Isozyme Specific Modification of Myosin ATPase by cAMP in Rat Heart

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The total ATPase activity of myosin and the values for the isozyme V1 have been measured in hearts from rats of different ages and with different levels of thyroid function. The contribution of V1 was calculated from the difference between total and V2 ATPase, neglecting the small contribution of V3. Hearts were quickly frozen after rapid removal from the animals in order to preserve the state of ATPase activity that existed in the intact animal, and ATPase activity was measured in thin sections of tissue by a microphotometric technique. In euthyroid hearts, although cAMP increases total myosin ATPase activity and the activity of V3, the cyclic nucleotide inhibits the ATPase activity of V1. In hearts from rats with developing hypothyroidism following thyroidectomy, the same occurs. After a sufficient period has elapsed after thyroidectomy for V3 to have practically disappeared, cAMP has no effect on ATPase activity, but the injection of thyroid hormone restores the effect. Total myosin ATPase activity is maintained relatively constant as the animal ages from 80 to 165 days and during the first 10–11 days following thyroidectomy even though the concentration of V3 is dropping. The explanation proposed for these observations is that myosin can exist in two different forms, only one of which can participate in the active generation of force. The transition between the two forms is regulated by a soluble factor that is itself controlled by the adrenergic system. The factor(s) involved in this regulatory mechanism is soluble and can be transferred between different thin sections cut from a frozen heart. (Circulation Research 1987;60:384–392)

The enzymatic properties of myosin in mammalian cardiac muscle do not remain constant throughout the lifetime of an organism. They change during the normal aging process, with different hemodynamic states and as a result of different levels of circulating thyroid hormone. This behavior has led to the hypothesis that different forms of cardiac ventricular myosin exist in the same animal and ultimately to the demonstration of isozymes of myosin in hearts of rats and several other small mammals.1,2 Modification of the genetic regulation can occur within hours and lead to changes in the isozyme pattern of myosin in an animal within days, particularly in response to changes in the concentration of thyroid hormone.1,3

There are, however, differences in both the properties of myosin and the hemodynamic performance of the heart that cannot easily be explained by changes in myosin composition of the heart. Either the time course of the change in function is too rapid, the change in the isozyme pattern is inappropriate for the associated change in function, or changes in the isozymic content of myosin have occurred without any alteration in function. For instance, Martin et al found that thyroid treatment increased myofibrillar ATPase activity in rabbit hearts by approximately 20% in 2 days, after which continued administration of thyroxine had no further effect on myofibrillar ATPase activity. Yet, during the 2-week period of stable myofibrillar ATPase activity, calcium-activated myosin ATPase doubled. If, as native gel electrophoresis indicated, the increase in calcium ATPase was the result of replacement of V3 by V1, an increase in myofibrillar ATPase would have been expected. Similarly, the administration of thyroid hormone to thyroidectomized rats caused an increase in the mean systolic force to a new stable level in 18 hours, but neither the change from V3 to V1 nor the increase in the maximum rate with which pressure rose during systole was complete for several days. Force and velocity appeared to change at different rates, but because these observations were made in the intact heart, other factors besides alteration of the contractile proteins could have been responsible. A relatively constant acromyosin ATPase activity has been observed in rats between 12 and 28 months old in spite of a steady replacement of V3 by V1.2 The maximum velocity of shortening and the myofibrillar ATPase activity of rat hearts decline by only a small amount between 2 and 10 months and between 3 and 30 months, respectively, even though V1 is being replaced by V3 over this period.

The properties of the contractile proteins in hyperpermeable cardiac muscle cells can be modified by activation of the β-adrenergic system.9,10 In these preparations, maximum calcium-activated force has been elevated by an average of about 150% in a period that was much too short for any significant changes in the expression of myosin genes. The ATPase activity of myosin in sections of frozen rat heart has also been shown to respond to β-adrenergic stimulation on a time scale of fractions of a minute.11 These results, along with other observations in the literature,12 indicate that a mechanism for the regulation of the functional properties of myosin besides changes in calcium sensitiv-
ity exists in mammalian hearts and may be important in the overall regulation of the function of the heart.

