Adrenergic Regulation of Myosin Adenosine Triphosphatase Activity

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SUMMARY. The amount of inorganic phosphate liberated by the adenosine triphosphatase activity of myosin in a thin section of cardiac tissue can be measured quantitatively by precipitation with calcium in an alkaline medium under a defined set of conditions. Specificity of the procedure for myosin adenosine triphosphatase has been confirmed by the response to inhibitors and to different degrees of contractile filament overlap. Precise quantitation of adenosine triphosphatase activity has been demonstrated by (1) constant rate over time, (2) linearity with amount of enzyme, (3) correct values for the $K_m$ of adenosine triphosphate, and (4) a similar value for $V_{max}$ to those determined by more traditional procedures. Stimulation of the $\beta$-adrenergic system by the release of catecholamines following injection of the animal with 6-hydroxydopamine causes a rise and then a fall of both calcium- and actin-activated adenosine triphosphatase in parallel with the changes in blood levels of the transmitter. Tyramine injection of rats produces a dose related increase in myosin adenosine triphosphatase. Perfusion of isolated hearts with isoproterenol increases myosin adenosine triphosphatase in dose-related manner. Addition of cyclic adenosine monophosphate and phosphodiesterase inhibitor to the solution bathing frozen, dried sections of heart increases both calcium- and actin-activated adenosine triphosphatase activity by almost 150%. The data show that the $\beta$-adrenergic system, through cyclic adenosine monophosphate, regulates the enzymatic activity of myosin, independent of the concentration of calcium. The possible role of this regulatory mechanism in the physiological modulation of cardiac contractility is discussed. (Circ Res 58: 83-95, 1986)

THE variable force and velocity with which the heart can contract depend on modulation of the contractility of individual heart cells. Either a change in the concentration of calcium ions triggering the contraction or modification of the response of the contractile proteins to a given concentration of calcium may be responsible (Winegrad, 1971; Fabiato and Fabiato, 1975a; McClellan and Winegrad, 1978). The concentration of activating calcium can be modified by adrenergically conditioned changes in calcium movements across the cell membrane and within the cell through alteration of the shape of the action potential, the number of calcium channels in the sarcolemma (Reuter and Scholz, 1977), or the function of the calcium pump in the sarcoplasmic reticulum (Tada et al., 1974; Fabiato and Fabiato, 1975b). The myofibrillar proteins may also be altered in a way that changes the characteristics of the contraction. By increasing the rate of release of bound calcium by troponin, cyclic adenosine monophosphate (cAMP)-regulated phosphorylation of the inhibitory subunit of troponin (TNI) increases the concentration of calcium necessary to activate contraction, and as a result, alters the time course of the contraction (Ray and England, 1976; Mope et al., 1978; Holroyde et al., 1979; Robertson et al., 1982). The maximum calcium-activated force produced by myofibrils is regulated, as well. In hyperpermeable cardiac cells, a several-fold increase in maximum force can be produced by activation of the $\beta$-adrenergic system through a mechanism independent of electromechanical coupling (McClellan and Winegrad, 1980; Winegrad et al., 1983). This change in the contractile performance of the myofibrils is sensitive to cAMP, although it does not involve phosphorylation of TNI (Weisberg et al., 1983).

The regulation of the enzymatic properties of myosin by cAMP has now been examined in cardiac cells in which the sarcolemma has not been modified to enhance its permeability. The adenosine triphosphatase (ATPase) activity of calcium- and actin-activated adenosine triphosphatase activity by almost 150%. The data show that the $\beta$-adrenergic system, through cyclic adenosine monophosphate, regulates the enzymatic activity of myosin, independent of the concentration of calcium. The possible role of this regulatory mechanism in the physiological modulation of cardiac contractility is discussed. (Circ Res 58: 83-95, 1986)

Methods

General Procedure

Young male rats weighing 150-250 g obtained from Charles River Breeding Laboratories were used in all studies. Hearts were studied either immediately after removal
from the rat or after a period of perfusion of the isolated organ. Some rats were injected with either 6-hydroxypindolol or tyramine into the jugular vein during a brief period of ether anesthesia. When possible, the animals were allowed to recover from the anesthetic before being killed by decapitation. After sacrifice, hearts were quickly removed, and the free walls of the right and left ventricles were isolated. The dissected tissue was quickly frozen in isopentane that had been cooled with liquid nitrogen. The entire process of isolation and freezing of the cardiac tissue can be accomplished in no more than 30 seconds. Animals that did not receive drugs were not anesthetized before their decapitation.

