and 5 mM theophylline.
TABLE I
PHOSPHORYLATION OF TROPONIN I

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Ca (μM)</th>
<th>Stoichiometry (mol PO₄/mol TNI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>High Ca sensitivity → low Ca sensitivity</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Theophylline in RR</td>
<td>4.2</td>
<td>0.06</td>
</tr>
<tr>
<td></td>
<td>4.0</td>
<td>0.08</td>
</tr>
<tr>
<td></td>
<td>4.5</td>
<td>0.11</td>
</tr>
<tr>
<td>Theophylline in RR → RR</td>
<td>7.9</td>
<td>0.24</td>
</tr>
<tr>
<td></td>
<td>9.5</td>
<td>0.33</td>
</tr>
<tr>
<td>Theophylline in RR → cAMP + theophylline in RR</td>
<td>8.9</td>
<td>0.51</td>
</tr>
<tr>
<td>Theophylline in RR → epinephrine in RR</td>
<td>9.2</td>
<td>0.34</td>
</tr>
<tr>
<td>Low Ca sensitivity → high Ca sensitivity</td>
<td></td>
<td></td>
</tr>
<tr>
<td>cAMP + theophylline in RR</td>
<td>8.8</td>
<td>0.34</td>
</tr>
<tr>
<td></td>
<td>8.9</td>
<td>0.31</td>
</tr>
<tr>
<td></td>
<td>8.6</td>
<td>0.52</td>
</tr>
<tr>
<td>cAMP + theophylline in RR → 1% Triton in RR</td>
<td>3.0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>3.5</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>3.8</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>4.0</td>
<td>0</td>
</tr>
<tr>
<td>cAMP + theophylline in RR → cAMP + theophylline + 1%</td>
<td>4.9</td>
<td>0.28</td>
</tr>
<tr>
<td>Triton in RR</td>
<td>4.4</td>
<td>0.06</td>
</tr>
<tr>
<td></td>
<td>4.4</td>
<td>0.17</td>
</tr>
<tr>
<td>cAMP + theophylline in RR → cGMP + theophylline in RR</td>
<td>3.7</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>3.5</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>3.4</td>
<td>0.02</td>
</tr>
<tr>
<td>cAMP + theophylline in RR → cGMP + theophylline + 1%</td>
<td>3.5</td>
<td>0</td>
</tr>
<tr>
<td>Triton in RR</td>
<td>3.4</td>
<td>0.02</td>
</tr>
<tr>
<td>cAMP + theophylline in RR → RR</td>
<td>8.6</td>
<td>0.42</td>
</tr>
<tr>
<td>cAMP + theophylline in RR → RR → cGMP + theophylline</td>
<td>3.6</td>
<td>0</td>
</tr>
<tr>
<td>in RR</td>
<td>3.7</td>
<td>0</td>
</tr>
</tbody>
</table>

Two groups of experiments were performed: conversion of high to low Ca sensitivity and the converse. In the first group hyperpermeable bundles were superfused with radioactive relaxing solution (RR) containing 5 mM theophylline for 20 min and then with one of the three solutions listed for an additional 15 min. In the second group hyperpermeable bundles were superfused with RR with 10⁻⁶ M cAMP and 5 mM theophylline for 20 min and then with one of the four solutions listed for an additional 15 min. The Ca concentration is the amount necessary to produce 50% of maximum Ca-activated force in two hyperpermeable bundles from each group that were tested for Ca sensitivity. The moles PO₄ per mole TNI was calculated from the measured radioactivity of the TNI band in the gel, the specific activity of the soak solutions, and the amount of undamaged tissue in the group of bundles used in each study. Each value for PO₄ is the result from six to seven individual hyperpermeable bundles studied together. See text for additional details.
were subjected to procedures that raise Ca sensitivity (Table I). Ca sensitivity was fairly high—4.2 \( \mu \text{M} \) Ca was required for 50% of maximum activation—and 0.06 mol \( ^{32}\text{P} \) was covalently bound per mol of TNI after 20 min in radioactive relaxing solution with theophylline. The Ca sensitivity was then reduced by superfusing the tissues with radioactive relaxing solution alone, radioactive relaxing solution with \( 10^{-6} \) M cAMP and 5 mM theophylline, or radioactive relaxing solution with \( 10^{-7} \) M epinephrine. These three solutions all increased both the concentration of Ca required for 50% maximum activation and the amount of TNI labeled with \( ^{32}\text{P} \). Superfusing the tissues with radioactive relaxing solution with cAMP and theophylline labeled approximately 45% of the TNI with \( ^{32}\text{P} \) and produced a low Ca sensitivity.

