Contractile Proteins in Myocardial Cells Are Regulated by Factor(s) Released by Blood Vessels

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The importance of perfusion of the coronary vasculature in the regulation of ATPase activity of myosin in rat myocardial cells has been studied. Quantitative histochemistry was used to determine the activity of the enzyme among cells in tissues that had been either perfused through the coronary system or superfused over the surface of the tissue. Enzymatic activity was measured in cryostatic sections from three different preparations: 1) hearts frozen immediately after removal from the animal; 2) isolated hearts frozen after they had been perfused through the coronary circulation; and 3) isolated papillary muscles or trabeculae that had been superfused after dissection and then frozen. ATPase activity was measured in the isolated tissues at different times after dissection. Both calcium- and actin-activated myosin ATPase activities were uniform among cells in both the ventricles of the hearts frozen immediately after dissection and those that had been perfused through the coronary system. In the superfused tissues, although calcium-activated myosin ATPase activity was uniform, actin-activated ATPase activity was not uniform for about 90 minutes after the dissection, the period required for stabilization of the contraction. The pattern of nonuniformity was complex. In all bundles the lowest enzymatic activity was found in the most superficial cells. In very thin bundles, the cells in the center had the highest activity. In the medium and thicker bundles, there were three concentric zones of actin-activated ATPase activity, the superficial zone with the lowest activity, an intermediate zone with high activity, and a central zone with lower activity. Within each zone, the activity was often greatest in myocardial cells immediately next to blood vessels even though the blood vessels had not been perfused. The transverse distribution of ATPase activity of myosin could be explained by a mechanism in which cells in blood vessels (presumably endothelium) release a substance that upregulates myosin ATPase activity, with the rate of release being related to the local oxygen tension. A downregulating substance may also be produced. The period of stabilization of the contraction coincides with the time during which the pattern of actomyosin ATPase activity is nonuniform. These data suggest that the contractile proteins are regulated by a substance produced by blood vessels in proportion to the local PO₂ and possibly in relation to shear force on the vascular endothelium. (Circulation Research 1992;70:787–803)

KEY WORDS • myosin • ATPase • endothelium • endothelin • coronary circulation • contractile proteins • regulation of contractile proteins

The mammalian heart can vary its output by a factor of almost five as a result of increasing stroke volume by about 50% and heart rate by about 300%. Because all of this occurs while the afterload on the heart remains approximately constant, cardiac power increases in proportion to cardiac output. The metabolic needs of the myocardium rise to an even greater extent since the efficiency of the heart declines as the heart rate increases. Work performed against internal elastic forces with each contraction is a major source of inefficiency.

Even in the resting organism coronary venous blood has a low oxygen tension as a result of the high degree of extraction of oxygen by the myocardium. Consequently, not much additional oxygen is available to the myocardium for further extraction of oxygen, and an increase in oxygen demand by the heart must be supplied by an increase in coronary blood flow. Without a significant rise in aortic blood pressure, any increment in coronary flow must come from vasodilatation. An association between a decline in the partial pressure of oxygen in the myocardium and coronary vasodilatation has been observed, but until recently the basis for this change in vascular tone has been obscure. It now seems likely that regulatory factors released by the vascular endothelium play an important role.

At least two different factors with important effects on vascular tone, endothelium-derived relaxing factor and the vasoconstrictor endothelin, are released by vascular endothelium. The former is probably nitric oxide or a substance related to nitric oxide and the...
latter, a 21-amino acid peptide whose sequence has been determined. These substances appear to be released in response to changes in the oxygen content of the blood and possibly in the mechanical characteristics of flow within the blood vessel.

Two different types of receptors for endothelin have been identified on the surface of myocardial cells, one related to the calcium channels in the membrane and the other clearly not part of the calcium channel. From their amino acid sequence, the receptors appear to be integral membrane, G-protein–related proteins within the cardiac myocyte. An increase in cardiac contractility is produced by endothelin. The positive inotropy may be associated with an increase in the inward calcium current. There are some indications, however, that all of the increase in contractility cannot be attributed to an enhancement in calcium available for activation and that a change in the performance of the contractile proteins themselves may also occur.

Studies of ATPase activity of actomyosin in the heart have demonstrated the presence of a regulatory system that can alter the contractile performance of the contractile proteins and measurement of mechanical properties and energetics of cardiac tissue have confirmed this conclusion. With the characterization of actomyosin ATPase activity by quantitative histochemistry, it is possible to achieve a very high degree of spatial resolution of enzymatic activity. The method provides the means for determining whether there is variability in the ATPase activity within cells related to either their proximity to blood vessels or the characteristics of the perfusion of the blood vessels. Changes in contractility caused by alterations in contractile proteins can be distinguished by this technique from those caused by modification of excitation–contraction coupling because the measurement of ATPase activity is conducted under conditions in which the concentration of calcium is not limiting.

In this study, we have used quantitative histochemical measurements of actomyosin ATPase activity to determine if change in the perfusion of the blood vessels in the myocardium alters the regulatory state of the contractile proteins. The results indicate that changes in the perfusion of coronary blood vessels alter actomyosin ATPase activity.

