An X-Ray Diffraction Study on Mouse Cardiac Cross-Bridge Function In Vivo: Effects of Adrenergic β-Stimulation

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ABSTRACT To investigate how β-stimulation affects the contractility of cardiac muscle, x-ray diffraction from cardiac muscle in the left ventricular free wall of a mouse heart was recorded in vivo. To our knowledge, this is the first x-ray diffraction study on a heart in a living body. After the R wave in electrocardiograms, the ratio of the intensities of the equatorial (1,0) and (1,1) reflections decreased for ~50 ms from a diastolic value of 2.1 to a minimum of 0.8, and then recovered. The spacing of the (1,0) lattice planes increased for ~90 ms from a diastolic value of 37.2 nm to a maximum of 39.1 nm, and then returned to the diastolic level, corresponding to ~10% stretch of sarcomere. Stimulation of β-adrenergic receptor by dobutamine (20 μg/kg/min) accelerated both the decrease in the intensity ratio, which reached a smaller systolic value, and the increase in the lattice spacing. However, the intensity ratio and spacing at the end-diastole were unchanged. The recovery of the lattice spacing during relaxation was also accelerated. The mass transfer to the thin filaments at systole in a β-stimulated heart was close to the peak value in twitch of frog skeletal muscle at 4°C, showing that the majority of cross-bridges have been recruited with few in reserve.

INTRODUCTION

Stimulation of cardiac β-adrenergic receptors has been known to enhance contractility of cardiac muscle through phosphorylation of various enzymes, including troponin-I, C-protein, and phospholamban (1–6). Although these effects have been extensively studied both physiologically and biochemically in isolated cardiac muscles and myocytes, they can be studied in a living body by only a limited number of methods. Lack of a molecular index that can be measured in vivo has made it difficult to study the mechanism of the β-stimulation. Since the overall influence of neurohumoral factors on functional properties of cardiac muscle can only be studied in vivo, it is important to develop a method to monitor contractility at the molecular level in a live animal. This is especially important when investigating the consequences of genetic alterations.

It is usually difficult to study murine, especially mouse, cardiac muscle in an isolated specimen under physiological conditions. Perfusion with oxygenated saline does not provide enough oxygen, and a heart tends to be in a hypoxic condition at physiological heart rates. Although an x-ray diffraction study on an intact rat papillary muscle has the advantage that force and sarcomere length can be measured simultaneously, such experiments have been made at a heart rate lower than 1 Hz (7,8). Since the heart rate is an important factor in cardiac physiology, which affects calcium handling and contractile force, it is necessary to make these experiments at higher heart rates. This is especially the case if the increase in the contractile force with increased heart rate (the staircase phenomenon) is due to a higher number of cross-bridges associated with the thin filament during diastole (9).

X-ray diffraction has been used to study contractility of cardiac muscle. It is a noninvasive method which enables us to study myosin cross-bridge activity in striated muscles (10). Two pieces of information can be obtained: one is the intensity ratio of the (1,0) and (1,1) equatorial reflections from the hexagonal lattice of myofilaments, which can be used as an index of the number of myosin cross-bridges formed during contraction (mass transfer from the thick to the thin filament). This correlates well with tension development in cardiac muscle, especially during the early phase of contraction under an isometric condition (9). Theoretically, the intensity ratio might be affected by a conformational change of cross-bridges. However, experiments on skeletal muscle showed that a force-generating conformational change does not affect the ratio (11). Thus, the change in the ratio is mostly caused by a mass movement from the thick filaments to the thin filaments due to cross-bridge formation. The other piece of available information is the (1,0) spacing of the lattice, which is equal to $\sqrt{3}/2$ of the distance between neighboring thick filaments. Since the volume of a cardiac cell remains approximately constant during a cardiac cycle (7), the lattice spacing can be used as an index of sarcomere length.

Recently, it was shown that diffraction patterns from a whole heart can be interpreted based on the orientation of muscle fibers in the heart (12), and a time-resolved x-ray diffraction study was made on a heart of a thoractomized rat (13). Here we applied this technique to a heart in a body of a

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living mouse to study physiological functions of myosin cross-bridges in vivo. Without thoractomizing, this is the first time the contractility in heart muscle was studied at a molecular level in a living body. Effects of β-stimulation by dobutamine were investigated.

