

# Cyclic AMP Regulation of Myosin Isozymes in Mammalian Cardiac Muscle

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**ABSTRACT** Hyperpermeable cells from rat heart contain a cAMP-dependent system that can increase the maximum Ca-activated force (contractility) of the contractile proteins. In two different conditions where the relative concentration of the myosin isozymes changes, i.e., hypothyroidism and aging, the size of the increase in contractility from activation of the cAMP-regulated system varies closely with the relative concentration of V<sub>1</sub>, the isozyme of myosin with the greatest Ca- and actin-activated ATPase activity. The existence of another system for the regulation of the slow isozyme V<sub>3</sub> has been demonstrated, and it may be inhibited by  $\beta$ -adrenergic activity. The possibility of cAMP-dependent myosin regulation of contraction in addition to Ca regulation of troponin is considered. Phosphorylation of the contractile proteins themselves is not required for the increased contractility.

## INTRODUCTION

The contractility of individual heart cells must be regulated because the force generated by a heart, in which every cell is believed to participate in each contraction, can vary over a wide range. The most important mechanism involved in modulating contractility of the normal heart under physiological conditions is neuroendocrine stimulation by catecholamines. Several important changes in mechanical performance are produced by catecholamines, including increases in maximum developed force, rate of rise of force, rate of relaxation, and energy required for the maintenance of tension. Any comprehensive explanation of the effects of catecholamine stimulation must account for these observations.

Some of the factors in the change in mechanical performance have already been identified.  $\beta$ -Adrenergic stimulation, by increasing the influx of calcium during the action potential (Reuter and Scholz, 1977) and accelerating calcium uptake by the reticulum (Katz, 1977), provides more calcium for activation of the contractile proteins and a larger tension. During  $\beta$ -adrenergic stimulation the inhibitory subunit of troponin (TNI) is phosphorylated by a cAMP-dependent protein kinase, which leads to a faster release of calcium bound to troponin and a more rapid relaxation (Robertson et al., 1982).

Detection of a change in the force-generating capabilities of the contractile proteins themselves, however, is difficult to make in an intact cell because of the inability to distinguish changes in excitation-contraction coupling from changes in the contractile proteins. To overcome the difficulty, a preparation of hyperpermeable cardiac fibers was developed that not only permits direct activation of the contractile sections with buffered concentrations of Ca in the bathing solution but also retains many of the cells' normal regulatory systems that involve membrane receptors. Direct probing of the contractile properties of the myofibrils in the presence of many normal membrane and intracellular functions is possible. With hyperpermeable fibers it was possible to show the relation of  $\beta$ -adrenergic stimulation to TNI phosphorylation and the concentration of Ca required for activation of contraction (McClellan and Winegrad, 1978; Mope et al., 1980).  $\beta$ -Adrenergic stimulation of hyperpermeable mammalian cardiac cells under the appropriate conditions could also increase the maximum Ca-activated force by a cAMP-dependent mechanism (McClellan and Winegrad, 1980). The mechanism responsible for this increase in contractility is not clear, but the physiological relevance of this change in the properties of the contractile system in intact hearts has been demonstrated (Winegrad et al., 1983). More recent studies of regulation of the contractile proteins in hyperpermeable heart muscle by cAMP indicate that the response may be dependent upon the specific isozyme or isozymes of myosin that are present. In regulating contractility, the cardiac cell may be able to distinguish the different isozymes of myosin. These studies are described below.

#### METHODS

##### *Production of Hyperpermeable Fibers*

Natural bundles of hyperpermeable cells from the right ventricle of the rat were produced as already described (McClellan and Winegrad, 1978) by an overnight soak in a solution containing 140 K propionate, 10 mM EGTA, 5 mM ATP, and 2 mM Mg acetate buffered to pH 7.2 with imidazole. They were mounted in a continuous perfusion chamber so that they were ~75% up the ascending limb of the length-developed tension curve (McClellan and Winegrad, 1978), and tension was recorded continuously.

##### *Combined Physiological and Myosin Isozyme Studies*

Hearts were removed from rats and washed free of blood with a modified Krebs (140 mM NaCl, 4 mM KCl, 2.5 mM MgCl<sub>2</sub>, 2.5 mM CaCl<sub>2</sub> buffered at pH 7.2 with 10 mM imidazole) solution, and the right ventricles were cut open and pinned down on Sylgard as has already been described in detail (McClellan and Winegrad, 1978). Trabeculae from the endocardial surface of the right ventricle were removed and pinned in the 10-mM EGTA solution at the same length they had in the open right ventricle. Then the remaining right ventricular free wall was removed and cut into two approximately equal pieces. Myosin was extracted from these two pieces of tissue by first mincing the tissue with a fine scissors at 4°C in a solution consisting of 0.6 M KCl, 0.05 M Tris, 0.01 M  $\beta$ -mercaptoethanol, 0.01 M sodium pyrophosphate, and 2