Additional studies, which are presented in this paper, have produced evidence of isozyme specificity for this regulatory mechanism as well as additional details about the nature of its function in the intact cells.

Materials and Methods

Hearts were quickly removed from rats after decapitation, the ventricular walls dissected free, and the tissue rapidly frozen according to procedures already described. Sections were cut with a Hacker cryostat, and the activity of calcium- and actin-activated myosin ATPase was measured by quantitative microphotometry. The principle of the method is the trapping of inorganic phosphate produced by myosin ATPase activity by calcium ions, and the quantitative replacement of the calcium phosphate by the more opaque cobalt sulfide. The optical density was measured at 1,800 points in each of 10 sections of a heart. Each value is a measure of ATPase activity, and because of the large number of measurements, standard errors for measurements to ATPase activity in a tissue section are less than 2%. The V3 isozyme of myosin was inhibited in the presence of V1 by exposure to alkaline pH in the absence of ATP according to a procedure already described. In at least 10 sections from each of 6 different rat left ventricles in which no V3 could be detected by native gel electrophoresis, myosin ATPase activity after preincubation at pH 10.5 for 10 minutes was 93 ± 2% of the value in serial sections from the same ventricles that had been preincubated at neutral pH. Native gel electrophoresis to measure the relative amounts of myosin isozymes was performed according to Hoh et al. Since ATPase activity was measured in 10 sections taken from different regions of the same chamber, it has been assumed that both isozyme and ATPase measurements are average values for the entire heart. The validity of the assumption has been substantiated by measurements of isozyme concentration in serial sections using a polyclonal antibody specific for V1 (Horowitz and Winegrad, manuscript submitted to Circulation Research). All rats were supplied by Charles River Breeding Company, Stoneridge, N.Y., which also performed the thyroidecomies. Triiodothyronine was administered to some thyroidectomized animals by subcutaneous injection of 5 µg/day.

Results

cAMP Increases V1 and Decreases V3 ATPase Activity

Addition of micromolar cAMP to the bathing solution increased the ATPase activity of myosin in sections from hearts that contain predominantly or exclusively V3. The specific responses of each of the two major isozymes of myosin, V1 and V3, to the inclusion of cAMP in the incubation solution were studied by using alkaline preincubation to inhibit the ATPase activity of V3 in sections serial to those incubated at neutral pH, in which total myosin ATPase activity was measured. Since the relative amounts of the myosin isozymes vary with age and thyroid function, hearts from rats of different ages and rats at different times after thyroidecomy were used to provide tissues containing different amounts of V3. The relative amount of the myosin isozymes was determined by gel electrophoresis.

In animals that had been killed at different times after thyroidecomy, the population of ventricles was divided into 3 groups according to whether the percentage of V1 was less than 20, 20-90, or 90-100% of the total myosin. Serial sections were preincubated at either neutral or alkaline pH, and the increase in myosin ATPase activity in the presence of cAMP was compared among the 3 groups for the 2 types of preincubation (Figure 1). The specific activity of V1 ATPase for each heart was calculated from the ATPase activity after alkaline preincubation and the percentage of myosin present as the V1 isofrom. In sections from 6 different ventricles in which the amount of V1 was 90% or more of the total myosin, the increase in myosin ATPase activity was the same for the 2 types of preincubation. The ratio of the increases with alkaline vs. neutral preincubation was 1.00 ± 0.06. In the 2nd (n = 8) and 3rd (n = 7) groups, in which the relative amount of V1 varied from 20–90% and 0–20% respectively, the specific activity of V1 after exposure to cAMP was the same as in the 1st group (4.8 ± 0.4, 4.4 ± 0.6, and 4.7 ± 0.5 µmol ATP split · mg·min−1). In the 2nd and 3rd groups, however, the change in total ATPase was less than the change due just to V1 ATPase. In the 2nd group, ATPase activity due to V1 increased by 1.36 ± 0.14 times the increase
in total ATPase, and in the 3rd group, the factor was 3.95 ± 0.95. The ATPase activity due to V₂ (neglecting V₃) must, therefore, have decreased as a result of the exposure to cAMP.