For studies of the effect of drugs in the isolated perfused heart, the heart was removed and the aorta was cannulated to permit continuous perfusion with oxygenated Krebs. A balloon was placed in the left ventricle and connected to a Statham pressure transducer for continuous recording of diastolic and systolic pressure. Fast paper speed allowed the shape of the systolic pressure wave to be observed, but rigorous measurement of dP/dt was not made. The perfusion fluid (140 mM NaCl, 4 mM KCl, 2.5 mM MgCl₂, 10 mM dextrose, 15 mM NaHCO₃, 2 mM NaH₂PO₄, 1 U/ml insulin, pH 7.3) was kept at 25°C because it was thought that the properties of the heart would remain more stable at this temperature than 37°C. Flow rate was 12 ml/min: contraction was initiated by the tissue’s own pacemaker or by stimulation with electrodes at 120 contractions/min. (The results of the ATPase measurements were the same in each case.) Diastolic pressure was set at 2–5 cm of water. The heart was allowed to recover from the dissection until its systolic force had stabilized. At this point, isoproterenol in concentrations from 10⁻⁹ to 10⁻¹⁰ M was added to the medium perfusing some of the hearts. When a new steady state of systolic force had been reached, the free walls of the right and left ventricle were removed quickly and frozen in isopentane.

The effects of β-adrenergic activity on myosin ATPase were also studied by measuring the changes induced by cAMP added to the solutions bathing sections of the heart, in contrast to producing β-adrenergic stimulation in the heart before sectioning. Sections cut from the frozen cardiac tissue with a cryostat (Hacker Instrument Co.) that was kept between −18°C and −22°C were picked up on coverslips. Alternate serial sections were used for control and experimental studies of calcium- and actin-activated myosin ATPase.

Microphotometrical Measurement of ATPase

A recently described modification (Weisberg et al., 1982) of the Padykula-Herman method (1955) was employed for measuring calcium-activated myosin ATPase after the conditions under which it could be used quantitatively had been ascertained. For measurements of calcium-activated ATPase activity, cryostatic sections were exposed in succession to solutions of the following composition: (1) 28 mM calcium chloride, 6 mM sodium barbital, 5 mM sodium azide, and 0.2 mM ouabain at pH 7.0 or 10.5 for 10 minutes at 25°C (preincubation solution); and (2) 18 mM calcium chloride, 20 mM sodium barbital, 5 mM ATP, 5 mM sodium azide, and 0.2 mM ouabain at pH 10.5 for 7–10 minutes at 37°C (incubation solution). In the absence of adenosine triphosphate (ATP), alkaline solution inhibits the ATPase activity of V₃ myosin; therefore only the activity of V₁ myosin is measured when the preincubation solution has a pH of 10.5. For actin-activated myosin ATPase, a modification of the Mabuchi-Sreter procedure (1980) was employed. Tissue sections were exposed successively to: (1) 28 mM calcium chloride, 6 mM sodium barbital, 5 mM sodium azide, 0.2 mM ouabain at pH 7.0 for 15 minutes at 25°C (preincubation solution); and (2) 10 mM magnesium chloride, 10 mM calcium chloride, 20 mM sodium barbital, 1.5 mM ATP, 5 mM sodium azide, 0.2 mM ouabain at pH 9.0 for 10 minutes at 37°C (incubation solution). The rest of the procedure is described in Weisberg et al. (1982).

The amount of cobalt sulfide, which is the final reaction product after substitution for calcium phosphate formed during the splitting of ATP, was measured with a photometer attached to a Zeiss Photomicroscope equipped with either 40× (n.a. 0.6) or 63× (n.a. 0.9) objectives. A scanning stage controlled by the Zeiss Zonax computer was used. The entire section was scanned, and the optical density and absorbance at spots either 5 or 3 μm in diameter were measured every 10 μm in a matrix of 60 × 30 points to give a total of 1800 measurements for each section. Because of the irregular shape of tissue sections, some areas outside of the section were occasionally measured, but they were eliminated during the data analysis, and the remaining points were statistically analyzed with a program furnished by Zeiss. The program averages the values for 1800 measurements made on each section (minus any deleted), and it calculates standard error, standard deviation, and variance. From these values, significance was determined by Student’s t-test.