**Materials and Methods**

Male rats obtained from Charles River Breeding Co. and weighing between 200 and 350 g were killed by accepted procedures (American Association for Accreditation of Laboratory Animal Care), and the hearts were removed immediately. The hearts were treated in one of three ways. In some hearts, left and right ventricles were separated and immediately frozen in isopentane precooled by liquid nitrogen. These hearts were then sectioned in a cryostat at −20°C to −25°C, and quantitative histochemical analysis of myosin ATPase activity was performed. A second group of hearts was quickly set up as isolated perfused working organs by using a minor modification of the Neely-Morgan technique. These hearts were perfused with a modified Krebs’ solution containing (mM) NaCl 118, KCl 4.7, CaCl2 2.5, MgSO4 1.2, KH2PO4 1.2, EDTA 0.5, NaHCO3 2.5, and glucose 11, equilibrated with 95% O2−5% CO2. At the end of a run with a perfused heart, the apical region of the left ventricle was isolated and quickly frozen in isopentane cooled by liquid nitrogen. After cryostatic sectioning, myosin ATPase activity was measured by quantitative histochemistry. In the third group of hearts, the right ventricle was opened in well-oxygenated Krebs’ solution. Papillary muscles and trabeculae with cross-sectional areas between 0.01 and 0.4 mm2 were removed from the endocardial surface and tied at each end. In the case of the papillary muscles, the tie at the end was placed on the chordae. The bundles of tissue were then suspended in a chamber with one end attached to a force transducer and the other end to a mechanical ground that could be moved to adjust resting tension. The tissue was continuously superfused with oxygenated Krebs’ solution. Diameter was measured twice at orientations perpendicular to each other for calculating cross-sectional area. This value was then compared with the value measured on the frozen section. The resting length was set at the point at which resting tension began to rise sharply with further increases in resting length. This length corresponds to approximately 2.2 μm sarcomere length. Some bundles remained at rest until frozen; others were electrically stimulated to obtain values for developed tension. The observed values were related to the cross-sectional area in a manner that has already been described. After 3–120 minutes of superfusion, the tissue bundles were quickly frozen with isopentane that had been precooled with liquid nitrogen, and then they were removed from the apparatus without disturbing resting length. The frozen bundles were carefully oriented on the stub of the cryostat so that transverse sections could be cut for measurement of myosin ATPase activity by quantitative histochemistry.

The procedure used for measuring ATPase activity has already been described. Briefly, it involves trapping the inorganic phosphate released by the hydrolysis of ATP and converting the precipitate to cobalt sulfide in a two-step substitution. The density of the cobalt sulfide is linearly related to the amount of inorganic phosphate produced. Under the conditions in which the measurements are made, the assay is specific for myosin ATPase, it can distinguish between calcium- and actin-activated ATPase activity, and it has sufficient spatial resolution to distinguish individual myofibrils in a cross section.

For the examination of ATPase activity in the ventricles from hearts that had been quickly frozen, at least 80 serial sections were cut. Sectioning generally began at the epicardial surface, but as a result of the orientation of the tissue with respect to the knife edge most sections included the full thickness of the ventricular wall. At least 10 sections were used for each type of measurement: actin-activated myosin ATPase activity, calcium-activated ATPase activity, and the two forms of ATPase activity in the presence of 1 μM CAMP (see Reference 13 for details of the specific protocols). Either every fourth or every eighth section was used for the same protocol, and the order of the protocols within the sequence of 4 or 8 was randomized among experiments. There was an element of chance in the way in which the ventricular wall was oriented with respect to the knife edge, but among the more than 150 hearts studied in
this fashion, a very broad sampling of the tissue should have been accomplished.

To study the ATPase activity of isolated trabeculae or papillary muscles, the tissue bundle was oriented on the cryostat so that the instrument cut transverse sections. Attachment to the stub of the arm of the cryostat was made with the base of the tissue bundle, that is, the portion of the ventricular wall at one end of the tissue bundle. Therefore, sectioning began at the chordae end of papillary muscles and one wall attachment end of the trabeculae. The tissue bundles were generally between 1 and 2 mm in length. Initially, 80 sections were cut from the bundle, the count starting after the connective tissue end of the bundle had been sectioned away. In later experiments, however, the entire bundle was sectioned. Because the sections were 4 μm thick in these studies, at least 25% of the tissue bundle was sectioned in every experiment.

When only 80 sections were cut, optical density was measured in every section, and the results of every section were included in the data. When bundles of tissue were entirely sectioned, the ATPase activity of every section was measured and the resultant density and distribution of reaction product was measured directly as well as being recorded on film for analysis of the distribution of reaction product. The average optical density within sections through the bundle was measured.

For the determination of optical density, sections were visualized with a Zeiss photomicroscope using a ×6.3 objective with 0.16 N.A. or a ×25 plano objective with 0.40 N.A. The entire transverse section of the tissue bundles could be included in the measurement, thus eliminating any possible bias. For the much larger sections cut from ventricles, regions from at least 10 sections taken every fourth or eighth in sequence were measured. Different regions of the tissue were chosen for study in each of the 10 sections, the regions chosen randomly. Because the standard deviation of the mean density of the sections from the ventricular wall was always less than 3% and generally less than 2%, there was great uniformity of ATPase activity in different parts of the ventricle. Consequently, bias in section or area could not have been a factor in the measurement.

Optical density was measured by digitizing a video image of the tissue section formed by the microscope and a video camera. The density could be measured either within a selected area or along a selected straight line. The software allowed the subtraction of any contribution of nonmyocyte space to the average density of the tissue section. The sensitivity of the density measurement at any point was 1/256 since each pixel could record 256 gray levels. The spatial resolution was determined by the resolving power of the microscope’s optics and could theoretically have been less than 1 μm. For measurement of the distribution of ATPase activities in the tissue bundles, a straight line was taken from the calculated center of the tissue section to the surface of the bundle. For measurement of the gradient of ATPase activity adjacent to a blood vessel, a straight line was used normal to the axis of the blood vessel.

In studying the effects of endothelin on the ATPase activity of myosin, the different concentrations of the peptide were added to the preincubation and incubation solutions. The histochemical protocols were otherwise the same as in the standard enzyme assays.

For measurements of the relation between coronary oxygen tension and the ATPase activity of myosin in the cells, larger rats (weighing between 450 and 600 g) were used because it was easier to introduce the oxygen electrode into the larger heart. Coronary sinus oxygen tension was determined by the placement of an oxygen electrode into the right atrium. The electrode was calibrated by exposure to flowing solution that had been equilibrated with 95% O₂, air, and 100% N₂. The solution used for perfusion of the heart contained (mM) NaCl 118, KCl 2.5, MgSO₄ 1.2, KH₂PO₄ 1.2, EDTA 0.5, NaHCO₃ 25, and glucose 11 at 37°C. The solution was equilibrated with 95% O₂–5% CO₂. Electrodes were placed on the right atrium, and the heart rate was set by rhythmic stimulation between 250 and 325 beats per minute. Hemodynamic parameters including coronary flow, cardiac output, heart rate, systolic and diastolic aortic pressures, and dP/dt were measured both continuously with a chart recorder and on-line at 10-minute intervals with a Labmaster analog/digital converter (Scientific Solutions Inc., Solon, Ohio). The hearts were perfused at a left atrial filling pressure of 5 cm H₂O and an afterload of 100 cm H₂O for 30 minutes, and then the heart was removed and the apical region of the left ventricles separated and quickly frozen in isopentane cooled by liquid nitrogen. Calcium- and actin-activated ATPase activities of myosin were measured in 4-μm cryostatic sections, and the contributions of myosin containing α and β heavy chains were separated by their different sensitivities to alkalinity.¹²²