The apparent difference in the effect of cAMP on the ATPase activity of V₁ and V₃ was not a consequence of the hypothyroid state per se, as hearts from animals of different ages with a mixture of isozymes responded in a similar way (Figure 2). Alkaline preincubation reduced ATPase activity an average of 21%, in keeping with the amount of V₃ present. Exposure of sections to cAMP, regardless of the pH of the preincubation, increased ATPase activity to the same value, the absolute increase being greater for sections that had been preincubated in alkaline pH to inhibit V₃. The final ATPase activity after cAMP was the same even when V₁ had first been inhibited by alkaline pH. These data indicate, therefore, that cAMP increases the ATPase activity of V₁ and probably inhibits the ATPase activity of V₃ in hearts from euthyroid rats.

**Changes in ATPase Activity of Myosin Following Thyroidectomy**

Hearts from a group of rats that had been thyroidectomized at the age of 45 days were studied. This series of experiments, each with its own controls, was repeated twice with the same results. During the ensuing 40 days, as V₁ was replaced by V₃, the ATPase activity was measured after neutral or alkaline preincubation and with or without cAMP in the incubation solutions. A portion of each ventricle was used for native gel electrophoresis to measure the relative amount of the myosin isozymes.

Following thyroidectomy, the relative amount of V₁ fell rapidly (Figure 3). By the 3rd day, it had decreased about 30%, and by the 5th day, the decline was about 50%. In spite of this replacement of the myosin isozyme by the more active ATPase by the isozyme with the less active ATPase, myosin ATPase activity measured 7 days after thyroidectomy had not changed from the values measured just before thyroidectomy, and total ATPase activity was insensitive to alkaline preincubation. Only the ATPase of V₁ was active, and the amount that was active was unchanged by the modification of the isozyme pattern. cAMP increased the ATPase activity of myosin by about 50% throughout this 7-day period regardless of the pH of the preincubation solution. By day 11 when total V₁ had fallen by 80%, ATPase activity also began to fall, but based on its lack of alkaline lability, it remained entirely due to the V₁ isoform.

The change between the 11th and the 17th day was dramatic. Total ATPase activity was altered very little, but most of the ATPase activity became alkaline labile. cAMP produced only a small increase in the total ATPase activity, but its large, positive effect on V₁ ATPase activity indicated that a strong inhibition of V₃ had...
occurred. Over the next 23 days, the relative amount of \( V_3 \), measured by gel electrophoresis fell to near zero, and the total ATPase activity was alkaline labile.

Between the 11th and 17th day after thyroidectomy, when the hearts appeared to shift from a "\( V_1 \) dominated" to a "\( V_3 \) dominated" state, the ATPase activity of cells after neutral preincubation was uniform. In sections that had been preincubated in alkaline solution so that only the ATPase activity of \( V_1 \) was active, cells had different degrees of ATPase activity. The difference was not eliminated by cAMP (Figure 4). The nonuniformity after alkaline preincubation disappeared by the 17th day, and the uniformity remained for at least 40 days, the longest period recorded. Apparently, the transition from the primarily \( V_1 \) to the \( V_3 \) state does not occur with the same time course in all cells, but some cellular regulatory mechanism maintains a constant total myosin ATPase activity. This observation has been already noted.16