mM MgCl<sub>2</sub> at pH 7.5. The minced tissue was homogenized at 4°C in ~6 vol of the same solution and then stirred for 1 h. After the stirring, the homogenate was centrifuged for 20 min at 19,000 rpm using a 5534 rotor in a Sorvall centrifuge (DuPont Instruments-Sorvall Biomedical Div., Wilmington, DE). The pellet was discarded and 5 vol of 2 mM MgSO<sub>4</sub> was added to the supernatant. The supernatant was then stirred for 20–30 min and centrifuged at 7,000 rpm for 10 min using an HB-4 rotor (DuPont Instruments-Sorvall Biomedical Div.). The supernatant was discarded, the pellet was redissolved in the original 0.6 M KCl buffer, and 5 vol of 2 mM MgSO<sub>4</sub> was added. The 20-min stirring and 10-min centrifugation at 7,000 rpm were repeated. After the pellet had been resuspended in a solution of 40 mM sodium pyrophosphate, 1 mM EDTA, and 0.025% mercaptoethanol at pH 8.5, the suspension was centrifuged in an ultracentrifuge at 29,000 *g* for 3 h. The supernatant was used for the electrophoretic determination of the relative concentration of myosin isozymes (see below).

The trabeculae were kept in the 10-mM EGTA solution at ~4°C overnight, and then they were used for the determination of maximum Ca-activated force under one of several sets of conditions that are described in the Results. When these measurements had been made, each trabecula was placed in a homogenizer with 50  $\mu$ l of a solution containing 10 mM Na<sub>4</sub>P<sub>2</sub>O<sub>7</sub>, 10% glycerol, 0.01%  $\beta$ -mercaptoethanol, and 1 mM EGTA. In view of the small size of a trabecula, which weighed <0.3 mg (wet wt), the entire homogenate was used for determination of the myosin isozyme content.

Electrophoresis of the myosin extracted from the right ventricle and the homogenates of the trabeculae was performed according to the procedure of Hoh et al. (1977). Before loading the gels, each sample of extracted myosin was analyzed for protein content using the Lowry et al. (1951) method and the myosin was diluted so that the final solution contained 0.1 mg/ml protein, 10 mM Na<sub>4</sub>P<sub>2</sub>O<sub>7</sub>, and 50% glycerol. 1–2  $\mu$ g of each sample of extracted myosin was loaded on a gel, and samples were run in duplicate. The entire homogenized trabecula was loaded on the gel. Electrophoresis was carried out for 22–24 h at a voltage gradient of 14 V/cm in 3.88% polyacrylamide gels. The buffer contained 20 mM Na<sub>4</sub>P<sub>2</sub>O<sub>7</sub> and 10% glycerol at pH 8.8 at a carefully controlled temperature of 1–2°C. The buffer was recirculated between anodal and cathodal baths to prevent pH changes. In every electrophoresis, a sample of myosin extracted from the right ventricle of a 6–8-wk-old euthyroid rat was included as a control. In each electrophoresis, specimens containing equal amounts of myosin from the experimental and the control were mixed and run together on the same gel to assist in identifying the myosin isozymes in the experimental heart. At the end of the electrophoresis, the gels were stained with Coomassie Blue and destained in 15% methanol and 7.5% acetic acid until the background of the gels was clear. After destaining, the gels were scanned twice with a Gilford spectrophotometer (Gilford Instrument Laboratories, Inc., Corning Glass Works, Oberlin, OH) at a wavelength of 550 nm with a slit of 0.05 mm, the second time after the gel had been rotated 90°. The areas under the different peaks in the gel were calculated by a reporting integrator (3390A; Hewlett-Packard Co., Palo Alto, CA). The areas were also measured with an electronically integrating planimeter (courtesy of Professor L. D. Peachey, University of Pennsylvania, Philadelphia, PA) as a check on the integrator. Two different conventions were used in calculating the areas for each isozyme with the planimeter: first, that the shape of any band was symmetrical around its peak, and second, that the areas occupied by the different bands in a given gel had the shapes of similar triangles. The reproducibility of calculation when the same gels were measured by two different people had a standard deviation of 6%. The percentage of each isozyme

was expressed as the ratio of the area attributed to that isozyme to the total area of myosin on the gel.

In the analysis of the data, the results from the two measurements of myosin from each right-ventricle strip were averaged. The differences between the averages of the two strips were never more than the differences between the duplicate measurements for a single strip. Therefore, the four values were averaged for comparisons between hearts.

In the gels containing some of the trabeculae, the amount of protein was too small for a good quantitative analysis of the scans to be made. In those gels where there was adequate protein staining, the distribution of protein among the three regions of the gel corresponding to the three isozymes was almost identical with that present in the gels of myosin extracted from the right-ventricular strips. There was however, a very faint additional staining that was present about one-third to one-half of the way back to the top of the gel. In view of the absence of adequate protein in all gels of trabecular homogenates and the unexplained faint staining above the myosin region of the gel, measurements of myosin isozyme distribution from myosin extracted from right-ventricular strips were used for comparison with the results of the mechanical studies.