METHODS

Animal preparation

Eight-week-old male mice (C57BL/6, purchased from CLEA Japan, Tokyo, Japan) were anesthetized with isoflurane (0.25–0.5%) and artificially ventilated (MiniVent, Hugo Sachs Elektronik, March-Hugstetten, Germany; stroke volume 200 μl, 250 strokes/min). To avoid strong x-ray diffraction spots from skeletal muscles (see below), part of the breast muscles was surgically removed. The mouse was fixed vertically in the x-ray beam, which entered the thorax from the third intercostal space. In this configuration, the x-ray beam passed through the upper part of the left ventricle, whose motion during a heart beat is smaller than in the lower part. The electrocardiogram was recorded with three electrodes (Fig. 1). Systemic blood pressure was measured by a computerized tail-cuff apparatus (MK-2000, Muromachi Kikai, Tokyo, Japan). The ventilator was stopped during an x-ray exposure (for ~2 s) to avoid movement due to respiration. Dobutamine was infused from carotid artery at a rate of 20 μg/kg/min. The animal experiments were conducted in accordance with the guidelines of SPring-8 for care and welfare of experimental animals.

X-ray diffraction methods

X-ray diffraction experiments were conducted at BL40XU in the SPring-8 third generation synchrotron radiation facility (Harima, Hyogo, Japan) (14). The peak x-ray energy was adjusted to 15.0 keV. This high energy ensured sufficient penetration of x-rays through the body of a mouse. The x-ray flux was adjusted by the front-end slits and aluminum absorbers to $\sim 3 \times 10^{12}$ photons/s. The beam size was ~0.10 mm vertically, 0.25 mm horizontally at the specimen.

The x-ray detector was an x-ray image intensifier with a beryllium window (V5445P, Hamamatsu Photonics, Hamamatsu, Japan) (15) coupled by a tandem lens to a fast charge-coupled device (CCD) camera (C4880-80-24A, Hamamatsu Photonics). The time resolution was 15 ms/frame. An x-ray shutter was opened for 1.1 s, and 70 successive frames were recorded.

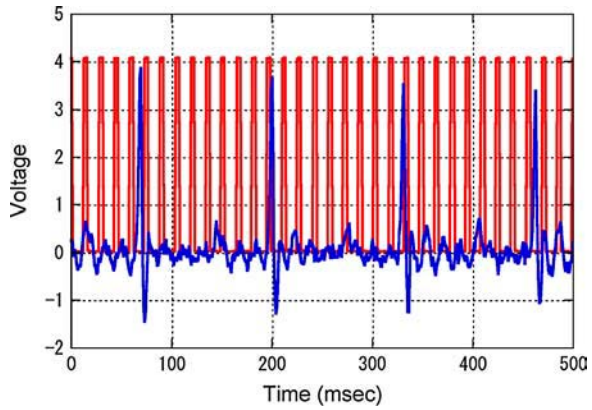

FIGURE 1 Electrocardiogram (*blue*) and frame timing of the CCD camera (*red*) during time-resolved x-ray recording. There is noise in the electrocardiogram from the power line (60 Hz) but sharp QRS peaks and preceding small Q peaks are clearly seen. The rising edge of the frame timing pulses corresponds to the beginning of a new frame.

The specimen-to-detector distance was 3.2 m. The lattice spacing was calibrated with the third-order meridional reflection from the thick filament of frog skeletal muscle at 1/14.34 nm^{-1}. Both the electrocardiogram and the frame timing signal from the CCD camera were recorded with a data acquisition system with 1-ms sampling.