Photomicrographs were taken using both the automatic exposure of the Photomicroscope and, when the same exposure times were desired, the manual control. Kodak Panatomic X film was used and developed with Microdol X.

Because of the striated structure of the muscle and the presence of extracellular space, it was important to demonstrate that significant errors were not made by measuring different amounts of extracellular space in different tissue sections. This was done by comparing the results of scanning the same general area of tissue sections six times at different sets of points. Five different experiments were evaluated this way. The standard error of the mean of such measurements was less than 2%. A second check involved comparing measurements with scans of the same region of the same section with five different spot sizes from 1.7–10 μm. Again, the standard error of the mean of measurements with different spot sizes was less than 2%. A final check was made by comparing the relative difference in ATPase activity of frozen sections from the different hearts by the automated scanning procedure and a manual procedure in which the recording spot was placed over 30 different myofibrils in each section. The two procedures gave the same results in six different experiments.

Studies were conducted to determine whether soluble calcium phosphate that is uniformly present throughout the cell is distributed according to any pattern when it forms a precipitate. This control was necessary to determine whether the location of staining was due only to the location of ATPase sites or, in addition, to the location of sites that preferentially trap precipitated calcium phosphate. Frozen tissue sections free of precipitate were incubated for 5 minutes in a solution of 2.0 mM calcium phosphate at pH 7.0. Precipitation was induced by raising the pH to 12.5 with sodium hydroxide. After 15 minutes at room temperature, the solution was changed to one

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containing 20 mM sodium barbital buffered at pH 12.5
and then to a solution of 50 mM calcium chloride. These
steps, which removed the previous solution and any un-
trapped precipitate, were analogous to the first wash step
after the incubation solution in the standard ATPase as-
says. After 15 minutes in this solution, the sections were
treated in the same way as sections after an incubation
with ATP. In one section of each serial pair, the formation
of calcium phosphate precipitate had been inhibited by
carrying out the reaction at neutral pH to avoid the
influence of the reaction product on the phase image,
which was used to identify the myofibrillar striations. The
correlation between the localization of reaction product
and the sarcomere pattern in serial sections was deter-
dined.

To calibrate the absorbance signal, different concentra-
tions of calcium phosphate from 1.0–2.5 mM were dis-
solved in water solutions containing 1–10% gelatin, ap-
proximately the concentration of protein in the cell. Care-
fully measured volumes from 100–500 µl were placed into
rectangularly shaped wells 1 cm² in area in tissue culture
chamber slides (Labtek, Miles Scientific Div. Miles Lab.
Inc.). The pH of the solutions was made alkaline to pre-
cipitate calcium phosphate, and then the slides were dried
slowly. The absorbance was measured by the same micro-
photometric procedure used in the ATPase assays. The
amount of calcium phosphate per unit cross-sectional area
was calculated from the measured volume of fluid, the
known concentration of calcium phosphate added to the
well, and the dimensions of the well. The concentration
of gelatin between 1% and 10% does not influence the
absorbance measurement, but in the absence of gelatin,
calcium phosphate forms large granules as the water
evaporates and produces an uneven distribution of precip-
itate. Inclusion of gelatin prevents the formation of the
large granules and the uneven distribution.

Separation of Myosin Isozymes by Native Gel
Electrophoresis

The relative amounts of the isozymes of myosin in each
heart were determined by native gel electrophoresis (Hoh
et al., 1978). Since the ATPase activities of the myosin
isozymes are different, it was important in making com-
parisons between animals to be sure that differences in
ATPase activity were not due to differences in relative
amounts of isozymes. Myosin was isolated from the heart
for the electrophoresis by a standard method of extraction
in 0.6 M KCl (Adelstein et al., 1972). In some cases, tissue
from the heart was minced and then gently homogenized
with Potter-type glass homogenizer in a solution contain-
ing 10 mM sodium pyrophosphate, 0.6 M potassium chlo-
ride, 0.015 M Tris, 0.01 M mercaptoethanol, and 2 mM
magnesium chloride at pH 7.5. The protein concentration
of the solution loaded on the gel was measured by the
Lowry method (Lowry et al., 1951). The relative amounts
of the isozymes of myosin in the gel were measured by
scanning the gel after the protein had been stained with
Coomassie Blue (Weisberg et al., 1982).