Fifty days after thyroidectomy, several animals were injected with triiodothyronine to reverse the hypothyroid state. Four days after the initiation of the injections, the relative amount of \( V_3 \) had risen to 20%, total ATPase had increased to the prethyroidectomy level, and once again the ATPase activity was almost entirely alkaline stable. The transition was essentially a mirror image, at an accelerated rate, of the changes with developing hypothyroidism, including a 1-day period during the transition to a "\( V_3 \) dominated" issue when the ATPase activity after alkaline preincubation was not uniform among the cells, even though uniform ATPase activity existed after neutral preincubation. Only a small increase in myosin ATPase activity from the addition of cAMP to the solutions was observed. This is probably due to the high level of circulating thyroid hormone from the injections and the hypersensitivity to adrenergic stimulation that exists under those conditions.26. The cell is in a mixed state, still hypothyroid regarding transcriptional regulation and possibly even hyperthyroid regarding certain post-translational regulation. Although cAMP inhibited the \( V_3 \) ATPase for 1–2 weeks after surgical removal of the thyroid gland, when the conversion of the myosin isozyme pattern was complete, the effect of cAMP on ATPase activity disappeared. Because the relative amounts of myosin isozymes change with age, the true control for the thyroidectomy series consists of hearts from euthyroid, age-related animals (Figure 5). These studies show that the ATPase activity rises between the ages of 45 and 80 days, and the increase produced by cAMP increases proportionately. The ATPase activity is due entirely to alkaline stable myosin. From 80–165 days of age, total ATPase activity remains constant, due to a combination of both alkaline labile and alkaline stable ATPase. The increment in ATPase activity produced by cAMP declines approximately in proportion to the size of the alkaline stable component. During the 2nd and 3rd months of life, myosin ATPase for a given isoform increases even without cAMP addition to the incubation solutions. This appears to be related to other developmental changes in the regulation of the contractile protein.23 (Horowits and Winegrad, submitted manuscript).

Effects on Actin-activated Myosin ATPase Activity

The relation of the response of calcium activated myosin ATPase to the response of actin activated myosin ATPase was studied by measuring the latter after neutral and alkaline preincubation and with and without cAMP during the period of developing hypothyroidism. The measurements were made at 5, 13, and 25 days after thyroidectomy because the response of cardiac myosin at these times exemplifies the 3 different stages observed with calcium activated ATPase: the period of maintained total and \( V_1 \) ATPase in spite of a large decline in the concentration of \( V_3 \); the transitional period of \( V_3 \) to \( V_1 \) dominated ATPase activity; and the period of only \( V_3 \) activity. The data are shown in Table 1. At 5 days after thyroidectomy, when the concentration of \( V_3 \) has declined substantially, ATPase activity is maintained and is entirely alkaline stable. Thirteen days postthyroidectomy, almost half of the ATPase activity is alkaline labile, but the ATPase activity during cAMP exposure is the same regardless of whether \( V_3 \) has been inhibited first by alkaline preincubation. At 25 days, all of the ATPase activity is alkaline labile and insensitive to cAMP. Actin activated ATPase responds, therefore, in the same way as calcium activated ATPase, as has been previously observed.18

Relation Between Effect of cAMP and Amount of \( V_1 \)

The results in the previous sections show that the increase in the ATPase activity of myosin produced by cAMP is not related to the total amount of myosin. On the other hand, there is a significant correlation between the size of the increase in myosin ATPase activity from cAMP and the amount of \( V_1 \) ATPase activity measured without cAMP in the solutions (Figure 6). The results, derived from studies of euthyroid animals of different ages and animals with developing hypothyroidism extrapolate to the origin of the graph. Values measured more than 3 weeks after thyroidectomy have not been included because data indicate that a change in the response to adrenergic stimulation occurs after this time.22 They also do not include values after the administration of \( T_3 \) because of the possibility of an abnormally high level of catecholamine stimulation at the time the animals were killed.

cAMP-Induced Changes in \( V_1 \) ATPase Due to Soluble Factor

The changes in ATPase activity of \( V_1 \) are due to a soluble factor that appears in the incubation solution. This conclusion was drawn from the following experiment: A small volume of incubation solution containing 1 \( \mu M \) cAMP was used with a large number of sections for the purpose of producing a relatively high concentration in the incubation solution of any substance released by the sections during their exposure to cAMP. The change in ATPase activity from the presence of cAMP was determined by comparison with
sections from the same region of the same heart exposed to an incubation solution containing no cAMP. The pH of the incubation solution was checked after its exposure to the sections, and any small change was corrected. The incubation solution was then divided into aliquots and diluted with standard incubation solution containing 1 μM cAMP. The original incubation solution, which had already been exposed to tissue sections, was diluted 0–90%. Two small sections from another frozen heart that contained only V₁ were incubated in the various dilutions of the original incubation solution (a different pair of serially cut sections for each dilution), and a pair of small sections was bathed in fresh incubation solution containing 1 μM cAMP.