The animals used for these studies were obtained from Charles River Breeding Laboratories, Inc., Wilmington, MA, and West Jersey Biological Co., Winonah, NJ. The thyroidectomized rats were received within 3–4 d of surgery that had been performed by the supplier. These animals were all 6–7 wk old, and since they were still in their growing period, the development of the hypothyroid state could be followed by comparing their rate of weight gain with euthyroid rats of the same age. During this study only one rat that had had surgery failed to show a substantially slower rate of increase in weight than euthyroid controls. Its cardiac myosin isozyme distribution was intermediate between those of euthyroid and hypothyroid rats.

Comparison was also made between the myosin isozyme concentrations and the contractility response to cAMP, theophylline, and detergent in rats of different ages. Animals of three different ages were studied: 4–10, 23–25, and >80 wk, as examples of young, mature, and elderly rats. All rats including the old ones had been obtained by age 4 wk and then maintained within the department animal facilities until they were killed.

Three rats from a group of known spontaneously hypertensive rats were studied. The exact age of these animals was not known, but they weighed between 250 and 350 g, a weight normally seen in young rats. These animals had spontaneous systemic hypertension, which resulted in substantial left-ventricular hypertrophy with little or no right-ventricular hypertrophy. They appeared healthy, with no signs of cardiac failure. Based on the ratio of ventricular to body weight in these animals compared with normal rats of the same weight, left-ventricular hypertrophy varied from 27 to 34% and there was no detectable hypertrophy of the right ventricle.

#### *Modification of Contractility with cAMP, Theophylline, and Detergent*

The sensitivity of the contractile system of hyperpermeable cells to  $\beta$ -adrenergic regulation was assessed in the following way. Hyperpermeable cells were superfused with a relaxing solution containing 5 mM theophylline as a phosphodiesterase inhibitor for 5 min, then with relaxing solution containing 5 mM theophylline and 1  $\mu$ M cAMP, and finally with a relaxing solution containing theophylline, cAMP, and 1% Triton X-100. After 30 min in the last solution, the cells were superfused with plain relaxing solution for an additional 30 min before they were exposed to contraction solutions. Change in contractility was inferred from the change in maximum Ca-

activated force after the detergent had been washed out. This protocol was used because Ca-activated force is reversibly reduced in the presence of detergent (McClellan and Winegrad, 1980).

#### *Estimation of Force per Unit Cross-Sectional Area*

The diameters of the middle portion of the trabeculae were measured twice through a stereomicroscope with an eyepiece gradicule, the second time at a right angle to the first measurement. The length of the trabeculae between the ties was also measured. At the end of the experiment, the portion of the trabeculae between the ties was isolated, blotted, and then weighed on a Cahn electrobalance (Cahn Instruments, Inc., Cerritos, CA). From the length, weight, and an assumed density of  $1 \text{ g/cm}^3$ , an average value for cross-sectional area was calculated. The estimates of cross-sectional area from the two methods were always within 20%, and their average was used in defining force per unit cross-sectional area.

#### *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 have almost the same 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 studies of the mechanical responses of the tissues. 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  $^{32}\text{PO}_4$  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 were continuously superfused by syringe pumps. The muscles were first exposed to normal relaxing solution (140 mM KCl, 7 mM  $\text{MgCl}_2$ , 5 mM ATP, 15 mM creatine phosphate [CP]), 3 mM EGTA, and 25 mM imidazole at pH 7.0) for 10–15 min. In one set of experiments the solution was then switched to a  $[\gamma\text{-}^{32}\text{P}]\text{ATP}$  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  $\mu\text{Ci/ml}$  of solution. The solutions were then changed according to the following protocol: relaxing solution with  $[\gamma\text{-}^{32}\text{P}]\text{ATP}$  for 10 min, relaxing solution with  $[\gamma\text{-}^{32}\text{P}]\text{ATP}$  and 5 mM theophylline for 10 min, and then relaxing solution with  $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ , 5 mM theophylline, and 1  $\mu\text{M}$  cAMP for 10 min. In four experiments, the cells were bathed in detergent with  $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ , theophylline, and cAMP as a final step after the three other radioactive solutions. Experiments were terminated by bathing the tissue for 10 min in a relaxing solution containing either 20 mM NaF or a solution with 50 mM  $\text{KH}_2\text{PO}_4$  and 70 mM NaF to inhibit dephosphorylation (Holroyde et al., 1979a, b). The results were very similar with the two solutions.

Experiments were also conducted by using  $^{32}\text{P}_i$  in the presence of succinate, ADP,  $\text{O}_2$ , and creatine to allow the mitochondria to synthesize radioactive ATP. The results of these experiments were not different from those with  $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ . 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 an experiment, the tissue was homogenized at room temper-

ature with a micro Potter-type homogenizer in a solution of 3 mM Tris-Cl buffer and 20 mM NaF at pH 8.9. 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 containing 10–30  $\mu$ g of protein and each was loaded on a different lane of 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. 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).

#### *Solutions*

(1) 10 mM EGTA solution: 10 mM EGTA; 140 mM potassium propionate; 5 mM ATP; 2 mM Mg acetate; and 5 mM imidazole, pH 7.2.