Results

The presentation of results is divided into two sec-
tions: (1) the microphotometric method, and (2) the
effect of β-adrenergic stimulation on myosin
ATPase activity. The first section defines the con-
itions under which the ATPase activity of myosin
can be measured specifically and quantitatively with
subcellular localization by precipitating the inor-
ganic phosphate with calcium. The second section
describes how the microphotometric method has
been used to detect changes in both calcium- and
actin-activated ATPase produced by β-adrenergic
stimulation of intact hearts or cAMP stimulation of
sections cut from quickly frozen hearts.

The Microphotometric Method

Specificity for Calcium- and Actin-Activated Myosin
ATPase

The specificity of the method for measuring my-
osin ATPase activity was based on the location
within the myofibril of the reaction product, the
results with inhibitors of the other major ATPases,
and the influence of the degree of overlap of myosin
and actin filaments on the amount of reaction prod-
uct deposited.

Under the proper conditions, activation of myosin
ATPase in a frozen section by either calcium or actin
causes the deposition of the opaque product of the
enzymatic reaction only in the A-bands of the myo-
fibrils (Fig. 1). The location was determined by com-
parison with the striation pattern in the serial section
visualized with phase contrast optics, since bright
field illumination does not reveal the striations.
There is no deposition of reaction product in the
absence of ATP or at neutral pH.

Localization of calcium phosphate in the A-bands
depends upon the site of ATPase activity, and not
on any special trapping properties of the region of
the sarcomere. This was demonstrated by determin-
ing the distribution of staining in sections that had
been exposed first to a neutral solution of calcium
phosphate to allow penetration into the section, and then to an alkaline pH of 12.5 to precipitate the calcium phosphate. There was no pattern of staining within the cells in these sections, and no correlation existed between distribution and any cellular structure.

The inclusion of ouabain and sodium azide in the preincubation and incubation solutions to inhibit, respectively, sarcolemmal and mitochondrial ATPase did not change the location or the density of reaction product when a comparison was made in serial sections. The presence of mM calcium blocked the ATPase of the sarcoplasmic reticulum. Ouabain and azide were nevertheless routinely included in the myosin ATPase assays. Neither location nor density of the reaction product was altered by pretreatment of the cardiac tissue with 1% Triton X-100, a non-ionic detergent that removes cell membranes.

Actin activation of myosin does not occur in the absence of magnesium, but under these conditions, calcium activates myosin ATPase. Magnesium ions inhibit calcium-activated myosin ATPase and permit actin activation. Therefore, the concentration of magnesium ions can be used to distinguish calcium-activated from actin-activated ATPase activity (Luchetti et al., 1964; Mueller et al. 1964; Katz et al., 1966). After calcium activation of myosin, there was a high degree of uniformity of sarcomere lengths (Fig. 1). Considerable non-uniformity existed in the same cells in a serial section after actin activation, and the non-uniformity could be substantially reduced by exposing sections to a relaxing solution. This indicates that the non-uniformity in the presence of magnesium was produced by activation of the contractile system with interaction between actin and myosin and filament movement.

When myosin ATPase was activated by calcium, the width and the extent of the staining in the A-band were different at longer sarcomere lengths that diminished the amount of overlap of thick and thin filaments than at shorter sarcomere lengths where overlap of cross-bridges was either complete or almost complete (Fig. 2). Insensitivity to the extent of overlap is good evidence that an interaction between the thick and thin filaments was not required for the hydrolysis of ATP in the magnesium-free solution.