For the monitoring of the size and shape of the contraction during the period of stabilization after dissection of trabeculae or papillary muscles, the tissues were mounted in a bath containing Krebs’ solution and continuously bubbled with 95% O₂–5% CO₂ through a fritted glass tube. Tension was continuously measured with a Grass transducer. The tissue was stimulated with punctate electrodes to avoid possible release of transmitters from nerve endings. However, the results were no different when massive stimulation with two parallel platinum electrodes was used. The level of stimulation was set at 15–20% above the value needed for maximum contractile force. Frequency was either 0.2 or 1 Hz. Tension was continuously recorded with a strip-chart recorder, and periodically, the output of the transducer for five consecutive contractions was fed into an IBM personal computer through a Keithley analog/digital converter and the averaged twitch was printed with a Hewlett-Packard plotter. The tissue bundles used for these studies came from littermates of those used for the ATPase measurements because the more rigorous method used for measuring force in these studies was less convenient for quick freezing of the tissue.

Endothelin-1, α chain of insulin, and β-endorphin were obtained from Sigma Chemical Co., St. Louis, Mo.

Data were analyzed by unpaired and where appropriate paired Student’s t tests. Statistical significance was accepted when p≤0.05.

Results

Stabilization of Function in Isolated Perfused Hearts

The performance of an isolated perfused working heart stabilizes relatively quickly after the organ has been removed from the animal. Hemodynamic mea-
TABLE 1. Stabilization of Isolated Perfused Hearts

<table>
<thead>
<tr>
<th>Time (Minutes)</th>
<th>O₂ saturation coronary venous</th>
<th>Hemodynamic work/unit time</th>
<th>Systolic pressure</th>
<th>Mean pressure</th>
<th>Diastolic pressure</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>96±4</td>
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<td>99±2</td>
<td>101±1</td>
<td>101±1</td>
<td>99±2</td>
</tr>
</tbody>
</table>

Values are mean±1 SEM. Values at 20 minutes have been arbitrarily assigned a value of 100 and values at other times are expressed relative to the value at 20 minutes for each heart.

Measurements and recording of coronary venous oxygen tension were made within 5 minutes of the removal of the heart from the animal. Within 10 minutes of the beginning of these measurements, mean aortic pressure, systolic and diastolic pressures, cardiac output, work, and coronary venous oxygen saturation have become constant. They are maintained for at least 120 minutes if the conditions under which the heart is functioning are not changed (Table 1).

Myosin ATPase Activity in Perfused Myocardium

Both calcium- and actin-activated ATPase activity of myosin were measured because the former, though a nonphysiological enzymatic reaction, does provide information about the catalytic function of myosin, and the latter, which is the physiological enzymatic activity, is sensitive to the interaction of actin with myosin. A previous study has shown that there is uniformity of both calcium-activated and actin-activated myosin ATPase activity in both the right and the left ventricles quickly frozen after rapid removal of the heart from the animal. In ventricles from over 100 rat hearts that have been studied in this way, ATPase activity has been essentially uniform throughout the myocardium except for small gradient that was sometimes observed in the first one or two layers of cells just below the endocardium. A montage of photomicrographs of sections of an entire right ventricular wall and a high magnification of a portion of a left ventricular wall taken from hearts frozen immediately after removal from the animal are shown in Figures 1 and 2.

The calcium- and actin-activated myosin ATPase activities of hearts that have been quickly removed from the animal and then immediately suspended as isolated, perfused working hearts are also uniform throughout the ventricular chambers. Even at high magnification there is no indication of any gradient or nonuniformity of enzymatic activity regardless of the distance from blood vessels (Figure 3). Papillary muscles and trabeculae have uniform ATPase activities, and the mild nonuniformity in the first and second layers of the subendocardial cells seen occasionally in hearts frozen immediately after removal from the animal was not observed in sections of isolated perfused hearts. Over 150 rat hearts have been studied in this manner.

Stabilization of Function in Isolated Ventricular Trabeculae

An isolated ventricular trabecula requires a relatively long period to achieve stable mechanical function. When stimulated continuously at 0.2 Hz in oxygenated Krebs' solution, trabeculae require 90–120 minutes for the shape of the isometric contraction to become constant. During this period, peak tension declines and relaxation begins progressively earlier in the contraction. During the stabilization of 28 preparations, the mean decline in peak force was 16±5% and the time to 50% relaxation decreased by 19±3% (Figure 4). There is essentially no change in the maximum rate of rise of tension during this period. When contractility has stabilized, it remains that way for at least 2 more hours.

Myosin ATPase Activity in Superfused Papillary Muscles or Trabeculae

The ATPase activity in transverse sections of 37 papillary muscles or endocardial trabeculae that had been superfused for 5–100 minutes in modified Krebs' solution was studied. Different patterns of myosin ATPase activities were observed among these tissues. Actin-activated ATPase activity was often not uniform even though calcium-activated ATPase activity was always uniform through the thickness of the bundle (Figures 5 and 6). The pattern of ATPase activity was related to the duration of the superfusion period after dissection. In bundles that had been superfused for

FIGURE 1. Montage of photomicrographs of the actin-activated ATPase activity of myosin in cells in the right ventricular wall of a rat. The heart had been quickly frozen immediately after removal from the animal. Enzymatic activity is directly proportional to optical density. Magnification, ×60.
8–60 minutes, there was always clearly visible nonuniformity of actin-activated ATPase activity. For purposes of quantitation, nonuniformity was defined as a difference of 20% or greater in the optical density among cells. In tissues superfused for less than 5 minutes or greater than 90 minutes, actin-activated ATPase activity was always uniform (Figure 7). For tissues superfused between 60 and 90 minutes, the results were mixed. The overall nonuniformity consisted of concentric zones of differing ATPase activity. To quantitate the results, the density of the reaction production of the ATPase activity was measured along a line from the approximate center of the transverse section to the surface of the bundle (Figure 8). The actin-activated ATPase activity of myosin varied according to the thickness of the bundles despite the fact that calcium-activated ATPase activity was uniform and did not vary among bundles of different thicknesses. Thinner bundles contained more active actin-activated myosin ATPase. This relation of actomyosin ATPase activity in superfused bundles of myocardial cells has already been noted and described in detail.21
FIGURE 4. Recording of the computer average of five consecutive contractions of an isolated trabecula measured at different times after the preparation had been set up. Upper panel: Relative tension; lower panel: tension normalized to peak value.