(2) Relaxing solution: 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 25 mM imidazole, pH 7.0.

(3) Contraction solution: same as relaxing solution with added CaCl<sub>2</sub>. The pCa was calculated using stability constants from Fabiato and Fabiato (1979).

#### RESULTS

The maximum Ca-activated force (defined as contractility) developed by hyperpermeable fibers can be increased by almost 200% as a result of treatment with the combination of cAMP, theophylline as a phosphodiesterase inhibitor, and 1% non-ionic detergent in a relaxing solution (McClellan and Winegrad, 1980). Other substances that stimulate the production of cAMP by endogenous adenylate cyclase, such as  $\beta$ -adrenergic agonists and guanine nucleotides, are effective, but they also require non-ionic detergent for the increase in contractility to occur. The most effective procedure is to superfuse the hyperpermeable fibers successively with theophylline, theophylline and cAMP, and finally theophylline, cAMP, and detergent (Fig. 1).

#### *Relation of cAMP-dependent Regulation to Myosin Isozymes*

The response of the contractile proteins to cAMP regulation is greatly influenced by the specific pattern of myosin isozymes present in the cardiac cells. Ventricles of rats contain three isozymes of myosin, which have been designated V<sub>1</sub>, V<sub>2</sub>, and V<sub>3</sub> by Hoh et al. (1977). One of the isozymes, V<sub>1</sub>, which comprises ~80–85% of the total myosin in the ventricles of normal young rats, has a Ca-activated ATPase activity that is several times the activity of the other two (Pope et al., 1980). The relative amounts of the isozymes, however, are sensitive to thyroid hormone (Hoh et al., 1977), to hemodynamic conditions (Mercadier et al., 1981), and to the age of the animal (Hoh et al., 1977;

Lompre et al., 1981). Hypothyroidism from thyroidectomy or hypophysectomy causes complete or almost-complete disappearance of  $V_1$  in  $\sim 5$  wk (Hoh et al., 1977), but administration of thyroid hormone to these animals reverses the isozyme concentrations to normal values. Excess thyroid hormone administration causes replacement of  $V_3$  by  $V_1$  myosin in previously euthyroid animals (Hoh et al., 1977; Martin et al., 1981).

Rats were made hypothyroid by removal of the gland and, in addition, chronic administration of propylthiouracil (0.1 g/100 ml in the drinking water) in three animals, and over the 5-6-wk period during which the isozyme

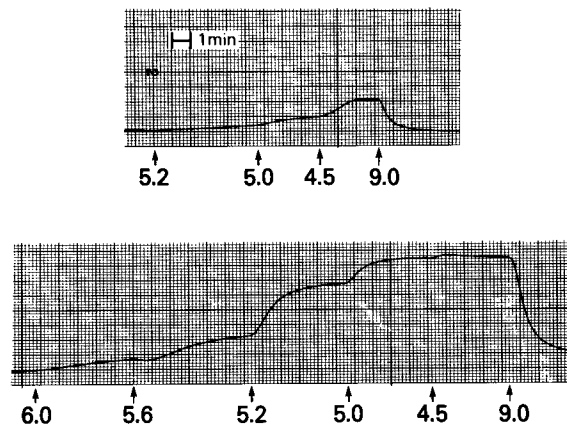


FIGURE 1. Tracing of tension produced by a hyperpermeable rat right-ventricular trabecula before (above) and after (below) exposure to 5 mM theophylline, 5 mM theophylline plus 1  $\mu$ M cAMP, and 5 mM theophylline plus 1  $\mu$ M cAMP plus 1% Triton X-100, followed by a 30-min wash in relaxing solution. Numbers below records indicate the pCa of the contraction solution.

pattern changes (Figs. 2 and 4), the effect of cAMP on the contractility of hyperpermeable cardiac fibers was examined. The method of producing hypothyroidism made no difference in the cells' behavior. The response to the combination of cAMP, theophylline, and detergent diminished in parallel with the relative concentration of  $V_1$  (Figs. 3, 4, and 7), and the effect of epinephrine and GTP changed in a similar fashion. Minimum values for the concentration of  $V_1$  and the increment in contractility were reached at the same time.

The response of contractility to detergent itself changed (Fig. 3). As  $V_1$  was replaced by  $V_3$ , detergent gradually began to enhance contractility until there was an almost 200% increase after  $V_3$  had reached its maximum value. However, even in hyperpermeable fibers with large amounts of  $V_3$ , the presence of epinephrine or cAMP prevented detergent from increasing contractility.