The ATPase activities of the myosin bathed in the various incubation solutions were compared (Table 2). The results show that the small sections bathed in the original undiluted incubation solution had higher myosin ATPase activities than those bathed in fresh incubation solution containing the same concentration of cAMP (and theophylline as a phosphodiesterase inhibitor). The difference gradually diminished with dilution.

Even though the experiment was carried out with the concentration of ATP in the range where myosin ATPase activity is essentially insensitive to changes in the concentration of ATP, and the direction of the observed difference — higher ATPase activity in a solu-

### Table 1. Neural Preincubation and Alkaline Preincubation in Thyroidectomized Rats

<table>
<thead>
<tr>
<th>Days post thyroidectomy</th>
<th>Neutral preincubation</th>
<th>Alkaline preincubation</th>
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<tbody>
<tr>
<td></td>
<td>−cAMP</td>
<td>+ cAMP</td>
</tr>
<tr>
<td>0</td>
<td>0.49±0.06 0.88±0.11</td>
<td>0.48±0.05 0.95±0.15</td>
</tr>
<tr>
<td>5</td>
<td>0.50±0.07 0.85±0.12</td>
<td>0.48±0.06 0.91±0.14</td>
</tr>
<tr>
<td>13</td>
<td>0.47±0.05 0.52±0.06</td>
<td>0.27±0.04 0.49±0.07</td>
</tr>
<tr>
<td>25</td>
<td>0.39±0.05 0.37±0.06</td>
<td>0.00     0.00</td>
</tr>
</tbody>
</table>

All values in μmol PO₄/mg myosin/min.
tion where the ATP concentration was slightly lower — was opposite to the effects of lower ATP concentration, the possibility of an influence of ATP concentration where the ATP concentration was slightly lower to be due to an increased concentration of inorganic phosphate from ATP hydrolysis because an increased phosphate concentration has been shown to decrease myosin ATPase activity and the rate of cross bridge turnover. 

Samples of the original incubation solution before and after exposure to the relatively large volume of sections and after dilution with fresh incubation solution have been electrophoresed on polyacrylamide gels after treatment with SDS. The gels showed that no myosin had been extracted from the tissue sections, and therefore a transfer of myosin could not have been responsible for the change in measured ATPase activity.

**Discussion**

β-Adrenergic stimulation of rat ventricular muscle cells increases the maximum calcium-activated force and the ATPase activity of both calcium- and actin-activated myosin. Some of the observations made with hyperpermeable fibers have suggested that the β-adrenergic stimulation is specifically stimulatory for the V1 isozyme of myosin and possibly inhibitory for the V3 isozyme. Measurements of the response of calcium-activated myosin ATPase to cAMP with hearts containing both isozymes indicate that in euthyroid hearts the stimulatory effect is specific for V1 and inhibitory for V3 at the same time. Since work has shown that cAMP produces similar changes in actin-activated ATPase to those observed for calcium-activated ATPase, it is likely that the changes in ATPase activity and maximum calcium activated force are reflections of the same regulatory event. cAMP may be able to inhibit V1 ATPase activity almost completely at the same time that it increases V1 ATPase activity. In hearts in which myosin is almost entirely V1, however, cAMP does not alter ATPase activity.

A mechanism in which adrenergic stimulation increases V1 and decreases V3 activity can have a major effect on the kinetics of contraction. The velocity of shortening appears to be a continuous function of the relative amounts of the isozymes when the amounts have been changed by modification of hemodynamic or hormone function. Alteration of the relative amounts of existing V1 and V3 that participate in a contraction should also change the velocity of shortening. The kinetics of the contraction in a cell containing both isozymes could be changed in seconds without any modification in the myosin composition. Whereas the change in the kinetics of contraction in response to alteration of the isozyme composition of the cell by age, thyroid function, or hemodynamics is dependent on gene expression and occurs over days, the same result can be achieved in seconds in a cell with a mixture of regulated isozymes of myosin. A mechanism of this type would endow a single cell with a multitude of force-velocity relations depending on adrenergic tone, and it would give maturing and adult rats, with their mixture of isozymes, a greater versatility of function. An increased rate of contraction from preferential activation of the fast isozyme of myosin would be an additional response of cardiac cells to the already identified cluster of β-adrenergically regulated changes in calcium conductance of the membrane, calcium release by troponin, and calcium accumulation by the sarcoplasm reticulum.

