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# Effect of ventricular stretch on contractile strength, calcium transient, and cAMP in intact canine hearts

KOJI TODAKA, KAZUHIDE OGINO, ANGUO GU, AND DANIEL BURKHOFF  
*Division of Circulatory Physiology, College of Physicians and Surgeons,  
Columbia University, New York, New York 10032*

**Todaka, Koji, Kazuhide Ogino, Anguo Gu, and Daniel Burkhoff.** Effect of ventricular stretch on contractile strength, calcium transient, and cAMP in intact canine hearts. *Am. J. Physiol.* 274 (*Heart Circ. Physiol.* 43): H990–H1000, 1998.— Isovolumic contractions were imposed by intraventricular balloon in 39 isolated, blood-perfused canine hearts to investigate the effects of myocardial stretch on contractile force. After stabilization at 37°C, left ventricular volume was increased so that end-diastolic pressure increased from 0 to 5 mmHg. After the immediate increase in developed pressure [DP; from  $37 \pm 14$  to  $82 \pm 22$  mmHg (means  $\pm$  SD)], there was a slow secondary rise in DP ( $97 \pm 27$  mmHg) that peaked at 3 min. However, DP subsequently decreased over the next 7 min back to the initial value ( $84 \pm 25$  mmHg). Light emission from macroinjected aequorin ( $n = 10$  hearts) showed that changes in intracellular calcium [3 min:  $124 \pm 15\%$  ( $P < 0.01$ ); 10 min:  $99 \pm 18\%$  of baseline] paralleled DP changes. Increases in myocardial adenosine 3',5'-cyclic monophosphate (cAMP) content ( $n = 12$ ) accompanied the secondary rise in DP. In contrast, the gradual elevation of DP after the stretch was not exerted during continuous  $\beta$ -adrenergic stimulation by isoproterenol. Thus, in contrast to isolated muscle, stretch only transiently increases intracellular calcium and contractile strength in intact hearts. The findings of changes in cAMP and abolition of the phenomena by  $\beta$ -stimulation suggest that a primary stretch-mediated influence on cAMP metabolism may underlie these phenomena.

adenosine 3',5'-cyclic monophosphate; contractility

WHEN MYOCARDIUM IS STRETCHED, there is an immediate increase in force generation that has been attributed to increased myofilament calcium affinity, decreased thin filament overlap, and decreased interfilament spacing (1). It has also been well documented that, in addition to this immediate effect of myocardial stretch, there is a more gradual, secondary increase in contractile strength; this phenomenon has been observed in isolated myocytes (46), isolated cardiac muscle strips, and intact isolated canine hearts (40). Studies in isolated muscle have shown that these secondary changes in contractile force are related to an underlying gradual increase in peak intracellular calcium (2, 3). The results of these studies have therefore suggested the existence of a mechanism whereby the myocardium can adjust its intrinsic contractile strength (contractility) to alterations in the hemodynamic load imposed on the heart (1).

The mechanisms underlying stretch-induced increases in peak intracellular calcium remain to be elucidated. Factors such as stretch-activated ion channels (21), stretch-induced alterations in sarcoplasmic reticulum function (5, 18), improved oxygenation of the muscle core (29), and stretch-induced catecholamine

release from intramyocardial nerve endings (29) have been studied in isolated muscle preparations. More recently, results of in vitro studies in various cell types suggest that stretch may directly activate adenylate cyclase and thus increase intracellular adenosine 3',5'-cyclic monophosphate (cAMP; Refs. 31, 43, 44), a factor that could potentially contribute to alterations in intracellular calcium (12); however, the potential role of this factor has not been extensively investigated in the context of myocardial stretch. For the intact heart, factors such as subendocardial ischemia and subsequent metabolic autoregulation of the coronary vasculature have been proposed as the primary mechanism underlying stretch-mediated changes in contractile force (41), shifting emphasis away from this phenomenon being a fundamental property of cardiac muscle.

In the present study, we used an isolated, blood-perfused canine heart preparation to more thoroughly define ventricular contractile responses to acute alterations in stretch, to test whether the basic phenomena relate to altered regional blood flow (RBF), and, in the absence of such an effect, to investigate other possible underlying mechanisms. Measurements of RBF, intracellular calcium (macroinjected aequorin), and tissue cAMP in response to altered myocardial stretch and the different receptor agonists and antagonists are used to accomplish these goals. The salient aspects of the results reveal new fundamental observations about myocardial responses to stretch under physiological conditions, confirm underlying changes in intracellular calcium, exclude a primary role of alterations in coronary blood flow, and implicate change in cAMP as a participant in the underlying mechanisms.

## METHODS

*Isolated heart preparation.* We employed a standard isolated, cross-perfused canine heart preparation that has been described in detail previously (39). Briefly, 39 mongrel donor dogs of either sex ( $23 \pm 2$  kg) were anesthetized by intravenous pentobarbital sodium (25–30 mg/kg); each heart was explanted and metabolically supported by blood provided from a second support dog. The femoral arteries of the support dog were cannulated and connected to a perfusion circuit consisting of two peristaltic pumps, a heater, a blood filter, and an air trap. The support dog received 3,000 IU of heparin intravenously before cannulation and an additional 15,000 IU over the next 6 h. The pressure in the aortic root of the isolated heart, which is the perfusion pressure for coronary flow, was measured and used as the feedback signal for a servo-system that regulated the speed of the peristaltic pump; experiments were performed with either constant perfusion pressure ( $\sim 80$  mmHg) or with constant coronary blood flow as detailed in RESULTS. Blood traveled through the coronary vasculature of the isolated heart and returned to the

support dog by gravity. Coronary flow was collected through a wide-bore cannula placed through the right atrium into the right ventricle and was measured by an in-line ultrasonic flowmeter (Transonic Systems model T108, Ithaca, NY) in some experiments. Anesthesia of the support dog was maintained by a continuous infusion of pentobarbital sodium (2 mg/min), and the depth of anesthesia was checked periodically.

A water-filled balloon was placed within the left ventricle (LV) through the mitral valve and was held within the chamber by an adapter that fixed the heart to a piston pump servo-system that controlled balloon, and therefore ventricular, volume. A micromanometer (Millar Instruments model SPC-360, Houston, TX) placed within the balloon was used to measure ventricular pressure. The heart was paced from the LV apex at a constant rate ( $136 \pm 18 \text{ min}^{-1}$ ) and was constrained to contract isovolumically. Blood temperature was kept constant at  $\sim 37^\circ\text{C}$  by a heat exchanger.