Experimental protocol

Since there are layers of skeletal muscles in the path of the x-ray beam, diffraction patterns from a mouse heart in vivo are always mixed with equatorial diffraction spots from skeletal muscles. With a mouse positioned vertically and an x-ray beam passing between the third and fourth ribs, usually two sets of spots were found in the directions of 10 o'clock and 4 o'clock, and 9 o'clock and 3 o'clock (Fig. 2). The areas between these spots were free from reflections due to skeletal muscles. Initially, the x-ray beam was positioned in the left side of a mouse thorax (Fig. 3, position *A*). Then, the mouse was moved across the beam horizontally so that its left ventricular free wall came into the beam (Fig. 3, position *B*). When the x-ray beam passed through the epicardium of the free wall, the equatorial reflections, which appear as spots (12), were observed in the directions similar to those of skeletal muscles. Thus, the epicardium surface plane seems to be approximately vertical in the mouse body. At this position, the movement of the heart due to beating shifted the heart in and out of the beam in every heart beat, making it impossible to observe the diffraction pattern continuously. When the mouse was moved further and the x-ray beam passed through the deeper layer of the free wall (Fig. 3, position *C*), the equatorial diffraction pattern became arcs (12). At this stage, it was still difficult to observe the diffraction pattern continuously because the beam passed through different regions of the wall in diastole and systole. When the mouse was moved further in the beam (Fig. 3, position *D*), the diffraction pattern appeared as a ring or a long arc with maximum intensity in the direction perpendicular to the diffraction from skeletal muscles (Fig. 2). The diffraction pattern was continuously observed during a heart beat, allowing us to study the molecular changes throughout a cardiac cycle. Thus, experiments were made under this condition. The x-ray beam is presumed to be passing through endocardium and left ventricle (12). Marking the recorded region after an experiment, with an x-ray flux three orders of magnitude higher than that used for the x-ray diffraction measurement, confirmed that the beam actually passed through the left ventricular free wall.

The x-ray recording was repeated up to 10 times (average 5.3 recordings) with a horizontal shift of a mouse by 0.2 mm. The data were analyzed only when distinct diffraction from cardiac muscle was observed throughout a cardiac cycle. After dobutamine infusion, the mouse was moved vertically by ~0.2 mm to avoid radiation damage, and the recordings were repeated. No sign of radiation damage, such as broadening or weakening of the equatorial intensity profile, was seen.

Since the x-ray recording was not in synchrony with the cardiac cycle, it was necessary to determine correspondence between the x-ray frames and the cardiac cycle. At the heart rate of 400–500 per min, there were 8–9 frames per each cycle, and 7–8 cycles were recorded in each x-ray recording, which lasted for $15 \times 70 = 1050$ ms. In this study, only the data with nine frames per cycle were analyzed. The frames that contained the R wave in the electrocardiogram (Fig. 1) were used as the first frame of a cycle (end-diastole). Then, frames in the same phase of the cycle were averaged. Thus, for each recording, a set of images was obtained. As the R wave could take place any time within a frame, the data had an ambiguity of one frame (15 ms) in time. This ambiguity is averaged out because, in total, data from 53 and 58 recordings were used for the baseline and dobutamine conditions, respectively.

Data analysis

Diffraction in the region of the x-ray pattern without contribution from skeletal muscles was circularly averaged. The background was fitted with a cubic spline function, and the area above the background was used as the

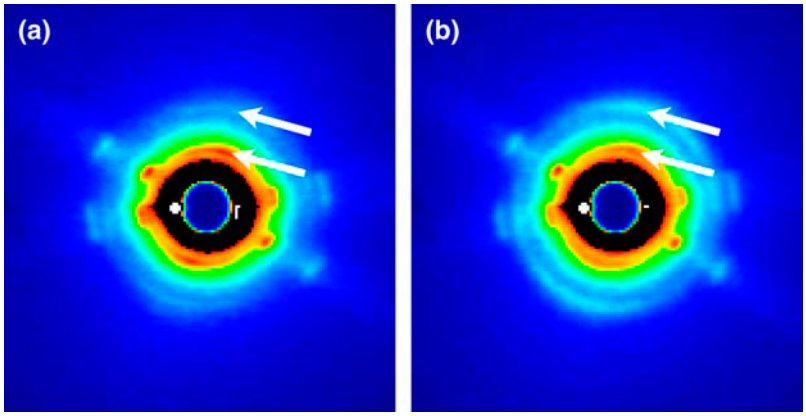

FIGURE 2 X-ray diffraction patterns from a heart in vivo. (*a*) In end-diastole (first frame), (*b*) in systole (fourth frame). The intensity distribution is shown in pseudocolor. The four spots are from skeletal muscles. The rings in the rest of the pattern (*arrows*) are from cardiac muscle: the inner one is the (1,0), and the outer (1,1) equatorial reflection. These are averages of six diffraction patterns from a normal mouse.