Four different sets of additions to radioactive relaxing solution were used to raise Ca sensitivity in these tissues: (a) 1% Triton X-100; (b) 1% Triton X-100 with \( 10^{-6} \) M cAMP and 5 mM theophylline; (c) \( 10^{-7} \) M cGMP and 5 mM theophylline, and (d) \( 10^{-7} \) cGMP, 5 mM theophylline, and 1% Triton X-100. In each case there was a decline in the moles of \( ^{32}\text{P} \) per mole of TNI that was proportional to the increase in Ca sensitivity. These results are shown in Table I and plotted in Fig. 4. Further evidence that the \( ^{32}\text{P} \)-labeled protein with a molecular weight of 28,000 daltons was TNI was provided from two experiments in which myofibrils were isolated before electrophoresis from hyperpermeable trabeculae that had been incubated in \( ^{32}\text{P}\gamma\text{ATP} \)-labeled relaxing solution containing cAMP and theophylline. In gels of these myofibrils only
the 28,000 dalton protein contained a significant amount of $^{32}$P, and there was, respectively, 0.39 and 0.36 mol of PO$_4$ bound per mol of protein in the two experiments. Since TNI is the only major myofibrillar protein that migrates as a molecule of 28,000 daltons during electrophoresis on polyacrylamide gel (7, 8, 15, 16), TNI is almost certainly the protein binding $^{32}$P.

Good correlation exists between the amount of TNI phosphorylated under the several conditions studied and the concentration of Ca required for 50% of maximum activation (Fig. 4). This relation offers strong support for the notion that Ca sensitivity is inversely related to TNI phosphorylation. Hyperpermeable cardiac cells with the lowest Ca sensitivity that has been observed in extensive studies of the point (10) require a Ca concentration of about $1.6 \times 10^{-5}$ M for 50% of maximum activation. Extrapolation of the plot of Ca concentration vs. TNI phosphorylation to that value yields a stoichiometry of almost 0.8 mol $^{32}$P per mol of TNI. In view of the approximations involved in the estimate of the amount of TNI, this value is not significantly different from one and indicates that minimum Ca sensitivity is probably achieved by phosphorylation of all of the TNI. Cyclic AMP with or without theophylline did not produce phosphorylation of TNI in hyperpermeable fibers treated with detergent. This is additional support for the inferred association of a membrane-dependent phosphorylation with a change in Ca sensitivity inasmuch as Ca sensitivity cannot be altered in hyperpermeable cells after treatment with detergent (10).

**DISCUSSION**

The present studies have focused on the question of whether the regulation of Ca sensitivity by cyclic nucleotides existing in hyperpermeable fibers is mediated through a phosphorylation of TNI that decreases the sensitivity. The production of a hyperpermeable state by EGTA (10) has been questioned by Miller in a paper in *Nature (Lond.*)* (11), which was in press before the full details of the properties of the hyperpermeable cells had been published (10). Although a formal response to Miller's questions has appeared (18), it is appropriate to list briefly several properties of EGTA treated cardiac cells that refute Miller's concerns:

(a) Removal of ATP from the bath rapidly increases tension, and restoration of ATP causes relaxation. This indicates that the membrane is highly permeable to ATP. ADP does not relax the cells but creatine phosphate with ADP does produce relaxation. Since there is no creatine kinase in the extracellular space (10), both the ADP and creatine phosphate must enter the cell.