**Protocol.** After the surgical preparation was completed, the heart was allowed to stabilize for 30 min. The LV volume (LVV) was set at a low value ( $17.8 \pm 5.3 \text{ ml}$ ) that was determined so that end-diastolic pressure (EDP) equaled 0 mmHg. After  $\sim 10 \text{ min}$  at low LVV, baseline measurements of isovolumic LV pressure, perfusion pressure, and, when measured, coronary blood flow were performed. LVV was then increased to a high value over 15 s at a constant speed provided by the servo pump. The high LVV ( $38.1 \pm 9.9 \text{ ml}$ ) was predetermined to provide an EDP of 5 mmHg. The same measurements that were performed at baseline were repeated immediately, 3 min, and 10 min after the volume increase. These two EDP values (0 and 5 mmHg) were chosen to provide a range of loading conditions sufficiently large to observe the phenomenon under investigation but small enough so that peak isovolumic LV pressure at the high volume setting was not excessively large, potentially inducing endocardial ischemia. The volume ramp was aborted if any extrasystolic contractions were noted during the ramp. After 10 min at the high volume, LVV was decreased back to the original low value. The same measurements were repeated again immediately, 3 min, and 10 min after the end of the ramp. At each time point of interest, 20 s of data were acquired digitally at a sampling rate of 1,000 Hz for off-line analysis. Special studies and measurements were performed in subsets of hearts.

**Aequorin macroinjection, collection, and analysis of light signal.** To evaluate load-dependent variations in intracellular free calcium, 10  $\mu\text{l}$  of an aequorin solution (1 mg/ml aequorin, 154 mM NaCl, 5.4 mM KCl, 1 mM  $\text{MgCl}_2$ , 12 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid, 11 mM glucose, and 0.1 mM EDTA, adjusted to pH 7.40; aequorin purchased from Friday Harbor Photoproteins, Friday Harbor, WA) were macroinjected (19) in the subepicardium of the anterolateral LV wall ( $n = 10$  hearts). A photomultiplier tube (9235QA, Thorn EMI, Fairfield, NJ) was placed against the injected site. The heart and part of the preparation were placed in a light-tight black box, and luminescence was measured by the photomultiplier tube. Luminescence measurements were normalized for aequorin loading, which was assessed in the usual manner by perfusing the hearts with 50 mM calcium-5% Triton X-100 (Sigma, St. Louis, MO) solution at the end of the experiment to lyse the cells and expose the remaining intracellular aequorin to saturating levels of calcium (19); total luminescence after this procedure was integrated and designated as  $L_{\text{max, Triton}}$ . Normalization of a particular transient (at time  $t$ ) also required assessment of total  $L_{\text{max}}$ , which accounts for aequorin consumption from  $t$  to the time when the Triton light measurement is made ( $t_{\text{Triton}}$ ). This

was also determined in the usual manner by determining the integral of the aequorin signal from  $t$  to  $t_{\text{Triton}}$  and multiplying by the rate constant for aequorin consumption ( $2.11 \text{ s}^{-1}$ ; Ref. 19)

$$L_{\text{max}}(t) = 2.11 \left[ \int_t^{t_{\text{Triton}}} L(\tau) d\tau + L_{\text{max, Triton}} \right]$$

where  $\tau$  is the time integration variable. The instantaneous  $L(t)/L_{\text{max}}(t)$  was then used as an index of the intracellular calcium transient.

**Regional coronary blood flow measurement.** To investigate the degree to which subendocardial ischemia may contribute to changes in LV pressure after a change in loading, colored microspheres [15- $\mu\text{m}$  diameter;  $3 \times 10^6$  microspheres/ml in a saline suspension with 0.01% Tween 80 and thimerosal (Dye-Trak, Triton Technology, San Diego, CA)] were used to estimate regional myocardial blood flow in three hearts. In each experiment, injections of four different-colored microspheres (white, yellow, red, and blue) were made under four different conditions (before and 0, 3, and 10 min after a volume increase); the colors were chosen in random order in each experiment. Calculation of RBF from the coronary circulation required knowledge of the total blood flow, which was obtained from the in-line flow probe described above. At each of the specified time points, 0.2 ml of vortex-mixed microsphere solution was injected into the coronary arterial perfusion line  $\sim 25 \text{ cm}$  from the heart. At the conclusion of the experiment, myocardial samples were obtained from the anterior and lateral walls of the LV. Each sample was divided into three pieces of roughly equal widths (epicardial, midwall, and endocardial regions) that were analyzed separately.

Retrieval and quantitative analysis of the microspheres were performed as described previously (20). In brief, tissue samples were digested with 4 N KOH, and the spheres were retrieved by filtration of the digestate. The dye on the microspheres was then itself digested into solution using dimethylformamide, and the photometric absorption of the resulting sample was measured by a diode-array spectrophotometer (model 8452A, Hewlett-Packard, Palo Alto, CA). The composite spectrum of each dye solution was resolved at the peak frequencies into the contributions from the individual colored spheres using a matrix-inversion technique (20). The number of spheres in each sample was calculated according to the absorbance of each dye color using standardization curves generated from known quantities of spheres from the same batch.

RBF from the coronary circulation was calculated by a standard technique modified for direct coronary injection of the microspheres (35)

$$\text{RBF} = \text{CBF}_{\text{total}} \cdot N/N_{\text{total}}$$

where  $\text{CBF}_{\text{total}}$  is the total coronary blood flow measured from the in-line flow probe,  $N$  is the number of microspheres per gram of tissue detected in the sample, and  $N_{\text{total}}$  is the total number of microspheres injected into the coronary perfusion line.

**Reserpization.** To confirm that release of catecholamines from the nerve endings was not the cause of length-dependent changes in ventricular performance, two dogs were given reserpine (1 mg/kg) subcutaneously for 7 days before the isolated heart experiment. Complete depletion of catecholamines from the nerve endings was confirmed on the day of the isolated heart experiment by an absence of heart rate and blood pressure response to bilateral stellate ganglia stimulation at 10 V with 2-ms-wide impulses at 10 Hz (15). In contrast, right and left stellate ganglion stimulation using these parameters in control animals showed 70% increase in

heart rate and 10% increase in systolic blood pressure, respectively.

*cAMP.* Because some studies have suggested that stretch may activate adenylate cyclase and increase intracellular cAMP (42, 45), we tested whether changes in cAMP are also associated with myocardial volume stretch. Three myocardial biopsies (total ~10 mg of tissue) were taken from the LV before and 3 and 10 min after ramp increases in LVV. The samples were rapidly frozen in liquid nitrogen and kept at  $-70^{\circ}\text{C}$  until analysis according to previously described methods (13). Briefly, the samples were thawed, weighed, and homogenized at  $4^{\circ}\text{C}$  with 6% trichloroacetic acid. The homogenate was centrifuged at  $2,500\ g$  for 20 min at  $4^{\circ}\text{C}$ . The supernatant was collected and extracted with water-saturated ether three times. The ether phase was discarded, and the aqueous layer was heated to  $55^{\circ}\text{C}$  in a water bath to remove the residual ether. cAMP content was assayed using commercially available radioimmunoassay techniques (Bio-medical Technologies, Stoughton, MA), with the assay protocol supplied by the manufacturer. cAMP content of myocardial samples was quantified from a standardization curve generated by standard cAMP supplied in the kit. All values are expressed as picomoles per milligram of wet tissue weight.