Of particular interest were the patterns of nonuniformity of actin-activated ATPase activity that were found in the superfused bundles having nonuniform ATPase activity. Invariably, regardless of the cross-sectional area of the bundles, the lowest actin-activated ATPase activity was found in the most superficial cells, those immediately below the endocardium (Figures 5 and 6). The thickness of this ring of low activity varied to some extent, but there was no obvious relation between the thickness of this ring and the overall thickness of the bundle. In thin bundles less than 0.1 mm², the cells with the highest actin-activated ATPase activity were found in the middle of the bundle (Figure 5). In thicker bundles there were three zones of actin-activated ATPase activity (Figure 6). The lowest activity was always in the cells just below the surface of the bundle. The cells with the highest activity were present in the middle zone of the transverse section of the bundle, and the central region of the bundle contained cells with an intermediate level of actin-activated ATPase activity. These three zones were found throughout the length of the myocardial bundle except for the damaged region where the bundle inserted into the wall of the cardiac chamber or, in the case of papillary muscles, where the chordae attached to the myocardial cells at one end of the bundle.

In many bundles, specific patterns of actin-activated ATPase activity were apparent within the zone with highest activity and sometimes within the zone with intermediate activity. Actin-activated ATPase was more active in the myocardial cells immediately surrounding small blood vessels than in cells more distant from the blood vessels (Figures 5, 6, and 9). This was seen regardless of whether the blood vessels ran perpendicular or parallel to the plane of the tissue section. Sometimes localized groups of cells within a section had a higher enzymatic activity even though no blood vessel was seen nearby within the section. In some cases it was possible to show that a blood vessel occupied an analogous position in an adjacent or nearby section (the thickness of each section is about 30% of the diameter of a single myocardial cell) (Figure 10). In general, the gradient in ATPase activity in cells adjacent to blood vessels disappeared over no more than two cells. Not all blood vessels were surrounded by cells with higher ATPase activity.

The density of the reaction product of actin-activated ATPase activity was measured along a line normal to the axis of the blood vessels, and the extent of the gradient in ATPase activity was measured. When gradients were present they generally were confined to the region within one to two cell diameters of the blood vessels. To determine the extent to which these gradients in enzymatic activity were present, criteria were established for four different degrees of gradient formation around the blood vessels within given tissue sections cut from 37 different trabeculae. The degree to which gradients of ATPase activity were found around blood vessels depended on the duration of the exposure of the tissue bundle to superfusion (Table 2). By 10 minutes, signs of gradients were beginning to appear. Between 10 and 40 minutes, the gradients were very prominent, and by 60 minutes of superfusion, the gradients had essentially disappeared. Even after the gra-
Gradients around blood vessels were no longer visible, nonuniformity existed in some tissue bundles for as long as an additional 30 minutes. From a comparison of the relative actin-activated ATPase activities in trabeculae frozen immediately after dissection with those superfused for greater then 60 minutes, there was a decline in ATPase activity of 14±4% (p<0.05) during the period of nonuniformity.

In these studies, the presence of blood vessels in unstained sections was detected by the ATPase activity of the myosin in the smooth muscle of the blood vessel walls. Detection of the thin-walled venules was more difficult. Therefore, conclusions about the existence of gradients around venules were not reliable. As regards gradients around capillaries, their presence is unlikely. Because each cell has more than one capillary at its surface, a gradient produced by proximity to a capillary would have to exist within a myocardial cell. There was no convincing evidence for ATPase gradients across the cross section of individual cells. Because a cell radius is 6-7 μm, the resolving power of the histochemical technique should be adequate to detect such a gradient.

<table>
<thead>
<tr>
<th>Class</th>
<th>0-10 Minutes</th>
<th>11-19 Minutes</th>
<th>20-40 Minutes</th>
<th>40-60 Minutes</th>
<th>&gt;60 Minutes</th>
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<td>3</td>
<td>3</td>
<td>7</td>
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<tr>
<td>Class II</td>
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<td>0</td>
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<tr>
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<td>1</td>
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<td>0</td>
</tr>
<tr>
<td>Class IV</td>
<td>0</td>
<td>4</td>
<td>4</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Values are numbers of trabeculae. Class I, no sign of gradient; class II, decline of optical density of less than 50% around one to two blood vessels; class III, decline of optical density of at least 50% around at least two blood vessels; class IV, decline of optical density of at least 50% around at least two blood vessels that are present in at least three serial sections.
FIGURE 6. Photomicrographs of serial transverse sections of a thicker bundle showing the highest actin-activated ATPase activity in the intermediate of three concentric zones. Magnification, ×156.
Relation of Actomyosin ATPase Activity to Coronary Sinus Oxygen Tension

The studies described so far indicate that during the period of stabilization of isolated cardiac bundles of tissue, which lasts 60–90 minutes in the case of rat trabeculae or papillary muscles, there is a period of transverse nonuniformity in actomyosin ATPase activity that temporally appears to coincide with the period during which the contractile response is changing. Because these tissues are supplied with oxygen by a superfusing medium flowing over the surface of the tissue, gradients of oxygen within the cross section of the bundle must exist. Gradients within tissue perfused through the coronary circulation should be very much smaller.

Isolated perfused working hearts were used to study the relation between oxygen tension and actomyosin ATPase activity under conditions in which the oxygen tension in the immediate environment of the myocytes was varied. The level of oxygen saturation in the effluent of the coronary sinus, as measured by an oxygen electrode in the right atrium, was used as an indication of oxygen tension in the microenvironment of the myocyte. To increase the level of actomyosin ATPase activity, 0.1 μM isoproterenol was added to the perfusion medium. The amount of work done per unit time by the heart was varied by changing heart rate to vary the amount of
oxygen extracted from the coronary perfusate and the oxygen tension in the coronary sinus fluid. Heart rate was chosen as the independent variable because it could be relatively easily controlled, and at the same time both the preload and the afterload on the heart could be maintained.