The intensity of staining from actin activation was progressively lighter in increasingly longer sarcomeres (Fig. 2), and in sarcomeres 3.2 µm long, the intensity was very light. Unfortunately, sarcomeres longer than 3.6 µm, in which thick and thin filaments did not overlap, were not seen, and could not be produced by stretching without accompanying damage to the tissue. The dependence of the intensity of staining on the amount of overlap of thick and thin filaments indicates that interaction between the two different sets of filaments had been involved in the enzymatic reaction. Presumably the interaction was between actin and myosin. This uncontrollable sarcomere length and non-uniform filament overlap in actin-activated myosin reactions increased the variability in the measured values of ATPase activity, but if a large enough number of small areas is measured, the error becomes tolerable, and meaningful comparison can be made. Whereas reproducibility for calcium-activated ATPase is ±2%, for actin-activated ATPase, it is approximately twice that value. In the same cells in which calcium activation caused reaction product to be uniformly deposited throughout the entire A-band, actin activation in the serial section produced precipitate in the central 50–65% of the A-band.

Quantitation

In order to use microphotometric measurement of cobalt sulfide as a quantitative determination of the ATPase activity of myosin in the tissue section, it was necessary to examine several properties of the technique. Specifically, the following questions were asked: (1) does the density of reaction product increase linearly with time? (2) how reproducible are measurements made on different days? (3) what is the relation between the concentration of ATP and the measured reaction rate? (4) what is the relation between the amount of enzyme and the rate with which reaction product is formed? (5) what is the effect of the conversion of calcium phosphate to cobalt sulfide on the quantitation? (6) can the procedure be calibrated so that absolute values for myosin ATPase activity can be determined?

Frozen sections cut from three different rat hearts were incubated with the reaction medium for periods from 10 seconds to 10 minutes (Fig. 3). Over this period, the rate of accumulation of phosphate...
during calcium or actin activation of myosin was constant. The ATPase activity of freshly cut sections from another group of frozen hearts was measured on three different occasions separated by 12 and 31 days. The values for absorbance, 0.203 ± 0.009, 0.197 ± 0.008, and 0.192 ± 0.004, indicate a very high degree of reproducibility. The relation between the concentration of ATP and the rate of formation of inorganic phosphate from its hydrolysis by myosin ATPase was examined by exposing tissue sections from young rat hearts containing almost entirely V1 to concentrations of ATP from 0.1 to 5 mM. Saturation, typical of enzymatic reactions, was observed with an apparent $K_m$ of approximately $6 \times 10^{-4}$ M, a value close to those measured by more traditional methods (Fig. 4) (Katz, 1970; Carey et al., 1979).

The influence of the amount of enzyme on the rate of production of inorganic phosphate was determined by varying the thickness of the tissue sections, since it is not possible to vary the concentration of myosin in the tissue section. Frozen sections from 2–10 μm were cut and incubated in the reaction solution for either 5 or 10 minutes (Fig. 5). The reason for using two different reaction times was to detect the possible effects of either the thickness of the section itself or the distribution of the same amount of reaction product within different thicknesses on absorbance measurements. There was a linear relation between section thickness and absorbance. The slope of the relation for 10-minute incubations is twice that for 5-minute incubations, indicating that the absorbance properties of the tissue and the reaction product distributed through the
tissue are unchanged by varying the thickness of sections.

In the microphotometric procedure, the calcium phosphate formed from combination of calcium ions with the inorganic phosphate liberated by the hydrolysis of ATP is replaced by the more optically opaque cobalt sulfide to produce a larger signal. The effect of this step on the quantitation of ATPase activity was examined. Tissue sections were incubated in reaction medium for different times up to 10 minutes. For half of the sections, the procedure was terminated before the replacement of calcium phosphate by cobalt sulfide, and in other sections serial to these, replacement by cobalt sulfide was effected. Absorbance increased linearly with time of incubation in both cases (Fig. 6). Since the only difference was a 2.5-fold increase when calcium phosphate was replaced by cobalt sulfide, the replacement of calcium phosphate acted as a linear amplifier with a gain of 2.5.

Successful calibration of the microphotometric procedure was achieved so that absorbance units can be converted into moles of calcium phosphate and then to enzymatic activity in terms of ATP split per milligram protein per unit time. The relation between the amount of calcium phosphate and the absorbance that it produced is shown in Figure 7. From this relation and the absorbance of a tissue section, moles of ATP split per milligram protein per unit time can be calculated by using the amplification factor of 2.5 from the replacement by cobalt sulfide, the dimensions of the tissue section, and the value of 3.5% of wet weight for the concentration of myosin (Katz, 1970).