The capacity of the cardiac cell to regulate the function of myosin is more complex, however, than a com-
bination of transcriptional control of the relative amounts of the isozymic forms$^{1,2,17,28}$ and modulation in response to β-adrenergic stimulation. This becomes apparent when one examines the changes in ATPase activity and relative concentrations of myosin isozymes during the maturation of the animal in its first months of life and even more strikingly in the period following thyroidectomy, when hypothyroidism is developing. There is a dissociation between myosin isozyme composition and measured ATPase activity.

Based on the amount by which the concentration of $V_3$ can decline before ATPase activity begins to drop, the simplest interpretation of these observations is that the heart in the euthyroid animal is operating with only about 25% of its myosin responsive to calcium-activation. Following thyroidectomy, the relative amount of $V_3$ that is calcium-responsive gradually increases as the total amount of $V_3$ decreases. Regulatory systems for both $V_1$ and $V_2$ that are sensitive to thyroid function are likely, and the control of their genetic expression seems to be closely associated with the expression of the gene for $V_3$.

Although β-adrenergic stimulation inhibits $V_3$ in the heart from euthyroid rats and in hearts from rats with developing hypothyroidism, it does not inhibit $V_3$ to any large extent in the well-established hypothyroid state, in which cardiac myosin is almost exclusively $V_2$. Inhibition of $V_3$ at that time would be disastrous for the animal. The same loss of $V_3$ inhibition from β-adrenergic stimulation during established hypothyroidism and its reversal with the administration of thyroid hormone have been detected with measurements of maximum calcium-activated force.$^{22}$

In hearts from maturing rats, in which the relative amount of $V_3$ is increasing, the tendency to maintain a relatively constant total myosin ATPase in spite of a changing mixture of the myosin isozymes has also been observed. Since the concentration of myosin remains approximately constant,$^{29,30}$ changes in the concentration of myosin cannot be responsible. Unlike the heart during developing hypothyroidism, the relatively stable total myosin ATPase of the aging heart is due to activity of both $V_1$ and $V_3$.

The tendency of the cardiac cells to maintain a relatively stable total ATPase activity of myosin is interesting. Since $V_1$ has a lower ATPase activity than $V_3$, substitution of $V_3$ activity for $V_1$ activity might mean that a larger number of myosin molecules would be active, and therefore a larger number of force generators would be calcium responsive. This last conclusion is derived from the observations that actin-activated myosin ATPase changes in parallel with calcium-activated myosin ATPase.$^1$ Since ATPase activity is a kinetic value, the stability of myosin ATPase, in spite of changes in the isozymic composition of the cardiac cells, suggests that the regulation of the kinetics of the contraction is important, possibly even more than regulation of force. This is not surprising in an animal with a high heart rate. Since an increase in cardiac output may be due more to an increase in heart rate than in stroke volume, modulation of the speed of the contraction may be even more important in regulating cardiac output than variation in the strength of contraction. There are some data derived from studies of intact hearts that support the conclusion that myosin regulation is important in the function of the intact heart. In addition to work already mentioned, there are the observations made by Lakatta et al$^{31}$ that the response of isolated perfused rat hearts to exogenously administered β-adrenergic agonists changes with age in a way that can be predicted by the existence of this mechanism. Both the increase in force and in the rate of development of force decrease with age (in the hearts from senescent rats, there is no increase in force) in spite of an unchanged contractile response to alterations in extracellular calcium concentration. Since these changes also occur in response to deoxyribonucleic acid (DNA) synthesis, the cause is unlikely to be changes in membrane receptors or adenylate cyclase.$^{32}$
chemical detection of specific isozymes in rat ventricular cells. 

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