diffraction peak (Fig. 4). The integrated area was used as the integrated intensity, and the center of gravity of the (1,0) reflection was converted to the (1,0) lattice spacing. The integrated intensity of the (1,1) reflection obtained this way is underestimated by $\sqrt{3}$ compared to that of the (1,0) reflection because the intensity is averaged, not summed, along an arc. Thus, the (1,1) intensity was multiplied by $\sqrt{3}$. Since the intensity of each equatorial reflection depends on the thickness of the sample, which changes considerably across a heart, the ratio of the intensities of the (1,0) and (1,1) reflections (I(1,0)/I(1,1)) was used as an index of the equatorial intensity change.

Then, data from different x-ray recordings were analyzed. Results from recordings on each mouse were averaged, providing a set of intensity ratios and lattice spacings for each mouse over frames. Then, data sets from 10 mice were treated statistically. The same procedure was used on the data taken after dobutamine infusion.

The half-time of contraction and relaxation was obtained by the following method: 1), The intensity ratio or lattice spacing in the first frame was taken as a diastolic level. 2), The smallest intensity ratio or the largest lattice spacing value was taken as a systolic level. 3), The average of the diastolic and systolic levels was taken as the midlevel. 4), The time when the intensity ratio or lattice spacing crossed the midlevel during the early or late phase of a heart beat was obtained by interpolation between frames and taken as the half-time of contraction or relaxation.

Using the ratio of the (1,0) and (1,1) intensities (I(1,0)/I(1,1)), the electron-density distribution in the transverse section of the hexagonal myofilament lattice was calculated by Fourier synthesis. From this, the mass associated with the thin filaments was estimated following the method of Haselgrove and Huxley (10) on the assumption that the lowest density in the electron-density map represented the background level. The ''apparent'' thin-filament mass thus calculated was interpreted as consisting of the thin filament and the myosin heads present in the vicinity of the thin filament. The mass of the thin filament was approximated by assuming that no myosin heads are present in the vicinity of the thin filament at the resting state. Then, by subtracting this from the apparent thin-filament mass, the mass of heads associated with the thin filament was obtained. The same procedure was done with the intensity ratio obtained in the rigor state, and the total mass of heads can be obtained by assuming that all heads are attached to the thin filament. From these masses of heads, the proportion of heads present in the vicinity of thin filaments was calculated.

All data are expressed as mean ± SD. Differences were analyzed using Student's paired *t*-test with $p < 0.05$ being regarded as statistically significant.

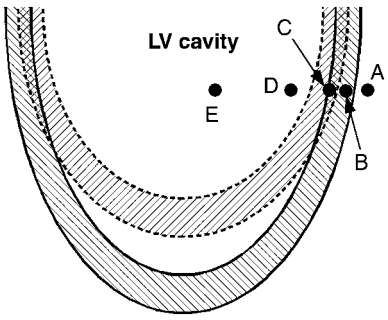

FIGURE 3 Positions of the x-ray beam relative to the left ventricle. The shaded areas represent a left ventricular free wall. The area between solid curves is a wall in diastole, and that between broken curves a wall in systole. At position *A*, the beam is out of the heart. At *B*, the beam is passing through the epicardium in diastole but out of the heart in systole. At *C*, the beam is always in the wall but it is in the epicardium in diastole and in the endocardium in systole. At *D*, the beam is always passing through the wall on both sides of the heart. At *E*, the beam also passes through the wall on both sides but absorption by blood in the cavity hampers the x-ray measurement. In the actual experiment, a mouse (hence a heart) was moved relative to a fixed x-ray beam.

RESULTS

Fig. 2 shows x-ray diffraction patterns from a mouse heart in vivo at end-diastole and systole. Although diffraction spots from skeletal muscles are superposed, arcs of equatorial diffraction from cardiac muscles are clearly seen. The origin of the reflection can be assigned without ambiguity because the skeletal muscle has a smaller filament lattice spacing and hence the reflections appeared at a larger radius. When the region where the equatorial reflections from cardiac muscle were strong, it was possible to subtract background from other tissues such as skin, lung, and skeletal muscle (Fig. 4). Fig. 5 *a* (*open squares*) shows the time course of changes in the (1,0)/(1,1) intensity ratio during a cardiac cycle at baseline. The diastolic intensity ratio was ~2.1 at end-diastole and decreased to a minimum of 0.8 in ~50 ms, which is comparable to the ratios observed in rat heart muscle (13).