Response to β-Adrenergic Stimulation

Injection of 6-Hydroxydopamine

Young rats were injected intravenously with 100 mg 6-hydroxydopamine/kg body weight, and their hearts were quickly frozen after the animals were killed 1, 3, 5, or 18 hours later. The times between injection and sacrifice were chosen to coincide with the early release of stored catecholamines that produce strong adrenergic stimulation of the cardiac cells and the period when catecholamine stimulation has been markedly diminished by the depletion of the transmitter (DeChamplain and Nadeau, 1971; Tabsh et al., 1982). (Serum catecholamine levels were not measured because the time course has been well documented in the literature.) Prior to depletion, some tonic sympathetic stimulation of the hearts of rats exists, however, even in resting animals. Calcium- and actin-activated ATPase activities were measured in serial sections of the hearts from injected and control animals. Photomicrographs were taken of sections of the right and left ventricles after staining for ATPase activity, and montages of
entire sections through the right ventricular wall were prepared for both calcium- and actin-activated myosin ATPase activity (Fig. 8 shows the results of calcium activation). The concentration of reaction product from both types of myosin ATPase activity was considerably greater 1 hour after injection than in the control, indicating that a large increase in myosin ATPase activity had occurred during the period of heavy catecholamine stimulation. Three hours after injection of 6-hydroxydopamine, the staining was even heavier. The degree to which cells had been stained 1 and 3 hours after injection was relatively uniform, except for the heavier staining of a small number of cells along the endocardial surface. By 5 hours after injection, the concentration of reaction product had diminished from the high levels observed 1 or 3 hours after injection, and marked differences in the degree of staining in neighboring cells had appeared (Fig. 9). Eighteen hours after injection, when over 95% of the catecholamine stores in the heart had been depleted, the intensity of staining was less than in the control hearts and the degree of heterogeneity was decreased. These results indicate that the activity of both calcium- and actin-activated myosin ATPases rises and falls with catecholamine release and depletion after the injection of 6-hydroxydopamine.

From the non-uniformity of staining of both calcium- and actin-activated myosin ATPases had been measured were examined, and in every case where it was possible to identify the same cell in both sections with a high level of certainty, the relative activities of the two ATPases correlated well (Fig. 9).

**Response to Exogenous β-Adrenergic Agonists**

Isolated hearts were perfused retrograde through the aorta with a modified Krebs solution, and their contractility was monitored with a balloon in the left ventricle. When the hearts had recovered from the dissection and had achieved a steady state of contractility, isoproterenol in concentrations from $10^{-9}$ to $10^{-6}$ M was added to the perfusion medium. After a new, stable state of enhanced contractility had been established, the hearts were quickly frozen, and the calcium- and actin-activated myosin ATPase was measured. Isoproterenol increased systolic pressure, $dP/dt$ and both ATPases in a dose-related manner (Fig. 10). The maximum increase in systolic pressure was 67%; $dP/dt$ was not measured quantitatively. The addition of $5 \times 10^{-6}$ M propranolol with $10^{-6}$ M isoproterenol completely prevented both the increase in systolic force and myosin ATPase activity. In the presence of $10^{-7}$ M isoproterenol, propranolol reduced the increment in systolic force by 73 ± 11%, and the increment in myosin ATPase activity by 64 ± 14%.

**Response to Endogenous β-Adrenergic Agonists**

Several rats were injected intravenously with 200 μg of tyramine per kilogram of body weight, and the hearts were frozen at times from 30 seconds to 10 minutes after injection. The tyramine, which causes a release of endogenous catecholamines without destroying the nerve endings as 6-hydroxydopamine does, produced an increase in both myosin ATPases (Fig. 11). Both calcium- and actin-activated ATPases rose over the first 6 minutes and then fell. An unequivocal increase was observed at 30 seconds, and the size of the increase was greater with a 10 times larger dose of tyramine.