The heart rate of the normal, well-functioning isolated perfused working heart was 254±6 contractions per minute. With a stimulus applied to the right atrium, it was possible to raise the rate to at least 325 contractions per minute and still maintain good function of the organ. Although preparations do not normally contract at rates below 250, occasionally, generally for reasons that are not clear, the spontaneous rate of contraction of the heart was below 240. Of the 12 hearts reported in this study, 10 were driven at rates between 250 and 325, and two hearts beat spontaneously at 210 and 220 beats per minute.

The hemodynamic work, defined as the product of pressure and volume, increased with heart rate to about 310 beats per minute and then declined at faster heart rates. There was no obvious change in the amount of work produced per unit of oxygen consumed over this range of heart rates. Coronary flow increased with frequency of contractions to about 280 beats per minute and then remained approximately constant with further increase in heart rate.

Among the group of stimulated hearts, as heart rate increased, the oxygen tension in the coronary sinus blood decreased from 40–50% of saturation at rates between 250 and 290 to significantly lower values at heart rates above 300. In the two hearts with low contractile rates, coronary sinus Po2 was low and may have been part of the reason for the unusually low spontaneous rate.

The contributions of myosin with α and β heavy chains (V1 and V3) to total actomyosin ATPase activity were distinguished by the alkaline lability of the enzymatic activity of myosin with the β heavy chain. The actin-activated ATPase activity of V1 declined as the Po2 in the coronary sinus effluent decreased over the entire range of 10–50% saturation (Figure 11). In cryostatic sections of the hearts that were quickly frozen, the response to cAMP depended on the particular value for Po2. The increment in ATPase activity of V3 produced in cryostatic sections of the heart by cAMP increased with lower Po2 to a maximum in hearts in which coronary sinus fluid was about 25% saturated (Figure 11). cAMP reduced without eliminating the differences in the V1 actomyosin ATPase activity found in untreated sections of hearts that had different coronary venous Po2 values. No increase in ATPase activity was produced by cAMP when oxygen tension was above 50% saturation. Because the perfusion medium of the isolated hearts contained an optimally stimulating concentration of isoproterenol, one would have expected little or no increase in ATPase activity from cAMP added to the incubation medium for ATPase assay. Further fall of coronary sinus Po2 below 25% saturation was associated with a progressive decrease in the increment in ATPase activity produced by cAMP activity in the cryostatic sections. The actin-activated ATPase activity of V, was independent of coronary sinus Po2 over the range of values from 10% to 50% saturation (Figure 11).

Relation of Actomyosin ATPase Activity to Coronary Flow

The ATPase activity of actin-activated myosin in which α heavy myosin chain was present (V1) declined as coronary flow increased (Figure 12). The increment in V1 actin–activated ATPase activity in cryostatic sections of the quickly frozen hearts when the sections were treated with cAMP increased with increasing coronary flow, so that the maximum value for V1 ATPase, achieved with cAMP stimulation, was independent of coronary flow within a twofold range of flow values. In contrast, the values for actin-activated ATPase activity of V3 were unchanged as coronary flow changed from 19 to 30 ml/min.

Effect of Endothelin on Myosin ATPase Activity

The effect of endothelin on both calcium- and actin-activated myosin ATPase was determined by exposing cryostatic sections of hearts quickly frozen immediately after removal from the animal to the peptide. No change in calcium-activated ATPase activity was observed with concentrations of endothelin as high as 50 nM. On the other hand, in all nine hearts that were studied, actin-activated ATPase activity of myosin was significantly increased (Figure 13). However, two different dose–response curves were observed. In sections from six hearts the intensity of the response increased with the concentration of endothelin from 1 to 50 nM. In the other three hearts, a significant increase in enzymatic activity occurred with 0.1 nM, a peak at 1 nM, and a decline to control levels at 10 and 50 nM. No reason for the two different types of dose–response curves was apparent. With seven of the nine hearts, there was a decline in the mean value for actin-activated ATPase activity with 0.05 nM endothelin, but the difference was not statistically significant at p=0.05. Two similar dose–response curves for the effect of endothelin on the inotropic state of isolated rat myocardial cells have already been reported.12

Because the endothelin was added to the assay solutions and the cryostatic section contains cut cells, it was possible that the endothelin exerted a nonspecific effect directly on the contractile proteins rather than through interaction with receptors on the cell membranes. To check this possibility, two peptides of similar size, the α chain of insulin and β-endorphin, were assayed under identical conditions and concentrations...
Figure 9. Photomicrographs of the myosin ATPase activity in transverse sections of bundles of cardiac cells that had been superfused for 30 minutes before having been quickly frozen. Note the higher density along some of the blood vessels and along what appear to be the pathways of branches of these blood vessels in the actin-activated (left panel) but not in the calcium-activated (right panel) ATPase patterns. Magnification, ×120.
as endothelin. No effect on actin-activated ATPase activity was observed with either of these peptides.

**Discussion**

The response of isolated cardiac tissue to in vitro conditions appears to be strongly influenced by whether the tissue is perfused through the coronary circulation or superfused over the surface of the tissue. When the in vitro preparation is perfused through the coronary circulation, very little time is required for the contractile performance to reach a stable state; this occurs within 10 minutes. In this type of tissue preparation, perfusion through the normal pathway is reestablished in less than 2 minutes after the animal is killed. If, however, the tissue preparation is an isolated bundle that is bathed over the surface of the tissue rather than through the coronary circulation, 90–120 minutes is required for a new stable level of mechanical function to be established. In this preparation, the pathway for oxygen supply to the tissue is changed from what existed in vivo, and the surface of the tissue bundle is exposed to physical forces that are different from those that existed in vivo.