Fig. 5 *b* (*open squares*) shows the time course of changes in the (1,0) lattice spacing at baseline. The lattice spacing at end-diastole was 37.2 nm and increased to 39.1 nm within

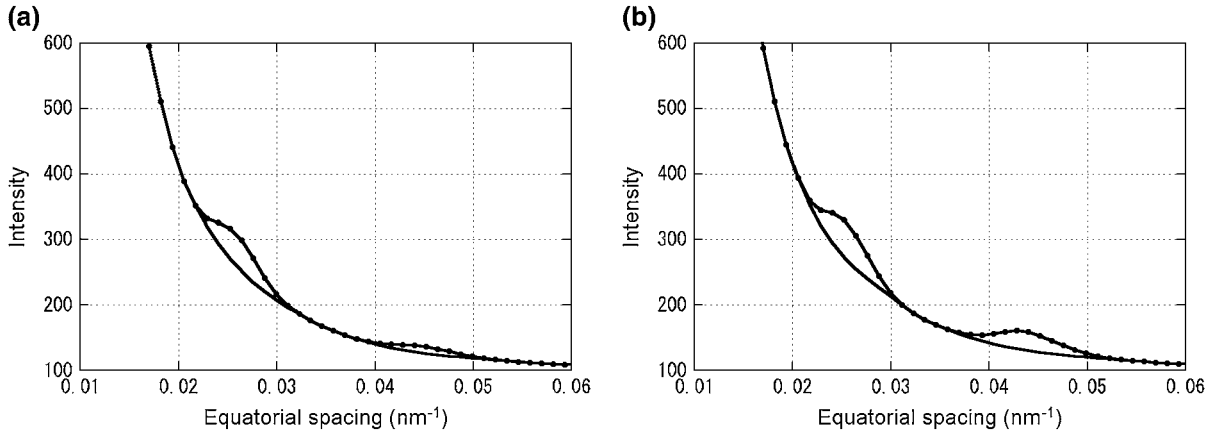

FIGURE 4 Intensity profiles of the (1,0) and (1,1) equatorial peaks. (a) in end-diastole, (*b*) in systole. The data were from Fig. 3, integrated within arcs of 10.5 o'clock through 2 o'clock and its opposite side. The background was drawn using a spline function.

~90 ms after the R wave in the electrocardiogram. If strict constant-volume behavior is assumed, this corresponds to ~10% shortening of sarcomere. The lattice spacing continued to increase after the intensity ratio started to increase (that is, after the muscle began to relax), until the mitral valve opened and the left ventricular volume began to increase.

The experiment was repeated after dobutamine had been infused for 10 min (20 μg/kg/min). The heart rate did not change significantly (from 446 $\pm$ 26 to 449 $\pm$ 26 beats per min, $n = 10$). The systemic blood pressure increased significantly from 83.9 $\pm$ 13.1 to 97.1 $\pm$ 14.9 mmHg ($n = 10$). Fig. 5 *a* (*solid circles*) shows the change in the (1,0)/(1,1) intensity ratio under the influence of dobutamine. The second, third, and fourth data points between 20 and 60 ms after the R wave were significantly lower than those in the control mouse, showing a faster and larger shift of mass of cross-bridges. Fig. 5 *b* (*solid circles*) shows the time course of the (1,0) spacing change. The third and fourth data points are significantly larger than those in the control mouse, showing a faster shortening of muscle. The half-time of the reduction in the intensity ratio was significantly shortened by dobutamine (24.4 $\pm$ 1.4 to 20.3 $\pm$ 1.3 ms, $n = 10$), and that of the lattice spacing was also shortened (52.8 $\pm$ 5.9 to 38.3 $\pm$ 1.8 ms, $n = 10$). The half-relaxation time of the lattice spacing was significantly shorter after dobutamine (112.6 $\pm$ 3.0 to 106.8 $\pm$ 2.8 ms, $n = 10$). Since the larger change in the lattice spacing suggests larger sarcomere shortening, the systolic intensity ratio might be affected by a larger filament overlap in the presence of dobutamine. However, since cardiac muscle normally works at a sarcomere length shorter than 2 μm where the thick and thin filaments are fully overlapped, this effect is not considered significant.