All procedures were approved by the Institutional Animal Care and Use Committee, Columbia University. Animals were handled and cared for in accordance with the "Principles of Laboratory Animal Care" formulated by the National Society for Medical Research and the National Institutes of Health (NIH) *Guide for the Care and Use of Laboratory Animals* [DHHS Publication No. (NIH) 85-23, Revised 1985, Office of Science and Health Reports, Bethesda, MD 20892].

*Statistical analysis.* All values are expressed as means  $\pm$  SD. Repeated-measures analysis of variance and Tukey's post hoc test were used for comparison of parameter changes;  $P < 0.05$  was regarded as statistically significant. Unpaired *t*-test on normalized values was used for the between-group comparisons such as drug effects.

## RESULTS

*Time course of change in LV contractile force after volume ramps.* Representative isovolumic LV pressure and volume curves during volume ramps (with constant coronary perfusion pressure), shown in Fig. 1, reveal a triphasic response. As was reported previously (40), LV pressure increased concomitantly with the increase in LVV (primary change) and continued to rise significantly toward a plateau for ~3 min after the end of the ramp, despite constant volume; this continued rise in pressure is referred to as the secondary rise. Although it was not reported in previous studies, we consistently observed that when examined over longer periods of time after the volume increase, peak LV pressure plateaued and subsequently decreased significantly back to about the same value as the initial pressure at high LVV; this decrease, which plateaued at ~10 min, is referred to as the tertiary decrease. When LVV was decreased back to the original low value, the opposite triphasic sequence of events followed; there was a primary instantaneous decrease of LV pressure, a secondary decrease, and a final tertiary increase.

Average results concerning the effects of volume changes on LV developed pressure (i.e., peak – end-diastolic pressure) from 32 hearts studied in the con-

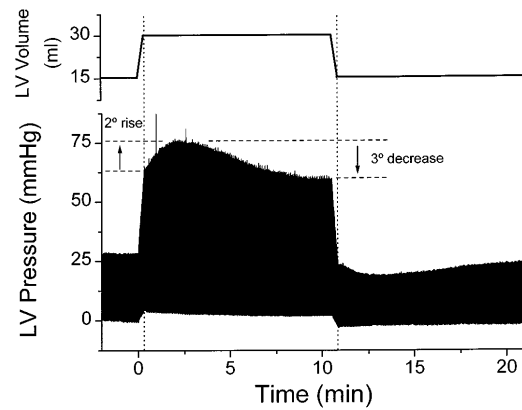


Fig. 1. Representative left ventricular (LV) pressure (LVP) changes after volume changes. Isovolumic LV volume (LVV) changes (*top*) and resultant LVP changes (*bottom*) from a representative isolated, cross-circulated canine heart. A typical triphasic response following a volume increase (primary instantaneous increase, secondary slow increase, and slow tertiary decrease) was observed. Note that a similar sequence of events, but in the opposite direction, followed a volume decrease. Vertical dotted lines indicate ends of volume ramps.

stant coronary perfusion protocol are summarized in Fig. 2. To account for interindividual variability in baseline contractile properties, developed pressures were normalized to the initial value at the high volume. As seen in the representative case, the finding of a triphasic response to volume ramps was a consistent finding. Statistical analysis revealed significant differences between 0 and 3 min after volume changes and between 3 and 10 min after volume changes for both volume increases and decreases.

To test whether any further changes in LV performance occurred after the 10-min observation period or whether this represented establishment of a true steady state, observations were made in a small subset of the

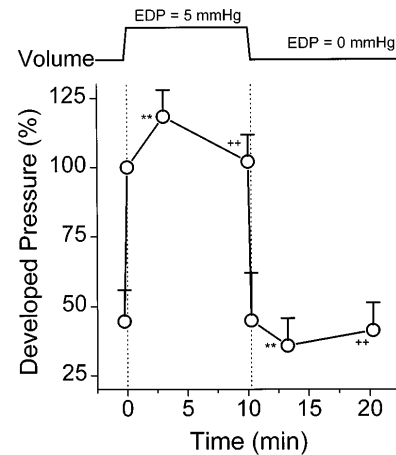


Fig. 2. Average normalized LV developed pressure in response to volume ramps. Average normalized developed pressure before, immediately after (0 min), 3 min after, and 10 min after volume changes ( $n = 32$  hearts) showed same triphasic pattern as seen in representative heart. Average LVV was  $17.8 \pm 5.3$  ml at low volume and  $38.1 \pm 9.9$  ml at high volume. Developed pressure was normalized to value at 0 min. Vertical bars represent SD. EDP, end-diastolic pressure. \*\*  $P < 0.01$  vs. immediately after volume changes; \*\*  $P < 0.01$  vs. 3 min after volume changes by repeated-measures analysis of variance with Tukey's post hoc test on raw values.

hearts up to 20 min after positive volume ramps. In these hearts, it was determined that there was a statistically insignificant  $0.8 \pm 3.1\%$  change in peak LV pressure between 10 and 20 min after the ramp. Thus, within this time frame, it appears that a steady state is reached and that a 10-min observation period is sufficient to study the phenomenon.

*Changes in aequorin luminescence transients after volume increases.* Representative aequorin tracings measured from the LV anterolateral wall surface of an isolated canine heart after volume increase are shown in Fig. 3. Each light transient was signal averaged over 40 beats to reduce ambient noise. As was reported in muscle studies (2, 3), the aequorin light transient did not change immediately after the volume stretch despite the large difference in LV pressure generation. (This finding indicates that mechanical factors, such as photomultiplier tube-heart coupling, heart motion, etc., did not contribute to observed changes in light transients described here.) The aequorin signal gradually increased over the next 3 min in parallel with the slow rise in LV pressure. When LV pressure decreased during the tertiary phase of the phenomenon, peak luminescence also decreased slowly. The aequorin signals in this range of  $L/L_{\max}$  ( $1.1-14.4 \times 10^{-5}$ ;  $n = 10$ ) roughly corresponded to peak intracellular calcium concentrations between 0.43 and 1.38  $\mu\text{M}$ , subject to the assumptions in the literature regarding aequorin consumption rates and calcium binding kinetic parameters (19).