(b) The membrane potential remains at -8 mV when all the KCl in the bath has been replaced by NaCl.

(c) Force from Ca-induced and caffeine-induced Ca releases from the sarcoplasmic reticulum is prevented by 3 mM EGTA in the bathing solution indicating that EGTA enters the cell.

(d) Treatment of EGTA-treated fibers with detergent for 30 min destroys the function of the cell membrane but does not alter either the rate of development of tension or the maximum Ca-activated force.
(e) The amount of force generated by a given concentration of Ca is independent of the concentration of Na in the bath.

(f) The amount of force and the rate of development of force at any given concentration of Ca are stable and reproducible.

These data indicate that molecules of at least 500 daltons easily pass into the cytoplasm. It is likely that the maximum size for readily permeant molecules or ions is less than 5,000 daltons since 32P-labeled proteolytic fragments are retained by the hyperpermeable cells but completely disappear with detergent treatment.

The results show that 32P from labeled ATP is incorporated into TNI when the Ca sensitivity is less than maximal and that an inverse relation exists between the Ca concentration for 50% of maximum activation and the amount of 32P-labeled TNI. Extrapolation to the minimum Ca sensitivity that has been observed in physiological experiments gives a stoichiometry of about 0.8 mol P for each mole of TNI. It is very unlikely that the phosphorylation of TNI involves 32P that has entered the cell after being split from [32Pγ]ATP in the bath because hyperpermeable cells superfused with a solution without suitable substrate cannot synthesize high energy phosphates (10). The 32P-labeled protein of 28,000 daltons is almost certainly TNI because it is present in the myofibrillar fraction, and its relatively large concentration in the cell indicated by the amount of PO₄ bound precluded other proteins of this size.

In the absence of added cyclic nucleotides or under conditions where hydrolysis of cyclic nucleotide occurs without any inhibition, very little 32P is incorporated into TNI. A phosphodiesterase inhibitor that raises the concentration of both cAMP and cGMP increases 32P incorporation but also produces an increase in Ca sensitivity. These coincidental findings may be explained by assuming that, while cAMP activates a kinase that phosphorylates TNI, cGMP activates a phosphatase that dephosphorylates TNI, the net result being an increase in 32P incorporation but a decrease in total phosphate content of TNI. The rise in 32P content when the tissues were returned to radioactive relaxing solution after radioactive relaxing solution with theophylline supports this assumption. A more direct demonstration was produced by adding cGMP to the solution bathing 32P-labeled tissues. The result was a loss of 32P in keeping with the idea that cGMP activates the phosphatase. In order to measure the amount of phosphate bound by TNI using [32Pγ]ATP, it was necessary first to label the phosphate already present by raising the turnover rate with either exogenous or endogenous cyclic nucleotides. This was done with theophylline or with cAMP plus theophylline to produce two populations of labeled tissues, one with high and the other with low Ca sensitivity, since theophylline alone apparently causes a greater inhibition of cGMP than cAMP hydrolysis (1). Either cAMP, epinephrine or removal of theophylline decreased Ca sensitivity and increased TNI phosphorylation while cGMP or Triton did the opposite. These observations support the model assumed above in which phosphorylation of TNI and Ca sensitivity are regulated by a cAMP-controlled kinase and a cGMP-controlled phosphatase.

The effect of the Triton is presumably due to the removal or inactivation of adenylate cyclase and protein kinase in the membrane (3), preventing phosphorylation and leading to dephosphorylation of TNI.

As discussed in a previous paper (10), the regulation of Ca sensitivity is not an important mechanism for regulating the amount of systolic force, but it may be useful to the heart in controlling diastolic tone and rate of filling of the ventricles.

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