After an experiment, a mouse was killed by overdose of pentobarbital and left for 30 min. Then, a diffraction pattern in the rigor state was recorded. The (1,0)/(1,1) intensity ratio was 0.30 $\pm$ 0.08 ($n = 8$).

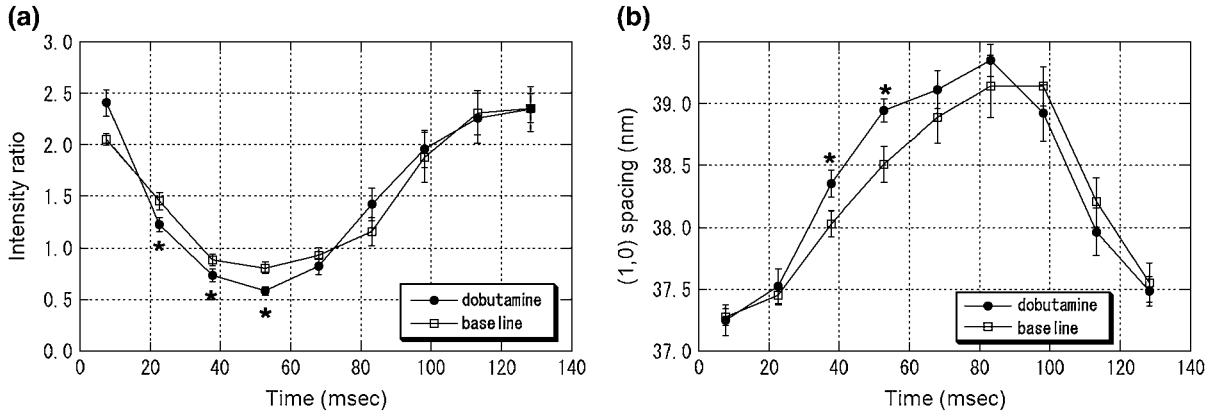

FIGURE 5 Effects of β-stimulation on the cross-bridge activity of mouse heart muscle. (*a*) The (1,0)/(1,1) intensity ratio, (*b*) the (1,0) lattice spacing. The abscissa is the time after the R wave in the electrocardiogram. Open squares represent data obtained before dobutamine infusion, and solid circles after dobutamine infusion (20 μg/kg/ml). The error bars represent the standard error of the mean of data from 10 mice. The asterisks indicate the data points that are statistically different in paired *t*-tests between baseline and dobutamine data.

DISCUSSION

This study is the first, to our knowledge, to investigate myosin cross-bridge activity in a heart in a living body. As stated in the Introduction, it is usually difficult to study murine, especially mouse, cardiac muscle in an isolated specimen under conditions that very closely match physiological. The technique here enables one to investigate mouse cardiac muscle under the most physiological condition, opening a way to study cardiac muscle functions of transgenic mice with x-ray diffraction.

The (1,0) lattice spacing observed in this study (37.2 nm in diastole) is close to that reported in an isolated rat heart with saline perfusion (12), as well as in an isolated intact rat papillary muscle at a sarcomere length of 2.1 μm (7). If we assume that a mouse heart in vivo has the same sarcomere length-lattice spacing relation as an isolated rat papillary muscle, the working range of sarcomere length is between 1.9 and 2.1 μm. On the other hand, a much larger lattice spacing has been reported in a thoractomized rat (13). This may be due to a change in osmolarity which is caused by exposure of the circulatory system to the air. Although the open-chest model of rat has the advantage that pressure and volume of left ventricle can be measured easily during the x-ray measurement, it may be too invasive to study the true physiological state of the heart. The present closed-chest method and the open-chest method should be regarded complementary. A lattice spacing of 34 nm was reported in isolated intact rat trabeculae at a sarcomere length of 2.2 μm (16), which is smaller than the present value. Lattice spacing may be important in cardiac muscle because the distance between myofilaments may affect the contractile tension and play a role in Frank-Starling's law. Previous studies to examine the effect of lattice spacing on tension development were made in skinned fibers which had a (1,0) spacing $>$40 nm (17,18). It is desirable to investigate the effect at smaller lattice spacings, which are more physiological.