Average results from 10 aequorin-injected hearts are summarized in Fig. 4; both  $L/L_{\max}$  and developed pressure are normalized to their respective initial values at the high LVV. Statistical analysis revealed that although peak light did not increase significantly immediately after the volume ramp, both light and developed pressure increased over the first 3 min and decreased back to the initial value by 10 min after the volume increase. Thus the changes in developed pressure following a volume ramp appear to be caused by underlying changes in intracellular calcium.

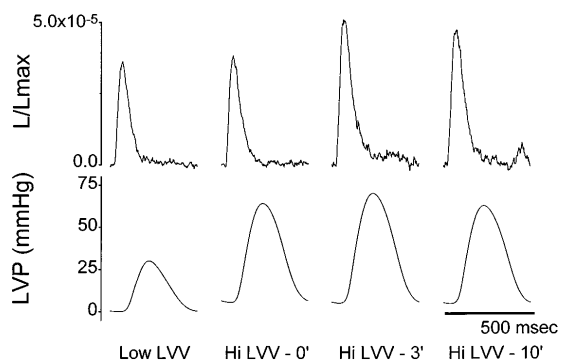


Fig. 3. Representative aequorin signals after a ramp increase in volume. Signal-averaged ( $\sim 40$  beats) tracing of aequorin luminescence ( $L/L_{\max}$ , top) and LVP (bottom) is shown. Aequorin signal amplitude was not changed immediately after increase in LVV (compare Low LVV and Hi LVV-0') despite large change in LVP. During 1st 3 min of secondary phase (Hi LVV-0' to Hi LVV-3'), aequorin signal gradually increased, as did LVP. When LVP decreased during tertiary phase (Hi LVV-3' to Hi LVV-10'), aequorin signal also decreased.

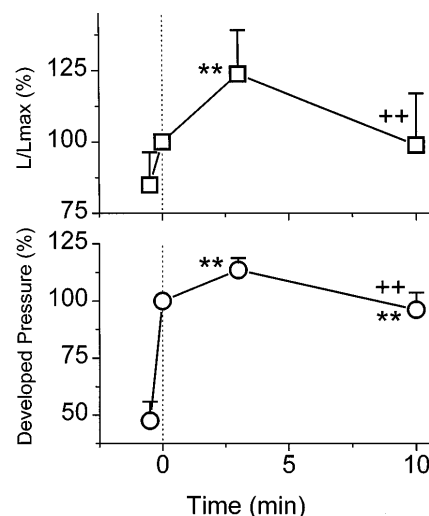
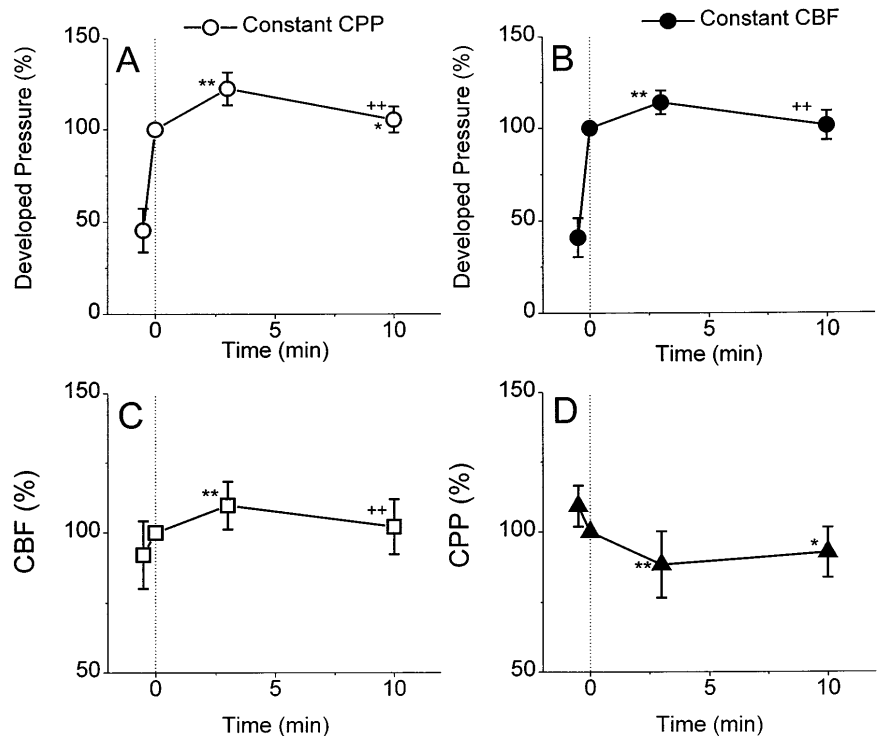


Fig. 4. Average aequorin luminescence and developed pressure. Average responses ( $n = 10$  hearts) of  $L/L_{\max}$  (top) and developed pressure (bottom) after ramp increases in volume (indicated by vertical dotted line). Values were normalized to corresponding values at 0 min. These average results confirm results presented for the representative heart, i.e., parallel changes in  $L/L_{\max}$  and developed pressure during secondary and tertiary phases. Vertical bars represent SD. \*\*  $P < 0.01$  vs. 0 min, ++  $P < 0.01$  vs. 3 min by repeated-measures analysis of variance with Tukey's post hoc test on raw values.

*Role of coronary blood flow.* With coronary perfusion held constant, total myocardial blood flow increased after an increase in LVV caused by metabolic regulation of the vascular bed (Fig. 5, A and C). To test whether the changes in LV performance after the volume ramps were related to this, we also measured the time course of LV pressure changes under conditions of constant coronary blood flow ( $n = 11$ ). Under these conditions, LV pressure generally showed the same triphasic response (Fig. 5B) despite the simultaneous decrease in perfusion pressure (Fig. 5D). However, there was a statistically significant decrease in the magnitude of the secondary rise ( $14 \pm 7\%$  with constant flow compared with  $22 \pm 9\%$  with constant pressure;  $P < 0.01$  by unpaired  $t$ -test). Thus changes in total flow may contribute partially to this phenomenon in the intact heart.

Another possible contributing factor proposed in the literature (41) is that volume stretch may cause subendocardial ischemia and that the secondary rise reflects recovery from that ischemia because of increased blood flow. To test this possibility, we measured regional myocardial blood flow with colored microspheres ( $n = 3$ ), and a representative result is shown in Fig. 6. Although there were differences among endocardial, midwall, and epicardial flows, increases in flow were similar in the three layers after the volume stretch when coronary perfusion was maintained constant. With total blood flow maintained constant, the microsphere analysis also revealed that RBF was also constant; specifically, there was not a redistribution of flow from endocardial toward epicardial regions. Thus neither an initial decrease nor a subsequent increase of subendocardial blood flow appears to contribute to these phenomenon.