In view of the effect of  $\beta$ -adrenergic stimulation on the increase in contractility produced by detergent, all experiments in which hyperpermeable bun-

dles from normal hearts had been treated with detergent without added cAMP, epinephrine, or GTP were re-examined. The amount of phosphorylation of TNI inferred from the pCa for 50% of maximum Ca-activated force (Mope et al., 1980) was used as a measure of the  $\beta$ -adrenergic activity that existed in each hyperpermeable bundle. In fibers with a large amount of  $\beta$ -adrenergic activity, detergent caused a decline in contractility, but in other muscles with little  $\beta$ -adrenergic activity, detergent produced an increase of as much as 50% in contractility (Fig. 5). In trabeculae from hearts that had only

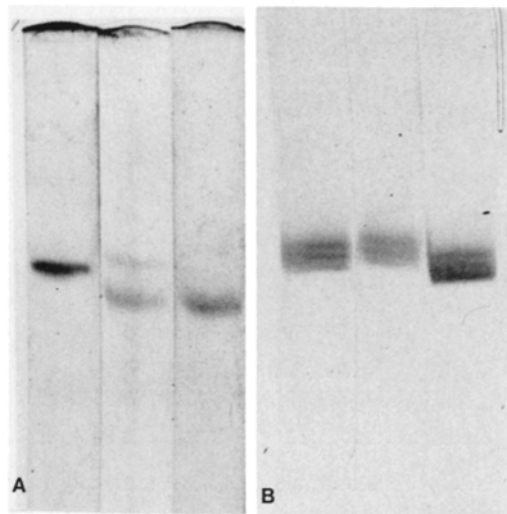


FIGURE 2. (A) Results of electrophoresis of myosin from a normal rat aged 5 wk (right tube); a rat 5 wk after thyroidectomy (left tube); and a mixture of myosin from euthyroid and hypothyroid hearts (middle). Polyacrylamide gels in pyrophosphate medium (Hoh et al., 1977) were used to prevent dissociation of myosin into subunits. Note the more rapidly migrating V<sub>1</sub> band from the normal euthyroid rat and the slower V<sub>3</sub> band from 5-wk post-thyroidectomy hearts. Note two separate bands in the mixture. (B) Electrophoresis of right-ventricular tissue from three mature (ages 23–25 wk) rats. Note the presence of significant amounts of V<sub>1</sub>, V<sub>2</sub>, and V<sub>3</sub> in contrast with the young euthyroid rat heart in A.

been washed free of blood before the tissue bundles were removed and placed into a 10-mM EGTA solution, detergent and detergent with theophylline decreased contractility by  $21 \pm 4$  ( $n = 5$ ) and  $18 \pm 7\%$  ( $n = 7$ ), respectively, but in hyperpermeable cells from hearts that had been extensively washed to remove bound catecholamine, detergent and detergent with theophylline increased contractility  $43 \pm 13$  ( $n = 7$ ) and  $74 \pm 9\%$  ( $n = 4$ ), respectively. All of these findings are consistent with the results in hyperpermeable fibers from hypothyroid hearts in the relation between  $\beta$ -adrenergic activity and the response of contractility to detergent. Detergent raised contractility in proportion to the amount of V<sub>3</sub>, and  $\beta$ -adrenergic activity inhibited the increase.



The relative concentration of isozymes of myosin in the heart changes with age of the animal. In very young rats,  $V_1$  predominates almost to the exclusion of the other two isozymes (Hoh et al., 1977; Lompre et al., 1981), but as the animal grows old the situation reverses and  $V_1$  has the lowest concentration (Fig. 6). The response of hyperpermeable cardiac cells to cAMP, theophylline, and detergent varied with the age of the animal in a manner analogous to the response in hypothyroid rats. When  $V_1$  was the predominant isozyme, the

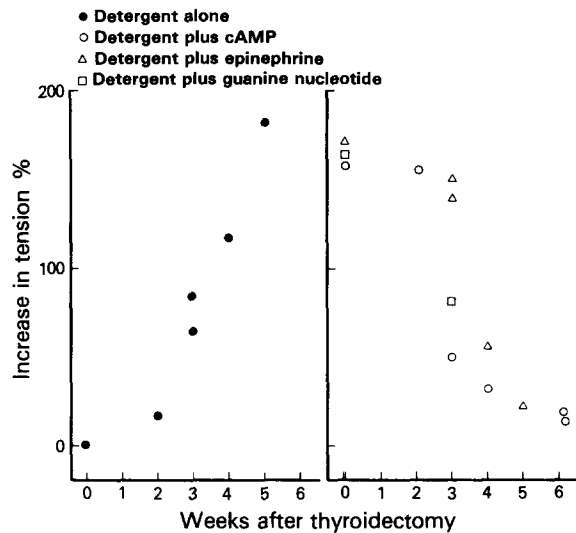


FIGURE 3. The change in maximum Ca-activated force in hyperpermeable right-ventricular trabeculae from rats that had been thyroidectomized up to 6 wk earlier. Each point represents the results of four different trabeculae from a single rat heart. Standard errors were never more than 15% of the mean and generally not more than 10% of the mean. Values at time 0 were from normal 4–6-wk-old rats. On the left are changes in contractility from relaxing solution with 1% Triton X-100. On the right are the changes in contractility after 1  $\mu$ M cAMP and 5 mM theophylline, 1  $\mu$ M epinephrine, or 10  $\mu$ M GTP each with 1% Triton X-100 in relaxing solution.

increment in contractility by stimulation with cAMP, theophylline, and detergent was large, and the change with detergent was either insignificant or negative (Table I, Fig. 7). As the fraction of  $V_1$  relative to  $V_2$  and  $V_3$  declined with age, the size of the increment with detergent increased. In mature rats, detergent caused a large increase in contractility (Table I).