The intensity ratios in diastole and systole found in this study are generally similar to those in previous studies. It has been reported that more cross-bridges are formed in heart muscle preparations that are perfused by blood than in those perfused by saline (9). However, comparison of the results on saline-perfused rat heart (12), on blood-perfused rat heart (13), and from this study on mouse heart under the normal condition shows only small differences. Since the early studies required many contractions to record an x-ray diffraction pattern on a photographic film, deterioration of the sample was probably the major cause of low contractility observed in previous studies on saline-perfused specimens.

In isolated cardiac muscles, it has been found that adrenergic β-stimulation enhances the contractile force and makes both tension development and relaxation faster. This is explained by acceleration of calcium uptake (through phosphorylation of phospholamban) and reduction of the binding affinity of troponin-C to calcium (through phosphorylation of troponin-I) (2–4). These lead to faster development of tension, larger twitch tension, and faster decline of tension in cardiac muscle. In this study, the shorter half-time of reduction in the intensity ratio shows a faster formation of cross-bridges with β-stimulation and the lower minimum intensity ratio shows that more cross-bridges are formed. Although the change in the intensity ratio during relaxation was not clearly accelerated, β-stimulation shortened the half-relaxation time of the lattice spacing. These results demonstrate that the changes of contractile properties previously observed in isolated muscle are actually taking place in a heart muscle in vivo.

In this study, the blood pressure and the amount of myosin cross-bridge formation was increased by dobutamine infusion. On the other hand, the heart rate did not change, indicating that the effects of dobutamine stress were modest. Under this condition, the most interesting finding is that β-adrenergic stimulation does not affect the end-diastolic state. Especially, the lattice spacing at end-diastole was unchanged (Fig. 5 *b*). Since the lattice spacing is related to sarcomere length, this suggests that the left ventricular volume at end-diastole was unaffected. The elevation of blood pressure by β-stimulation was simply due to recruitment of more cross-bridges in cardiac muscle of the free wall. The Frank-Starling mechanism, which would be relevant if the end-diastolic volume were larger and the sarcomere length longer, did not play a major role.

By assuming that all myosin cross-bridges are in the vicinity of the thick filaments in a resting state and that all are bound to the thin filaments in the rigor state, it is possible to calculate the fraction of cross-bridges that are transferred to the vicinity of the thin filaments during contraction (see Methods). In canine cardiac muscle, the resting (quiescent) state may not be the same as the diastolic state (9). However, in rat heart muscle, no such evidence has been found. Thus, the diastolic ratio (the first frame in Fig. 5 *a*) is taken as the resting value. Then, the peak cross-bridge mass transfer ratio under the normal condition is 56%, which increases to 74% by dobutamine infusion. The increase is by 34%, whereas the systemic blood pressure increased by 16%. If we take the resting ratio as an average of those in the absence and presence of dobutamine (2.23), the increase is by 26%. In both cases, the increase in the mass transfer ratio is larger than that in the systemic blood pressure. Since the mass transfer ratio with the β-stimulation (75%) is close to the maximum level observed in tetanus of frog skeletal muscle, the major fraction of cross-bridges is recruited for contraction. Thus, even at this modest level of stress that causes an increase in blood pressure but not in heart rate, most of the potential cross-bridge attachments have been made with few in reserve in the β-stimulated heart.

Weisberg and Winegrad (19) showed by electron microscopy that the heads of the α-isoform of myosin heavy chain are more extended from the backbone of the filament when C-protein was phosphorylated by protein kinase A. Since the

α-isoform is dominant in adult mouse ventricle (20) and β-stimulation causes C-protein phosphorylation (1,3,6), a change in the equatorial intensity ratio is expected. Although the results here do not support this observation, more studies at higher dobutamine stress are needed to clarify the effects of C-protein phosphorylation on the behavior of myosin heads.

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