Fig. 5. Effects of coronary blood flow (CBF). Total CBF was measured in 16 hearts after ramp increases in LVV with coronary perfusion pressure (CPP) held constant. As noted previously, developed pressure exhibited secondary and tertiary changes (A). As a consequence of metabolic autoregulation, CBF also exhibited similar secondary and tertiary increases and tertiary decreases (C). In 11 hearts, total CBF was fixed constant; developed pressure still exhibited secondary and tertiary phases (B) despite reduction in CPP due to metabolic autoregulation (D). All values were normalized to their respective 0-min values. Vertical bars represent SD. \* $P < 0.05$ , \*\* $P < 0.01$  vs. 0 min; ++ $P < 0.01$  vs. 3 min by repeated-measures analysis of variance with Tukey's post hoc test on raw values.



*Changes in cAMP after volume increases.* The observed simultaneous changes in developed pressure and peak intracellular calcium were reminiscent of changes observed with  $\beta$ -agonist treatment of myocardial tissue. In addition, there have been reports of increased cellular cAMP content after stretch in non-

myocyte cell types (31, 42, 44) and myocytes (32). Accordingly, we measured cAMP content at different time points after ramp increases in LVV. The results from 12 hearts are summarized in Fig. 7. Baseline cAMP content at low volume was  $1.83 \pm 0.47$  pmol/mg. Three minutes after volume increase, cAMP had increased significantly to  $2.60 \pm 0.61$  pmol/mg ( $P < 0.01$ ).

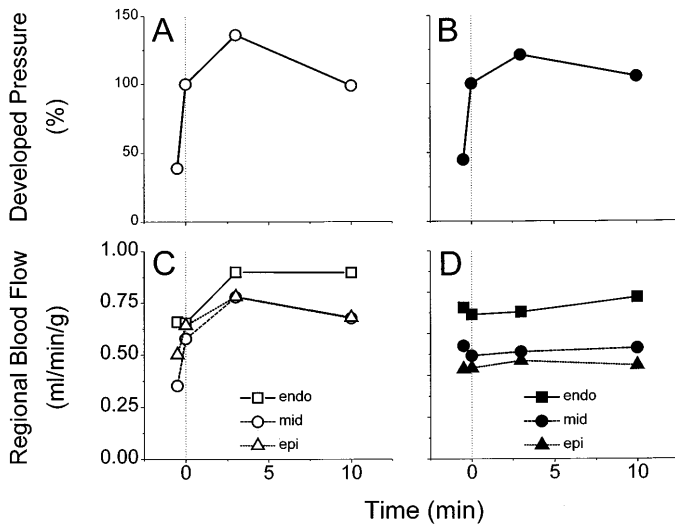


Fig. 6. Regional myocardial blood flow changes. Representative results concerning regional blood flow, from a heart in which CPP (A and C) was held constant and a heart in which total CBF (B and D) was held constant, in which regional CBF was measured in 3 myocardial layers using colored microspheres before and after ramp increases in LVV. Developed pressure showed secondary and tertiary changes (A and B) as seen in the other protocols. With constant CPP, regional blood flow in each layer (C), although slightly different in magnitude, showed the same response as was seen in total CBF (Fig. 5C). In contrast, in the constant total CBF protocol, regional blood flow was also nearly constant (D). endo, Endocardial; mid, midwall; epi, epicardial regions.

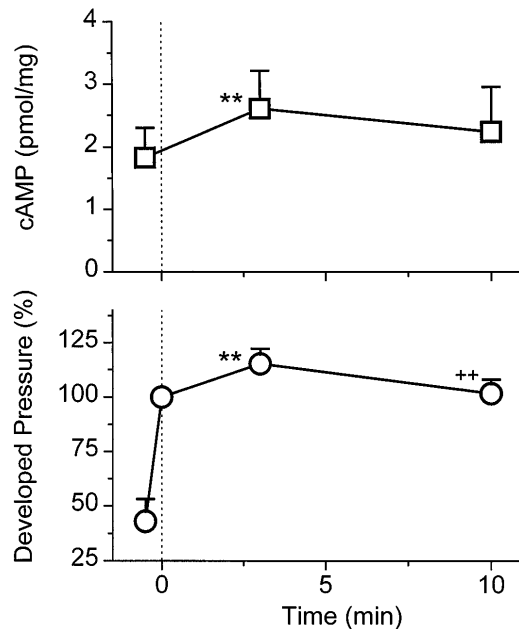


Fig. 7. cAMP changes. Myocardial cAMP content (top) was measured before, 3 min after, and 10 min after ramp increase in LVV ( $n = 12$  hearts). cAMP content paralleled variations noted in developed pressure (bottom). Vertical bars represent SD. \*\* $P < 0.01$  vs. before or 0 min; ++ $P < 0.01$  vs. 3 min by repeated-measures analysis of variance with Tukey's post hoc test on raw values.

As with developed pressure and peak calcium, myocardial cAMP levels had a tendency to fall back towards the baseline values by 10 min after the stretch, although this did not reach statistical significance.

To test for other physiological signs of increased cAMP, we also examined whether there was any effect of volume stretch on the rate of relaxation. Only high-volume conditions were examined in this analysis because of the inherent dependence of a relaxation parameter like time to half-pressure relaxation ( $t_{1/2}$ ) on preload (7). Consistent with a relatively modest rise in cAMP,  $t_{1/2}$  decreased by a small but statistically significant extent from its baseline value of  $32.7 \pm 8.9$  ms to  $29.2 \pm 7.6$  ms 3 min after the volume stretch ( $P < 0.01$ ; Fig. 8); in contrast to peak pressure and peak intracellular calcium, which returned toward their values immediately at the end of the volume ramp,  $t_{1/2}$  remained at the lower value at 10 min after the volume ramp.

**Effect of reserpization.** To test whether ventricular responses to volume increases could have related to stretch-induced release of catecholamines from intramyocardial nerve terminals, we examined hearts of animals that had been reserpinized for 7 days. As detailed in METHODS, we demonstrated in these animals a complete lack of stellate ganglion stimulation effects on heart rate or blood pressure, which provided physiological evidence of catecholamine depletion. Responses of these hearts to volume ramps are compared with those of normal hearts in Fig. 9. As shown in the figure, there was no difference in pressure responses, suggesting that stretch activation of neuronal nerve endings was not involved in this phenomenon.