A fundamental difference in the enzymatic activity of the contractile proteins in myocardial cells was found depending on whether the tissue was perfused, either in situ or in vitro, through an intact vascular system immediately before being frozen or suspended as a thin isolated bundle and superfused over its surface. In the perfused myocardium, both calcium- and actin-activated ATPase activity of myosin were essentially uniform throughout the tissue, regardless of whether the perfusing medium was blood in the heart in situ or well-oxygenated electrolyte solution in an isolated working heart. Within the intact heart, ventricular trabeculae of the type used for the isolated preparations have uniform calcium- and actin-activated ATPase activities in these perfused hearts.

Cells in the isolated superfused bundle of myocardium behave differently. During the period after dissection when the contraction is stabilizing, actin-activated ATPase activity of myosin is not uniform although the calcium-activated form of the enzyme is uniform. The nonuniformity in actin-activated ATPase activity in superfused bundles is not random. There are two major patterns depending on the thickness of the bundle. In thin bundles, the lowest activity is in the cells closest to the surface of the bundle, and the cells with the highest activity are in the center of the bundle. Thicker bundles have three concentric zones with the lowest activity in the most superficial cells and the highest in the middle zone. These distributions cannot be simply caused by limitations in diffusion of oxygen into the bundle from its surface inasmuch as the most superficial cells have the lowest activity. Most of the bundles used in this study were sufficiently thin for oxygen diffusion not to have been a limiting factor for oxidative phosphorylation at the mitochondria. A second important characteristic of the actin-activated ATPase activity was its greater intensity in thinner bundles in which only two zones of activity were identified. This observation was incompatible with the possibility that the observed patterns of enzymatic activity in bundles of different

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**Figure 10.** Two sections separated by four sections of a thicker bundle showing higher ATPase activity in one section and a blood vessel in the analogous position four sections removed. Magnification, ×150 (right) and ×117.5 (left).
thickenesses were produced simply by the loss of a
substance that was important in regulating the contractile
proteins.

Whatever mechanism was modifying enzymatic activity, it appears to have had a similar effect on the
development of force. During the same time span in
which nonuniformity of ATPase activity was observed, the peak force was declining and the onset of relaxation
occurred progressively earlier. At about the same time
that the contraction had stabilized and remained stable
for at least another 2 hours, the nonuniformity of
ATPase activity disappeared and ATPase activity be-
came stable as well.

The general patterns of ATPase activity in super-
fused bundles can be reproduced by a model with the
following four assumptions: 1) a factor is produced by
blood vessels (presumably the endothelial cells) that
regulates the calcium responsiveness of cardiac acto-
myosin so that actomyosin ATPase activity and force
generation are proportional to the concentration of the
factor; 2) the rate of production of the putative factor decreases as the concentration of oxygen falls and possibly as perfusion of the blood vessels declines as well; 3) the factor diffuses from the bundle into the superfusing solution, resulting in a concentration that is zero at the surface of the bundle; and 4) the factor regulates the attachment of the myosin head in the crossbridge to the thin filament.

When the equations for this model, taken from Hill’s
theoretical analysis of the diffusion of substances through
a muscle,22 are derived and solved (see “Appendix”), the
predicted relative ATPase activity across the transverse
dimension of the tissue bundle reproduces some of the
important characteristics of the patterns of ATPase activity that were observed experimentally (Figure 14). In thin bundles the pattern is dominated by the effects of diffusion from the bundle. The highest activity is in the center and
declives to zero at the surface. The pattern in thicker
bundles is the result of the gradually increasing effect of
the lower concentration of oxygen in the center of the
bundle on the production of the putative regulatory
substance. A zone of lower enzymatic activity gradually
appears in the center of the bundle as the thickness of the
bundle increases. The rise in enzymatic activity in the
center of thin bundles as they become thinner is also
predicted by the model.

An alternate model, in which there are two cardioac-
tive factors, one increasing and the other decreasing myocar-
dial contractile and enzymatic function, can be described
by the same equations and will produce the same results if
it is assumed that the downregulating factor is secreted by
the endocardial endothelium. As discussed below, there
are experimental reasons for considering the existence of
two factors with opposite effects.

Within the different zones of enzymatic activity, it is
possible to see some special characteristics. ATPase
activity is often substantially higher in the layer of cells
that immediately surrounds the small blood vessels but
not the capillaries. In view of the apparent absence of
any effect of capillaries on ATPase activity, it is relevant

![Figure 11](image1.png)

**Figure 11.** Left panel: Graph showing the relation between the relative actin-activated ATPase activity of V1 myosin in isolated perfused hearts and the oxygen saturation of the coronary venous effluent. Middle panel: Graph showing the relation between actin-activated ATPase activity of V1 myosin and O2 saturation in coronary venous effluent in the same hearts. Right panel: Graph showing the size of the increment in actin-activated ATPase activity of V1 produced by cAMP in frozen sections of the perfused hearts as a function of O2 saturation in the coronary venous effluent of the hearts.

![Figure 12](image2.png)

**Figure 12.** Graphs showing the relation between rate of coronary flow in isolated perfused hearts and the ATPase activity of V1 myosin (left panel), the increment in V1 ATPase activity produced by cAMP (middle panel), and the maximum ATPase activity under cAMP stimulation (right panel).
to note that immunocytological studies of endothelin-1 and endothelin receptors indicate the presence of both in small arteries and veins but their absence in capillaries of mammalian heart (Davenport, personal communication, and presentation at the Third Congress of Comparative Physiology and Biochemistry, Tokyo, August 1991). This is true for vessels oriented both perpendicular and parallel to the plane of the section. In some sections, one observes a row of cells with higher ATPase activity in the absence of a blood vessel. Because the thickness of each tissue section is about 30% of the diameter of a myocardial cell and the influence of a blood vessel may extend over at least two adjacent cells, a transversely oriented blood vessel present in one section may affect the ATPase activity in cells as far away as six or seven sections. In cases in which it was possible to trace the cells through a sufficient number of serial sections, the presence of blood vessels in the appropriate region of a nearby serial tissue section was identified.

Although nonuniform function among myocardial cells in superfused bundles of cardiac tissue, in contrast to the uniformity when the coronary circulation is perfused, can be considered to be an artifact of this type of in vitro preparation, the underlying mechanism that produces it should be of physiological significance. The presence of an endothelium-derived factor that modifies the enzymatic and mechanical properties of the contractile proteins is likely to be important in regulating the contractile behavior of the myocardial cells, particularly since it appears that either its release or the extent of its action on the myocardial cell appears to be sensitive to the local oxygen tension.