Hypertrophy of the left ventricle from a chronic increase in the resistance to blood flow increases the relative amount of slow myosin (Mercadier et al., 1981; Gorza et al., 1981; Rupp, 1981). In spontaneously hypertensive rats the left ventricle but not the right ventricle must work against an increased resistance. The increment in contractility from cAMP, theophylline, and detergent was  $78 \pm 14$  and  $165 \pm 23\%$  in hyperpermeable fibers from,

respectively, the hypertrophied left ventricle and the normal right ventricle of three rats. Hyperpermeable fibers from normal left and right ventricles showed no significant difference, although the mean of the left-ventricle response is somewhat smaller than the right. Myosin isozyme studies were not done on these hearts.

#### *Absence of Phosphorylation*

To determine whether phosphorylation of myosin or any other contractile protein was involved in this regulatory mechanism, hyperpermeable cells were

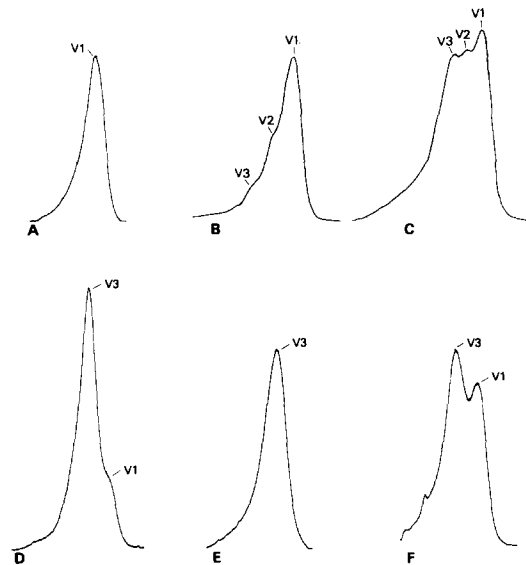


FIGURE 4. Scans of gels in which myosin from the right ventricle was electrophoresed. Direction of the electrophoresis is from right to left. There are five stages in the transition of primarily  $V_1$  myosin to primarily  $V_3$ , corresponding to 0, 2, 3, 4, and 5 wk after thyroidectomy (A-E). F shows the results of mixing the protein from a 5-wk post-thyroidectomy heart with that of a euthyroid animal of approximately the same age. This procedure was used in each electrophoresis to distinguish clearly between  $V_1$  and  $V_3$  in hearts where the myosin content was relatively homogeneous.

exposed to cAMP and theophylline in the presence of  $[\gamma^{32}\text{P}]\text{ATP}$ , and the proteins that had been phosphorylated were identified with autoradiographs of gels after SDS polyacrylamide gel electrophoresis. Four major sites of  $^{32}\text{P}$  concentration could be seen that corresponded to proteins with molecular weights of 150,000, 60,000, 41,000, and 28,000 (Fig. 8). In addition, there was  $^{32}\text{P}$  at the front of the gel, probably caused by the proteolytic fragments and phospholipids. These results are very similar to those found with isolated perfused hearts and isolated cardiac myofibrils (England, 1976; Jeacocke and England, 1980; Onorato and Rudolph, 1981; McCullough and Walsh, 1979).



## DISCUSSION

The change in the contractile protein that alters contractility is not yet known, but it seems to involve selection of a specific isozyme of myosin for force generation. In two different conditions where the relative amounts of myosin

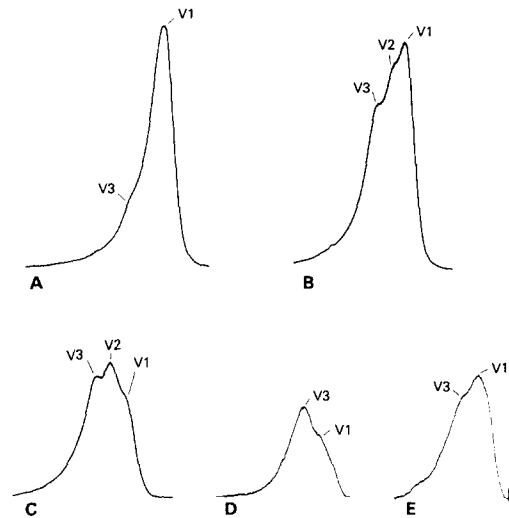


FIGURE 6. Scans of gels in which myosin from the right ventricle from rats of different ages were electrophoresed in pyrophosphate medium. The ages of the animals were 4, 23, 25, and 82 wk (A-D). E shows the scan of right-ventricular tissue from the same heart as D (>80 wk old) to which some myosin from a 5-wk-old, euthyroid rat heart has been added. Electrophoresis runs from right to left.