**Effect of  $\beta$ -agonist and  $\beta$ -blocker.** To further investigate the potential role of cAMP-related pathways, we assessed the impact of  $\beta$ -adrenergic agonism and  $\beta$ -

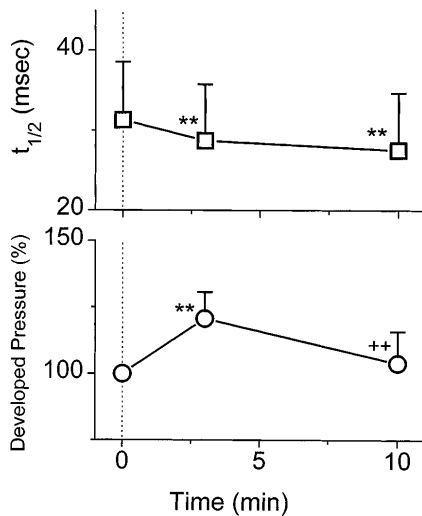


Fig. 8. Ventricular relaxation. Time for LVP to decrease by 50% ( $t_{1/2}$ ; top) was shown to shorten by a small but statistically significant amount as developed pressure (bottom) increased during secondary phase ( $n = 7$  hearts).  $t_{1/2}$  stayed decreased while developed pressure decreased in tertiary phase. Vertical bars represent SD. \*\*  $P < 0.01$  vs. 0 min; ++  $P < 0.01$  vs. 3 min by repeated-measures analysis of variance with Tukey's post hoc test on raw values.

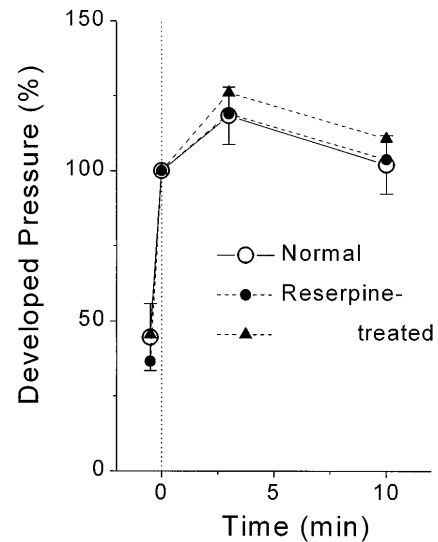


Fig. 9. Effects of reserpine treatment. To test role of stretch-induced catecholamine release from nerve endings, ramp increases in LVP were studied in hearts isolated from dogs pretreated for 7 days with reserpine (● and ▲ are individual responses of 2 dogs). Responses of developed pressure in these hearts were indistinguishable from those of normal hearts studied in this experiment.

adrenergic antagonism on the phenomenon. Figure 10A shows the triphasic pattern of developed pressure changes after volume increase under baseline conditions. Isoproterenol was then infused into the coronary perfusion line at a rate of  $0.256 \mu\text{g}/\text{min}$  (estimated final concn  $0.88 \pm 0.33 \text{ ng}/\text{ml}$ ), which increased developed pressure by  $180 \pm 60\%$  at starting low volumes. As shown in Fig. 10B, the secondary rise was completely lost. After withdrawal of the isoproterenol, the triphasic pattern returned (Fig. 10C). Esmolol was then injected at a rate of  $5 \text{ mg}/\text{min}$  into the perfusion line (estimated final concn  $25.2 \pm 5.4 \mu\text{g}/\text{ml}$ ), which decreased the starting developed pressure at low volumes

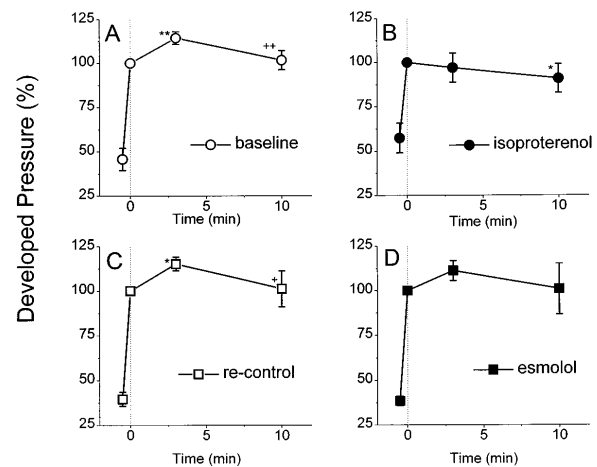


Fig. 10. Effects of  $\beta$ -agonism and  $\beta$ -antagonism. Triphasic response of developed pressure to ramp increases in LVP observed under baseline conditions (A;  $n = 5$  hearts) was completely lost during intracoronary injection of isoproterenol ( $0.256 \mu\text{g}/\text{min}$ , B;  $n = 5$ ). After washout of agent, triphasic pattern was recovered (C;  $n = 4$ ) and it was not apparently affected by esmolol injection ( $5 \text{ mg}/\text{min}$ , D;  $n = 4$ ). All results normalized to respective values at 0 min. Vertical bars represent SD.

by  $50 \pm 5\%$  of the recontrol value. However, esmolol did not affect the triphasic response to an increase in volume (Fig. 10D). Thus the time course of this phenomenon was altered by isoproterenol but not by esmolol.

In three additional isolated canine hearts, we studied the effects of BAY y 5959, a new calcium agonist that increases contractility by increasing peak intracellular systolic calcium (34). Developed pressure at low preload was increased by  $107 \pm 84\%$  by BAY y 5959, an increase that was similar to that achieved in the isoproterenol studies. Results obtained using the same volume-stretch protocol after administration of BAY y 5959 showed that, although blunted, the secondary and tertiary phases were still present; the secondary rise was  $5.1 \pm 3.8\%$ , and during the tertiary phase, pressure returned to the baseline value immediately after the stretch.

## DISCUSSION

It has been appreciated for a long time that acute stretch alters intrinsic myocardial strength and intracellular calcium in a manner reminiscent of what is seen with inotropic agents (2, 3). Although documented in a variety of preparations (9, 40, 46), the underlying mechanisms have not been elucidated. Furthermore, when myocardial adaptations to acute alterations in load under the more physiological conditions that exist in intact hearts are considered, questions are raised as to whether the phenomenon reflects fundamental cardiac muscle properties or whether it reflects factors related to organ physiology such as alterations in regional coronary blood flow (41). Although the existence of such factors provides the rationale for studying isolated muscle preparations in which they can be eliminated, it must be acknowledged that cellular processes may be altered in superfused muscle strips or isolated single muscle cells, making it important to define the phenomenon and elucidate the mechanisms under more intact conditions. The results of the present study not only suggest new insights into possible mechanisms underlying myocardial adaptations to acute stretch but also reveal phenomenological aspects that have not been observed previously in isolated muscle.