Previous work has shown that nonuniformity can be markedly reduced by the enhancement of ATPase activity in the more enzymatically weak cells by cAMP. Apparently, the putative endothelial factor acts on the myocardial cells by modulating the activity of a cAMP-dependent regulatory mechanism that modifies the function of the contractile proteins.

There are at least two major differences in the environment of endothelial cells in the isolated trabeculae from those in the perfused heart: the endothelial cells within the trabeculae are exposed to lower O2 tension than the cells in a perfused heart, and both the endocardial and vascular endothelial cells in the trabeculae are exposed to different physical forces from those in the normal in situ heart or in the isolated perfused heart. It is theoretically reasonable for the endothelial cell in the isolated trabeculae to behave differently from those in perfused hearts and for there to be a period of changing function as they adjust from the in vivo to the in vitro environment. It is possible to evaluate this notion experimentally since it appears that the cAMP-sensitive regulation of actomyosin ATPase activity is involved in the response to the putative endothelial factors with cardiac activity. To this purpose, the response of the cAMP regulation of the contractile proteins to changes in PO2 and to changes in the physical forces on the vascular endothelium was examined. As the coronary sinus oxygen tension declines, the actomyosin ATPase activity also declines. However, the response of actomyosin ATPase activity in frozen sections of these hearts to cAMP increases with declining O2 tension so that there is little difference in the cAMP-stimulated ATPase activity over a wide range of oxygen tensions. These results suggest that in the intact myocardial cell, reduction of PO2 decreases the ability of the cAMP-regulated mechanism to raise ATPase activity, but in the cryostatic section in the presence of a normal energy supply in the form of ATP, the responsiveness to cAMP returns. Below 20% saturation, the cAMP mechanism itself seems to break down. The existing data do not eliminate the possibility that the response of the enzymatic activity of actomyosin to the β-agonist in the presence of declining oxygen tension in the perfused heart results from a desensitization of the β-receptors.

A relation between the rate of coronary flow and cAMP regulation of actomyosin ATPase was observed. Increase in coronary flow, which would raise the shear force on the blood vessel endothelium, decreased actomyosin ATPase activity but increased the increment produced by cAMP. Although there was an inverse relation between actomyosin ATPase activity and coronary flow in the absence of cAMP, in the presence of the cyclic nucleotide, the enzymatic activity was independent of coronary flow. It is interesting that myosin with β heavy chain is not sensitive to the low PO2 and high coronary flow rate as is myosin with the α heavy chain. In the fetus, which is perfused by blood containing a low PO2, the predominant isoform of myosin heavy chain is the β form.

The pattern of ATPase activity and the proximity of cells with high actin-activated ATPase activity to blood vessels suggest that factors released by endothelial cells may be involved. Endothelin has been shown to increase
contractile tone in vascular smooth muscle and to increase contractility in cardiac cells.2,12,23 The peptide is effective in enhancing force at a concentration of nanomolar or less. An action on calcium channels has been observed, but all of the activity of endothelin cannot be produced by modification of calcium movements.12 First, endothelin binds to at least two different membrane receptors, one of which appears to have no direct connection to the calcium channel.25-29 Second, in myocardial cells in which the concentration of intracellular free calcium was measured with either fura-212 or aequorin,24 substantial increments in contractility were observed either without any change or with an inadequate change in intracellular calcium concentration to account for the enhancement of mechanical function. Third, the two endothelin receptors that have been cloned11-25 have sequences that are consistent with integral membrane proteins that interact with G proteins.

In this study, the effect of endothelin on the contractile proteins themselves has been measured under conditions in which the membrane receptors were still present but the concentration of calcium was maintained at a level at which it was not limiting. Under these conditions, endothelin in the nanomolar range increased actin-activated but not calcium-activated ATPase activity of myosin. Two different dose–response curves were seen, one in which the enzymatic activity continued to rise with increasing endothelin concentrations up to at least 50 nM and the other in which the effect reached a peak at 1 nM. Similar observations on contractility in isolated heart cells were made by Kelly et al.12 They found that in some cells the enhancement of contractility continued to increase above 1 nM and in other cells the effect began to fall above 1 nM. No reason was given by them for these observations.

A mechanism in which the level of contractility of the contractile proteins was regulated by a substance released from vascular endothelium within the coronary circulation according to oxygen tension and possibly perfusion rate would be very useful to the function of the heart. The amount of work done by myocardial cells could be adjusted by coronary blood flow, resulting in a balance between energy supply and work performed. Studies with tracheal smooth muscle indicate that endothelial cells respond to changes in oxygen tension in their environment before oxygen tension becomes limiting for oxidative phosphorylation in the mitochondria.26 Such a mechanism in the heart would regulate contractility in response to energy supply before the rate of oxidative phosphorylation became limiting.

In isolated preparations of tissue containing smooth muscle, release of endothelin may occur with a decrease in oxygen tension. In cardiac muscle it has been associated with coronary vasospasm.4,27 This is the opposite direction to what seems to be occurring in the studies reported here although no direct correlation with PO2 has been measured. It is difficult, however, to understand what utility the organism there would be in a mechanism that causes coronary vasocostriction in the presence of lower oxygen tension.27 In fact, this is the opposite of what has been seen in studies of coronary flow in in situ hearts. Clarification of this conundrum may come with an understanding of the factors producing the two different dose–response curves for endothelin or of the mechanical effects of flow within the vessel on the function of the endothelial cells.