**Direct Stimulation of Tissue Sections**

The calcium-activated myosin ATPase activity at 37°C, measured in at least eight different sections from each of 14 hearts from 2 to 3-month old rats, was $2.67 \pm 0.19 \mu$mol ATP split per milligram myosin per minute. The effect of β-adrenergic stimulation on the ATPase activity of myosin was studied by using the second messenger cAMP rather than β-agonists, because the cells in the tissue section were essentially skinned by the sectioning process. With 1 μM cAMP, 5 mM theophylline as a phosphodiesterase inhibitor, and 5 mM ATP added to the preincubation and incubation solutions, calcium- and actin-ATPase activity of myosin increased significantly compared to control serial sections. The addition of theophylline alone did not increase...
FIGURE 8. Montages of photomicrographs to form sections through most of the right ventricular wall of three different hearts. Panel A is the control, panel B is 3 hours after injection of 6-hydroxydopamine, panel C is 18 hours after injecting 6-hydroxydopamine. Because of the very large increase in ATPase activity produced by the massive release of catecholamines, the incubation times in these studies were shorter than in those shown in other figures, and therefore the relative densities are not directly comparable. Magnification is approximately 50x.
Calcium-activated ATPase activity increased by 143 ± 7% to 6.49 ± 0.52 μmol ATP per milligram myosin per minute, and actin-activated ATPase of myosin was increased by 145 ± 18% by cAMP from 0.67 ± 0.08 μmol of ATP split per milligram myosin per minute to 1.64 ± 0.30 μmol.

cAMP was most effective in increasing ATPase activity when it was added with theophylline and ATP to both the preincubation and the incubation solutions, and the exposure time of the sections to the preincubation solution was at least 5 minutes. The response was significantly smaller in 10^{-7} M cAMP and absent in 10^{-8} M cAMP; no consistent difference was observed when the cAMP concentration was varied from 10^{-6} to 10^{-4} M. The addition of 0.5 mg/ml protein kinase (the holoenzyme from Sigma Chemical Co.) with cAMP did not increase the increment in ATPase activity. Endogenous protein kinase, the almost universal target for cAMP, is either not limiting in the response of the tissue section, or it is not involved in the reactions modifying ATPase activity. The data do not distinguish between these possibilities.

**Discussion**

Conditions under which the precipitation of inorganic phosphate by calcium can be used as a specific, quantitative assay for myosin ATPase activity have been defined. Specificity has been shown by intracellular localization, response to inhibitors of other cellular ATPases, sensitivity to overlap between myosin and actin filaments, apparent $K_m$ for ATP binding, and kinetics of the enzymatic reaction. Quantitation has been confirmed by the following facts: (1) an insignificant amount of phosphate is lost from the tissue section during the assay (Mahbuchi and Sreter, 1980); (2) the phosphate that is liberated is trapped in the immediate vicinity of the enzyme; (3) the amount of phosphate trapped by calcium at the enzymatic sites and measured microphotometrically increases linearly with reaction time; (4) the replacement of the calcium phosphate by cobalt sulfide acts as a linear amplifier to produce a larger signal for microphotometric measurement; (5) the rate of ATP splitting increases linearly with the amount of enzyme; and (6) the rate of ATP splitting as a function of ATP concentrations conforms to standard enzyme kinetics with values for the $K_m$ and $V_{max}$ that are in close agreement with values in the literature derived from measurements using more traditional methods (Katz, 1970).

The distribution of reaction product within a sarcomere from calcium-activated myosin ATPase corresponds to the location of the catalytic sites. However, activation by actin does not distribute reaction product according to the location of ATPase sites. Staining occurs preferentially in the center of the A-band. It is unlikely that activation is restricted to cross-bridges in the central 50–65% of the A-band, for in that case there should have been a bare zone in the center of the A-band that became broader as sarcomeres were lengthened. It is tempting to attribute the unexpected distribution of staining during actomyosin ATPase activity in the rat heart to motion and/or mixing produced by the sliding filaments. In agitated cytoplasm, calcium phosphate might be mobilized toward the center of the A-band as it is formed, and become trapped on the fine network of the M-line. Cytosolic mixing of this type might be responsible for the ability of creatine ki-
nase, which is concentrated in the center of the A-band, to be so effective in rephosphorylating ADP produced at the cross-bridges during muscle contraction (Bessman and Geiger, 1981).