*The tertiary phase.* The first new observation relates to the gradual tertiary changes following a change in myocardial stretch. The tertiary phase follows the originally described secondary and plateau phases, becoming apparent  $\sim 3$  min after a change in volume and reaching a true steady state after  $\sim 10$  min. The tertiary phase may have eluded detection previously for several reasons. First, previous studies in isolated muscles were performed at lower temperatures. Because the mechanisms underlying this phenomenon likely involve enzymatic reactions, lower temperatures may substantially slow its time course, thus prolonging the secondary and plateau phases and making it less likely to reach the tertiary phase within the time period over which observations have been made. Second, the low stimulation rate typically used in isolated muscle studies (as low as  $12 \text{ min}^{-1}$  compared with  $>120 \text{ min}^{-1}$

in isolated canine hearts) may also extend the duration over which the secondary rise occurs, making it less likely to observe the tertiary phase; this is because Lakatta and Jewell (22) demonstrated that the secondary rise is quicker when stimulation rate is increased, suggesting that this and related phenomena may be beat dependent as opposed to strictly time dependent. Finally, in contrast to the consistent observations in the isolated, blood-perfused canine hearts, some studies have shown that even the secondary changes are not always seen in isolated cardiac muscle preparations and were therefore considered by some investigators to be an artifact (4, 28). Other investigators demonstrated that the secondary changes were more likely to be observed after specific muscle preconditioning regimens (2, 22).

The tertiary phase has also eluded detection in previous studies of isolated canine hearts (8, 37, 38, 40). The reason for this appears to be simply that the duration of observation was not sufficiently long in previous studies. Previous studies have uniformly reported that a plateau is reached between 3 and 5 min after a stretch, with no consideration of what happens at longer times; if our observations had been restricted to that time frame, we would have also only observed the secondary and plateau phases. Moreover, researchers who study isolated, cross-perfused canine hearts are generally inclined to neglect slow decreases in LV function because they may reflect a time-dependent deterioration of the preparation. However, the consistent time course of the phenomenon and the observation that after a decrease in volume the tertiary phase consists of a gradual increase in pressure indicate that the tertiary phase is not an artifact of the preparation. Finally, we extended the observation period to 20 min after a volume increase in several experiments (data not shown) and demonstrated that LV pressure did reach a true plateau with no demonstrable decline as would be seen if the tertiary phase simply reflected a steady deterioration in the preparation.

Lew (23) reported that contractile strength exhibited a steady increase over a 10-min period after intravenous volume expansion in denervated, anesthetized intact dogs. Although this may seem contradictory to the above results, there are several important differences in methodologies between the two studies. First, in the intact circulation, increased systolic pressure may exert a positive effect on contractile strength via coronary perfusion (Gregg's phenomenon; see Ref. 14). Second, in the Lew study (23), myocardial stretch could not be maintained, as evidenced by a decrease in diastolic length 10 min after volume infusion despite the sustained increase in systolic pressure. Therefore, there is a possibility that although our study and that of Lew appear to have investigated a similar phenomenon, this may not be the case.

The discovery of the secondary phase of progressive change in contractile strength after an abrupt stretch or release led to the hypothesis that it represented a mechanism whereby the heart could acutely up- or downregulate its contractile strength to provide a



rapid, reflex-independent manner of adapting to acute changes in hemodynamic load. However, identification of the tertiary phase, which renders this aspect of myocardial adaptations a transient phenomenon, raises questions about the potential role of this phenomenon in the intact circulation; this question can only be addressed in studies in the intact circulation. Independent of its physiological role, however, stretch-induced alterations in contractile performance provide a convenient and simple system for studying the fundamental question of how mechanical forces are transduced into intracellular signals that modify cellular biochemical processes.

*Macroscopic mechanisms excluded.* Two additional new sets of observations suggest for the intact heart that the observed acute adaptations to altered load reflect fundamental muscle properties. First, several pieces of data excluded the possibility that the load-dependent changes in performance were totally due to factors related to altered coronary blood flow. As for the heart in situ, coronary vasodilation occurs after an increase in work load. Accordingly, total blood flow increases after a ramp increase in volume when coronary perfusion pressure is maintained constant, and it has been suggested that this may underlie the secondary rise in contractile force. However, both secondary and tertiary phases were observed in hearts when total coronary blood flow was held constant, despite the vasodilation-induced decrease in coronary perfusion pressure. In addition, regional myocardial blood flow measurements revealed no transient subendocardial ischemia after a ramp increase in volume. Therefore, the previously advanced hypothesis that changes in LV performance after an increase in hemodynamic load simply reflect recovery from subendocardial ischemia (or hyperemia in the case of a ramp decrease in volume) (41) is not validated in this preparation.

Stretch-induced catecholamine release from nerve endings was also excluded based on our findings in reserpinized animals. We demonstrated that reserpine completely abolished hemodynamic responses to stellate ganglion stimulation in situ, indicating depletion of endogenous catecholamine stores. Consistent with previous observations in muscle isolated from reserpinized cats (29), this did not alter the myocardial responses to acute alterations in load in any detectable way.

*Intracellular calcium and cAMP.* Not surprising, but nevertheless demonstrated for the first time in an intact heart preparation, changes in intracellular calcium appear to underlie the load-induced changes in contractile performance during both the secondary and tertiary phases. As reported previously in isolated muscle (2), the calcium transient did not change immediately after a ramp increase in ventricular volume but did increase gradually in parallel with changes in performance for ~3 min. Similarly, the tertiary decrease in contractile strength was also accompanied by a decrease in peak intracellular calcium back toward the original value, indicating that the tertiary change was not due to myofilament calcium desensitization.

Two findings have implications for elucidating the mechanisms underlying the phenomena. First, we documented the new observation that changes in cAMP parallel the changes in intracellular calcium and contractile force during the secondary phase of the phenomenon. Second, consistent with a previous observation in isolated muscle preparation (18), stretch-induced changes in contractile performance were eliminated by  $\beta$ -agonist administration. The  $\beta$ -antagonist esmolol, which does not have "downstream effects" in the  $\beta$ -receptor cascade, did not modify the phenomenon. The strongly correlated changes in intracellular cAMP, the elimination of the phenomenon by preactivation of the  $\beta$ -receptor cascade, and the lack of effect of  $\beta$ -receptor blockade suggest involvement of components of this pathway in the underlying mechanism(s) but (consistent with the reserpine experiments) suggest that direct  $\beta$ -agonism is not involved.

There are well-established links between changes in cAMP and intracellular calcium that could be involved in the phenomena. Increased cAMP activates protein kinase A, which phosphorylates several regulatory proteins involved in calcium handling. Phosphorylation of L-type calcium channels increases their open probability, leading to increased transsarcolemmal calcium flux. Phosphorylation of phospholamban enhances sarcoplasmic reticular calcium adenosinetriphosphatase activity, leading to an increased rate of calcium pumping and sarcoplasmic reticular calcium loading. Phosphorylation of thin filaments decreases myofilament calcium sensitivity, which, together with the phosphorylation of phospholamban and enhanced SR  $\text{Ca}^{2+}$  uptake, helps increase the rate of relaxation that was also observed in the present study.

However, the relaxation rate has been shown to be dependent on multiple factors (6). In intact hearts, asynchronous relaxation was reported to increase relaxation constant when afterload was increased abruptly (26). This mechanism may have increased the  $t_{1/2}$  immediately after the volume increase in our study and exaggerated the effect of increased cAMP thereafter.