Relevant to the data reported here may be the observations of Brutsaert and coworkers9,28 and Lewis et al29 dealing with the effect of the endocardium on the function of the contractile cells. They have found that damage of the endocardium causes a decrease in the peak force of contraction and the time to peak tension. In the thin papillary muscles generally used by investigators in mechanical and biophysical studies of the heart, the endocardium makes a very substantial contribution to the total population of endothelial cells. If all endothelial cells contribute to the production of a factor that upregulates contractility, then inactivation of the endocardium could produce a substantial decline in the concentration of the substance and loss of contractility of some myocardial cells, particularly in those cells located nearest the endocardium. The presence of such a population of very weakly or noncontracting cells would decrease force production and, by constituting an

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**Figure 14.** To explain the patterns of ATPase activity in bundles of cardiac cells of different thicknesses, a model is proposed with the following characteristics: 1) A factor is produced by blood vessels (presumably the endothelial cells) that regulates the calcium responsiveness of cardiac actomyosin so that actomyosin ATPase activity and force generation are proportional to the concentration of the factor; 2) the rate of production of the putative factor decreases as the concentration of oxygen decreases and possibly as perfusion of the blood vessel declines as well; and 3) the factor diffuses out of the bundle into the superfusing solution so that its concentration at the surface of the bundle is zero. The expected concentration profiles of the factor are transverse sections of ellipsoidal bundles of different thicknesses calculated using the diffusion equations of Hill. These profiles should be compared with the experimentally measured profiles shown in Figure 8.
internal load on the normally contracting cells, terminate the rise in force at an earlier time in the contraction. If this explanation is correct, then when the endocardium has been damaged, the subjacent cells should have a low actin-activated ATPase activity.

In the work reported here, the myocardial cells that are immediately below the endocardial endothelium have the lowest ATPase activity during the period of stabilization of contractile activity, a result that would not be expected if these endothelial cells are the source of a factor that upregulates contractility. This apparent inconsistency can be explained in at least two different ways. First, it is possible that adjustment of endocardial endothelial function is an important part of contraction stabilization after isolation of the superfused tissue bundle; second, endothelial cells may release both up-regulating and downregulating factors as occurs for smooth muscle, and the relative amounts of the two factors released by the endocardial endothelium may be influenced by the dissection and/or the physical forces on the endothelium during superfusion. The response of ATPase to the rate of coronary flow favors this possibility.

There are other reasons for considering that at least two cardioactive factors, one upregulating and one downregulating, are released by endothelial cells. In the case of endothelial regulation of vascular smooth muscle tone, this has already been shown.30 The low ATPase activity in the most superficial cells reverses either with superfusion time or with cAMP. If the decline is due to the washing out of an upregulating substance, it is difficult to see how a longer period of superfusion would result in the reversal of this washout. It is more reasonable for a downregulating substance released by the endocardial endothelium in response to the unusual shear forces experienced by these cells in the in vitro preparation to be responsible. With prolonged superfusion the rate of release of this substance could decline or disappear. cAMP would be capable of preventing the downregulating effect of the factor.

In summary, we feel that the data presented indicate that endothelial cells in cardiac muscle release an upregulating and probably a downregulating factor acting on the contractile proteins in the myocytes, presumably through surface receptors on the myocytes. The rates of production of these factors are sensitive to local Po2 and physical forces acting on the endothelial cells. The action of these factors may involve modulation of a cAMP-sensitive mechanism for regulating the contractile proteins.

Appendix

Assume that a papillary muscle is of approximately elliptical cross section with the long axis of the ellipse being twice the short axis. If we assume that an inotropic substance is released uniformly across the cross section of the papillary muscle at a constant rate in response to the concentration of a second substance and that the inotropic substance is degraded by the tissue at a constant rate, then the relative concentration of the inotropic substance at any point in the cross section of the papillary muscle can be estimated from the solution of the diffusion equation with chemical reaction in two dimensions as given by Crank31 and Hill32:

$$\frac{\partial C}{\partial t} = D \left( \frac{\partial^2 C}{\partial x^2} + \frac{\partial^2 C}{\partial y^2} \right) + \frac{\partial S}{\partial t} \frac{\partial A}{\partial t} \quad (A1)$$

where C is the concentration of inotropic substance at any point x,y; D is the diffusion coefficient through the tissue; \(\frac{\partial S}{\partial t}\) is the rate of production of inotropic substance by the cells; and \(\frac{\partial A}{\partial t}\) is the rate of degradation by the cells.

If we further assume that the concentration of the inotropic substance can achieve a steady state in the tissue and that the production and degradation are both first-order processes depending only on the concentration of the inotropic substance for degradation and a second substance for production, then

$$\text{limit } t \to \infty \quad \frac{\partial C}{\partial t} = 0$$

$$\frac{\partial S}{\partial t} = VO$$

$$\frac{\partial A}{\partial t} = aC$$

where O is the concentration of the second substance, V is the first-order rate coefficient for production of inotropic substance, and a is the first-order rate coefficient for decrease of the inotropic substance.

Substituting these into Equation A1 results in

$$D \left( \frac{\partial^2 O}{\partial x^2} + \frac{\partial^2 O}{\partial y^2} \right) + VO - aC = 0 \quad (A2)$$

To solve this equation, it is necessary to determine the concentration of the second substance as a function of x and y in the cross section of the papillary muscle. If we assume that the second substance is a diffusible molecule like oxygen that can be considered to be at constant concentration in the external solution and used by the tissue at a rate proportional to its concentration, an equation similar to Equation A2 can be derived. Hence

$$D_O \left( \frac{\partial^2 O}{\partial x^2} + \frac{\partial^2 O}{\partial y^2} \right) - kO = 0 \quad (A3)$$

where O is the concentration of the second substance at any point x,y; Do is the diffusion coefficient of the second substance through the tissue; and k is the first-order rate coefficient for uptake of the second substance by the tissue.

Equations A2 and A3 were solved numerically using standard finite-difference formulations.31,32 The solution of Equation A3 was substituted into Equation A2 to provide the concentration of the substance diffusing in from the outside. The solution to Equation A2 is shown graphically in Figure 14 for bundles of three relative sizes. For this solution, we have taken a value of 2.33×10^-3 mm^2/sec for the diffusion coefficient of the substance diffusing in from the external solution. This is the value given by Hill for the diffusion of oxygen. The rate of utilization, k, was set at 1.5×10^-4 mm^2/sec, which is compatible with oxygen utilization by mammalian cardiac tissue at 20–25°C. The diffusion coefficient for the inotropic substance was set at 1.0×10^-6 mm^2/sec and the rate coefficient for breakdown was set at twice the rate coefficient of production (a=1.0×10^-6/sec and V=5.0×10^-4/sec). The basic shape of the curve is relatively insensitive to variations in these three rate coefficients over about an order of magnitude range.

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