TABLE I  
EFFECT OF AGE ON REGULATION OF CONTRACTILITY

Age	Percent change in contractility cAMP, theophylline, and detergent	
	Detergent	Detergent
<i>wk</i>	%	%
4-10 (young)	+167±7 (21)	+8±9 (20)
25 (mature)	+ 80±12 (5)	+60±20 (5)
80 (elderly)	-32±6 (3)	+12±2 (4)

Myosin from four hearts from each age group was characterized by gel electrophoresis, and the relation between the change in contractility in response to cAMP, theophylline, and detergent and the relative amount of V<sub>1</sub> is shown in Fig. 7. Values given ± 1 SEM. The numbers in brackets indicate the number of hearts.

isozyme change—that is, hypothyroidism and aging—a close relationship exists between the relative amount of V<sub>1</sub> and the size of the increment in contractility that is produced by cAMP. Changes in the concentration of  $\beta$  receptors are unlikely to be the basis of the correlation since cAMP acts beyond the membrane in modifying contractility of the proteins. It is inter-

esting to note in this context that  $\beta$ -adrenergic agonists cause a much larger increase in myocardial contractility in euthyroid than hypothyroid animals (Hashimoto and Nakashimi, 1978; Nakashima et al., 1971), which corresponds to the larger concentration of  $V_1$  than  $V_3$  in the hearts of young, euthyroid rats.  $\beta$ -Adrenergic stimulation may selectively convert the fast isozyme of

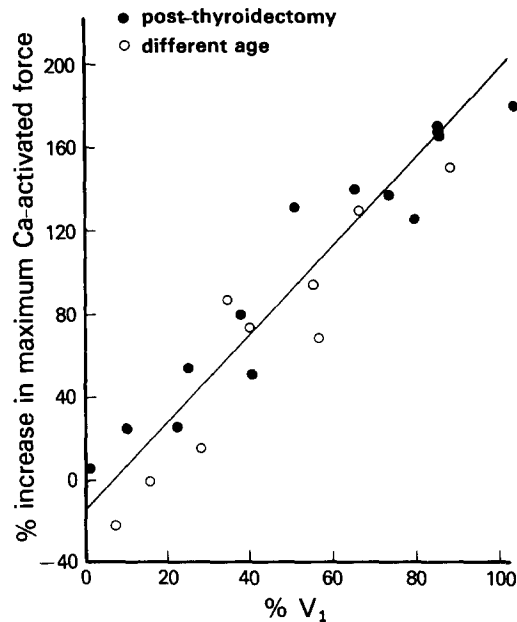


FIGURE 7. The relation between the relative amount of  $V_1$  and the relative increase in contractility (maximum Ca-activated force) from treatment with the combination of cAMP or epinephrine or GTP, theophylline, and detergent. Each point represents the average change in contractility of four trabeculae from a single right ventricle and the percentage of the total myosin that is in the  $V_1$  isomeric form as determined from electrophoresis in pyrophosphate medium of two right-ventricular strips. Two different factors, age and thyroid function, were responsible for the variability in  $V_1$  content, but only one factor was responsible in any given heart. Young animals were rendered hypothyroid by surgical thyroidectomy with or without administration of propylthiouracil to suppress function of any aberrant thyroid tissue. Mature and elderly animals were euthyroid, but varied in age from 23 to >80 wk. Standard error brackets for each point are not included because of the large number of points. The largest standard errors, however, never exceeded  $\pm 15\%$  of the mean and most did not exceed  $\pm 10\%$  of the mean.

myosin,  $V_1$ , from a Ca-nonresponsive or a low contractile state to a Ca-responsive or high contractile state. In essence, this would constitute a form of thick filament regulation of contraction in addition to calcium regulation of troponin on the thin filament. In the presence of strong stimulation of the  $\beta$ -adrenergic system, force generators would be primarily fast and more numer-

ous. Double regulation has already been described for many invertebrates (Lehman and Szent-Gyorgyi, 1975).

Cyclic AMP regulation of myosin isozymes is consistent with several properties of the catecholamine-stimulated heart. In addition to increasing the maximum developed force, catecholamine stimulation of the heart increases the rate of rise of tension and the amount of energy required to develop and maintain a given amount of tension (Alpert and Mulieri, 1982). (The increased