The average values obtained for calcium- and actin-activated V1 myosin ATPases at 37°C at pH 10.5 and 9.0, respectively, were 2.63 and 0.67 μmol phosphate per milligram myosin per minute. These values compare favorably with those in the literature, although some assumptions must be made because of different conditions of temperature, ionic strength, and pH in the published studies. Pope et

**Figure 10.** Response of calcium (panel A) and actin-activated (panel B) myosin ATPase to different concentrations of isoproterenol. The changes in systolic pressure of the hearts from which the sections were taken is also shown in panel C. Values are expressed relative to the control. The effect of propranolol added with isoproterenol is shown by the open circles.
tivity of phosphate precipitated by Mabuchi and Sreter (1980), measuring the radioactivity of relative ATPase activity of myosin. Lompre et al. (1981) have both found the precipitation of inorganic phosphate with calcium in alkaline medium to be a valid method for making measurements of relative ATPase activity of myosin. Mabuchi and Sreter (1980), measuring the radioactivity of phosphate precipitated by $^{45}$Ca, found values for actin-activated ATPase activity similar to those obtained with more conventional methods.

**β-Adrenergic Regulation**

Three different drugs that produce adrenergic stimulation of the intact heart increased both calcium- and actin-activated ATPase activity. The relative values of calcium- and actin-activated ATPase in individual cells were always directly correlated, and it can be tentatively concluded that the same change in the myosin molecules was responsible for the modification of enzymatic activity under both conditions of activation.

An increase in both forms of myosin ATPase activity was also produced in tissue sections by adding cAMP to the incubation medium. The amount by which cAMP increased myosin ATPase activity in tissue sections was less than the increment produced by raising serum catecholamine levels in the intact heart. The discrepancy probably was the result of dilution of a soluble component of the regulatory system that is released by cAMP, since cells in the tissue section do not have the intact sarcolemmas to retain the component (Weisberg et al., 1983; Lin et al., unpublished observation).

**Mechanism of cAMP Effect**

From this work alone, it is not possible to say whether the increase in the ATPase activity of cardiac myosin is due to an increase only in the enzymatic activity or to an increase in the total number of enzymatically active myosin molecules. The same problem exists for the interpretation of the changes produced in maximum calcium-activated force with hyperpermeable fibers. The data do not distinguish between changes in the properties of existing force generators and increase in the percentage of force generators that are activated by calcium ions. The change in the function of myosin could result from either release of an active co-factor or activation of an enzyme producing a change in the contractile proteins. In the former case it would be necessary for the co-factor to exist in relatively high concentration in the cell. So far, the only well-characterized proteins that are known to exist in high enough concentrations are phospholamban, creatine kinase, or the subunits of myosin itself. Another possible model is one in which an enzyme is activated to form a small co-factor that alters the function of myosin. From this work alone, it is not possible to say whether the increase in the ATPase activity of cardiac myosin is due to an increase only in the enzymatic activity or to an increase in the total number of enzymatically active myosin molecules. The same problem exists for the interpretation of the changes produced in maximum calcium-activated force with hyperpermeable fibers. The data do not distinguish between changes in the properties of existing force generators and increase in the percentage of force generators that are activated by calcium ions.

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unlikely in a direct enzymatic alteration of the contractile proteins.

Studies by other investigators have failed to detect significant changes in maximum ATPase activity of purified myosin induced by catecholamines (Ray and England, 1976; Dowell, 1976), although the concentration of calcium ions required for activation varied with the degree of cAMP-induced phosphorylation of troponin. The discrepancy between studies in the literature and those reported here may be due to changes in myosin that occur during extraction of the protein or the isolation of the myofibrils. A difference between the ATPase activity of isolated myosin and unextracted myosin in a frozen section has already been noted by Morkin et al. (1977). The increase in ATPase activation induced by catecholamine may be labile and easily lost during procedures generally employed in purifying contractile proteins.

The existence of a mechanism for regulating myosin in rat heart does not alter the conclusions, derived from a large amount of evidence, that calcium triggers contraction by binding to troponin, and the amplitude of the contraction is related to the amount of activating calcium. It does, however, indicate that the cell has an additional mechanism for regulating contractile force besides modification of the transmembrane calcium currents, the rate of calcium accumulation by the sarcoplasmic reticulum, and the calcium sensitivity of the contractile system. The possible role of such a regulatory system in the normal physiological function of the heart has been discussed in some detail in a recent review (Winegrad, 1984).

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INDEX TERMS: Myosin • ATPase • Contractility • Actomyosin • Adrenergic regulation
Adrenergic regulation of myosin adenosine triphosphatase activity.
S Winegrad, A Weisberg, L E Lin and G McClellan

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