Accordingly, it can be hypothesized that the secondary rises in calcium and contractile force following increased stretch could be direct consequences of increased cAMP (possible mechanisms whereby stretch could cause an elevation of cAMP are discussed further below).

It is also noteworthy that Matsuda et al. (25) reported that mechanical stretch of rabbit ventricular myocytes increased open probability and conductance of L-type calcium channels. These authors concluded that this was a direct effect of stretch on the channels because neither forskolin nor a large dose of cAMP affected the phenomenon. On the other hand, Sasaki et al. (33) found no increase in L-type calcium current when stretching ventricular myocyte. Thus there is not yet a consensus about the effect of stretch on L-type calcium channels.

Several possible mechanisms could be involved in the tertiary changes. First, increased intracellular calcium is known to inhibit the adenylate cyclase subtypes

found in myocytes (types 5 and 6) (17), and it has also been shown to suppress cyclase activity in rabbit and canine myocardium (11, 27). In addition, calcium-calmodulin-dependent phosphodiesterase activity has been shown to be enhanced by calcium in certain cell types (10). In light of the interactions among calcium, cAMP, and enzymes involved in cAMP production and breakdown, Cooper et al. (12) demonstrated that the dynamic interactions among these elements can result in calcium oscillations. Although theoretical, the frequency and magnitude of the oscillations depend on the values of the rate constants of the various reactions, and it was also specifically noted that under certain circumstances, the oscillations could be damped. This latter point is of particular interest because the responses of ventricular pressure, calcium, and cAMP to a ramp increase in volume (consisting of the secondary and tertiary phases) may be regarded as a damped oscillation.

As noted, although there were trends in the right direction, neither cAMP nor  $t_{1/2}$  changed statistically significantly during the tertiary phase. Therefore, it may be that other as yet undefined mechanisms may be contributing to altering contractile performance during the tertiary phase.

Although the links among calcium, cAMP, and enzymatic reactions that regulate cAMP metabolism are well established, the results of the present study do not prove a primary, direct role of these processes in the stretch-mediated phenomena that have been detailed in this study. The finding that an increase in contractility induced by a primary increase in systolic calcium independent of cAMP mechanisms did not abolish the phenomenon in the same manner as did isoproterenol suggests that the noted changes in intracellular calcium after an increase in volume may be primarily related to an increase in cAMP rather than the changes in cAMP being due to a primary increase in intracellular calcium. However, these simple comparisons cannot truly distinguish which is the primary factor. Furthermore, the mechanoreceptors remain to be identified. As implied above, the results of the present study (specifically, alterations in cAMP and abolition of the phenomena by preactivation of the  $\beta$ -agonist cascade) have led us to the hypothesis that components of the  $\beta$ -receptor cascade may serve as mechanoreceptors.  $\beta$ -Receptors and adenylate cyclase are large, membrane-spanning proteins; G proteins are also integral membrane components. It may be possible that mechanical forces imposed on the membrane may influence enzyme activities or protein-enzyme interactions. This is not an original hypothesis; a number of previous studies in different cell types have indicated that membrane stretch may directly activate adenylate cyclase and alter intracellular cAMP (31, 42–44). In contrast, however, it was found in one recent investigation that although elevated by hyposmolar swelling in S49 cells, cAMP concentrations were decreased in response to hyposmolar swelling in rat cardiac myocytes, a finding that was attributed to stretch activation of the inhibitory protein  $G_i$  (16). The authors concluded that a

difference in adenylate cyclase subtype between the different cells was likely to be responsible for the opposite cAMP responses. The discrepancy of these results with those of other investigations conducted in cardiac tissue (32, 36, 45, 47), as well as with the current observations that cAMP is increased by volume stretch in the intact heart, will need to be resolved. Factors that may contribute to differing results could include different methods used to induce stretch [e.g., increased coronary perfusion pressure (45), cell swelling by hyposmolar perfusate (16), or length changes (present study)]. Nevertheless, even these contrary findings support the general hypothesis that the effects of stretch may be mediated by well-known integral membrane proteins.

*Limitations.* One potential limitation of the present study reflects the fact that the aequorin signals and samples for cAMP measurements were each obtained from single epicardial sites that may not be representative of other sites. Epicardial-to-endocardial and base-to-apical gradients in properties may exist. On the other hand, it has been demonstrated that stresses and strains are fairly evenly distributed throughout the ventricle (30), so that changes in load imposed on the whole heart may be fairly equally experienced by cells of different sites. Accordingly, although absolute values of various parameters may vary between sites, it is possible that events occurring at the sample site may be generally indicative of changes occurring at other sites. Although this is an unavoidable limitation of this type of study, it should be balanced against the ultimate need to understand the behavior of the intact heart under physiological conditions. Additionally, cAMP was measured in myocardial samples that include cells other than cardiomyocytes. Therefore, some of these nonmyocardial cells may be stretch sensitive, so that the results may not be completely reflective of changes occurring in myocytes (24).

With the use of macroinjected aequorin and luminescence measured by the photomultiplier tube pressed against the heart's surface, estimation of absolute calcium levels (particularly diastolic values) has not been validated; thus no emphasis was placed on estimating absolute values in the present study. Motion artifacts affecting the luminescence signal can be excluded for several reasons. The light transients did not change significantly between the low and high volumes or during the time when the volume ramp was being performed. The changes in light only occurred during the times when there were much more subtle changes in contractile performance and at a constant volume, a period during which the coupling between the heart and the photomultiplier tube would be expected to be much more stable than during the ramps.

In summary, an abrupt increase in ventricular volume is associated with an immediate increase in pressure generation that is followed not only by a secondary rise in pressure but also a previously unappreciated tertiary decrease, with final steady-state pressures not significantly different than before the secondary rise. Data concerning RBF show for the first time that these

phenomena cannot be explained on the basis of subendocardial ischemia or metabolic autoregulation of the coronary bed but rather are likely to reflect fundamental muscle properties. As expected, and in agreement with previous studies in isolated cardiac muscle, the changes in contractile force are associated with parallel changes in peak intracellular calcium. It is shown for the first time that tissue cAMP levels also change in parallel with the changes in contractile strength, at least during the secondary phase. This, combined with additional confirmation of the previous observation that the phenomena can be abolished by treatment with a  $\beta$ -agonist, suggests involvement of components of the  $\beta$ -adrenergic cascade in the underlying mechanism.

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Address for reprint requests: K. Todaka, Research Inst. of Angiocardiology and Cardiovascular Clinic, Faculty of Medicine, Kyushu Univ., 3-1-1 Maidashi, Higashi-Ku, Fukuoka, Fukuoka 812-82, Japan.

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