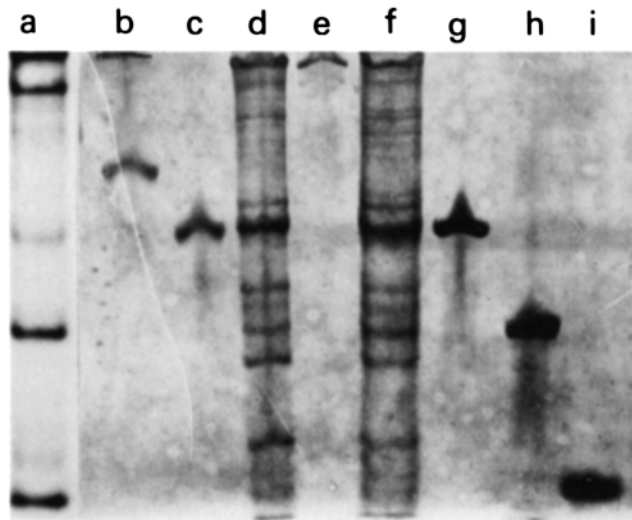


FIGURE 8. (A) Polyacrylamide slab gel electrophoresis of homogenate of trabeculae that had been exposed to theophylline and cAMP in the presence of [ $\gamma$ - $^{32}$ P]ATP in a relaxing solution. Lanes *d* and *f* are duplicates of the homogenate; lane *a* is an autoradiograph of lane *d*. The four very dark bands are 150,000 and 28,000 daltons, top and front. Lighter bands are present between 150,000 and 28,000, especially at 41,000. Markers in lanes *b*, *c*, *e*, *g*, *h*, and *i* are, respectively, serum albumin (66,000), ovalbumin (45,000), skeletal myosin and skeletal actin (courtesy of Dr. Annemarie Weber), carbonic anhydrase (29,000), and myoglobin (17,200). Gel 10% polyacrylamide. (B) Electrophoresis on a 6% polyacrylamide gel to increase separation of bands. The left lane is the gel and the right lane is an autoradiograph of the gel. Note that the 41,000-mol wt band labeled with  $^{32}$ P is clearly different from actin.

rate of relaxation can be explained by a combination of the increased rate of Ca uptake by the sarcoplasmic reticulum and the increased rate of release of bound Ca from TNI as a result of catecholamine-induced phosphorylations of phospholamban [Katz, 1977] and TNI [Ray and England, 1976; Holroyde et al., 1979a; Mope et al., 1980]). Although the increased rate of rise of tension has generally been attributed to a greater influx of Ca during the action potential and a larger release of Ca from the sarcoplasmic reticulum, there is a good correlation between the speed of contraction of the heart and the relative amount of the fast  $V_1$  myosin isozyme in different animals (Carey et

al., 1979). When the rate of rise of tension is high, as in rats or mice, the major fraction of the myosin is  $V_1$ , whereas in large animals like the cow, the rate of rise of tension is slow and most of the myosin is  $V_3$ . This relation is similar to the original correlation between myosin ATPase and the speed of contraction described in a variety of skeletal muscles by Barany (1967). It is therefore an attractive notion that catecholamine-induced changes in the rate of rise of tension in a given heart may be at least in part determined by the ATPase activity in the Ca-activated isozyme of myosin. This shift to faster myosin during adrenergic stimulation could also explain the increase in the energy required to develop and maintain tension since it is known that the rate of heat production during isometric contraction with the faster myosin isozyme is greater than with slow myosin (Alpert and Mulieri, 1982). Faster myosin produces more rapid cycling of cross-bridges and a greater rate of hydrolysis of ATP during the isometric phases of contraction. This control may give the organism the capacity to select the efficiency with which the heart contracts both acutely and chronically (Mercadier et al., 1981).

This hypothesis for the effect of catecholamines on cardiac contractile proteins must be incomplete because it only accounts for changes in rate and force of contraction in hearts that contain primarily fast myosin. It does not explain the changes in contractility that occur with adrenergic stimulation of hearts in animals like guinea pig, in which most of the myosin is slow (Lompre et al., 1981). The experimental results, however, show an additional mechanism for regulation of slow myosin that is activated by detergent and inhibited by  $\beta$  agonists. Based on results from the literature, it is tempting to suggest that the regulation of slow myosin may be dependent on the  $\alpha$ -adrenergic system. A number of observations are consistent with the suggestion: (a) the changes in the kinetics of contraction that are associated with the increased contractility after  $\alpha$ -adrenergic stimulation are much smaller than after  $\beta$ -adrenergic stimulation (Brückner et al., 1978; Osnes et al., 1978); (b)  $\alpha$  stimulation produces a larger increase in force of contraction in hypothyroid rats than  $\beta$  stimulation and the reverse is true in the euthyroid rat heart (Nakashima et al., 1971; Osnes et al., 1978); (c) in physically trained cats, where the percentage of fast myosin is increased (Resink et al., 1981; Rupp, 1981), there is an enhanced response to catecholamines (Wyatt et al., 1978); (d) in guinea pigs, which have much more slow than fast myosin, the increase in peak force is greater with  $\alpha$ - than with  $\beta$ -adrenergic stimulation (Ledda et al., 1975); (e)  $\alpha$  blockers increase the contractile response to  $\beta$ -adrenergic agents (Wenzel and Su, 1966). On the other hand, most of the increase in contractility in the cat heart, which primarily contains slow myosin or  $V_3$ , is produced by  $\beta$ -adrenergic stimulation. Although  $\alpha$  and  $\beta$  regulation of slow and fast cardiac myosin remain an attractive and testable hypothesis for regulation of rat myosin, additional work is necessary to determine whether it can be applied in modified form in hearts in which  $V_3$  is the predominant isozyme.

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