Phosphorylation of C-Protein in Intact Amphibian Cardiac Muscle

Correlation Between ^{32}P Incorporation and Twitch Relaxation

H. CRISS HARTZELL

From the Department of Anatomy, Emory University School of Medicine, Atlanta, Georgia 30322

ABSTRACT The molecular mechanisms by which neurotransmitters modulate the force of contraction of cardiac muscle are incompletely understood. Hartzell and Titus (1982. *J. Biol. Chem.* 257:2111–2120) have recently reported that C-protein, an integral component of the thick filament, is reversibly phosphorylated in response to ionotropic agents. In this communication, C-protein phosphorylation (as measured by isotopic labeling with ^{32}P) is correlated with changes in the rate of relaxation of twitch tension. On the average, isoproterenol simultaneously increases peak systolic tension twofold, decreases twitch relaxation time from a control value of ~450 to ~300 ms, and increases C-protein phosphorylation two- to threefold, with a maximum effect occurring <60 s after addition of 1 μM isoproterenol. Carbamylcholine, in contrast, decreases peak systolic tension more rapidly than it affects relaxation or C-protein phosphorylation. The maximum decrease in peak tension (60%) occurs within 1 min of addition of 0.5 μM carbamylcholine, but relaxation time increases slowly to 800 ms over ~6 min. The increase in relaxation time correlates well with the decrease in ^{32}P incorporation into C-protein ($r = 0.94$). Changing beat frequency between 0.2 and 1/s has no effect on C-protein phosphorylation but does alter relaxation time (relaxation time decreases ~100 ms when beat frequency is changed from 0.5 to 1/s) and thus alters the quantitative relationship between C-protein phosphorylation and relaxation rate. These results suggest that two separate processes affect relaxation. It is proposed that the level of C-protein phosphorylation sets the boundaries over which relaxation is regulated by a second process that is dependent upon beat frequency and probably involves changes in intracellular Ca.

INTRODUCTION

Although cardiac muscle contracts spontaneously without any nervous input, the nervous system exerts a strong influence on the mechanical performance of the heart (Katz, 1977, 1979; Tsien, 1977; England, 1980; Creazzo et al., 1983). Norepinephrine (NE) increases the maximal contractile force developed during

a beat and also increases the rate of relaxation after peak force has been reached (Katz, 1977). Acetylcholine (ACh) produces the opposite effects.

The molecular mechanisms by which these transmitters produce their effects are only partly understood. From studies on mammalian cardiac muscle, however, the following picture has emerged. The increased force produced by NE is due partly to an increased intracellular [Ca] during systole that results from increases in Ca influx into the cell and Ca release from the sarcoplasmic reticulum (SR) (Reuter, 1973; Van Winkle and Schwartz, 1976; Fozzard, 1977; Fabiato and Fabiato, 1979; Katz, 1979; Chapman, 1979). In addition, changes in maximum force are also probably due to changes in the functioning of the contractile apparatus, although the relevant target molecules have not been identified (Winegrad et al., 1983; Weisberg et al., 1983). The increased relaxation rate is thought to be due to an increased rate of Ca pumping by the SR (LaRaia and Morkin, 1974; Tada et al., 1974, 1980; Wray and Gray, 1977; Wollenberger and Will, 1978; Kranias et al., 1980; Tada and Katz, 1982) and by an increased dissociation rate of Ca from troponin (Solaro et al., 1981; Robertson et al., 1982), which results in a decrease in the Ca sensitivity of the contractile apparatus (Mope et al., 1980). These effects of NE are all apparently mediated by cyclic AMP-dependent phosphorylation. Convincing evidence now exists in intact cells for cyclic AMP-dependent phosphorylation of the Ca channel (or a regulatory subunit) (Reuter, 1974*b*; Tsien, 1973; Trautwein et al., 1982; Osterreider et al., 1982; Nargeot et al., 1983), a protein termed phospholamban that regulates the Ca pump of the SR (Kranias and Solaro, 1982), and troponin I (England, 1975, 1976). The decreased contractile force produced by ACh is due to a decrease in Ca influx (Giles and Noble, 1976; Nargeot et al., 1981) and the effects of ACh on relaxation rate are probably partly caused by decreases in phospholamban and troponin phosphorylation (England, 1976).

In addition, other proteins such as myosin light chain and C-protein are also phosphorylated and dephosphorylated in response to NE and ACh in the heart (Stull, 1980). The role that these proteins play in regulation of cardiac contraction is unknown. I have been particularly interested in C-protein, an integral component of the thick filament. C-protein becomes phosphorylated in response to NE and dephosphorylated in response to ACh (Jeacocke and England, 1980; Hartzell and Titus, 1982). Since C-protein binds to actin and myosin (Moos et al., 1975, 1978) and can inhibit actin-activated myosin ATPase (Moos and Feng, 1980), it seems very likely that this protein is involved in regulation of cardiac muscle contraction. In an earlier study (Hartzell and Titus, 1982), I showed that C-protein phosphorylation in response to β-adrenergic agonists correlated well with contractile force. Dephosphorylation produced by ACh, however, occurred much more slowly than the decrease in force, which suggested that C-protein phosphorylation was not involved in regulation of peak force. In the present study, I provide evidence to support an alternative hypothesis that C-protein phosphorylation plays a role in regulating relaxation rate of the heart.

In these studies I have used frog cardiac muscle. Frog heart offers an advantage over mammalian cardiac muscle for these studies because in frog heart release of Ca from intracellular pools does not contribute significantly to the develop-

ment of tension (Morad and Orkand, 1971; Anderson et al., 1977; Fabiato and Fabiato, 1979; Chapman, 1979; Winegrad, 1982; Morad et al., 1983), and relaxation is determined largely by Ca efflux into the extracellular space by Na/Ca exchange and not by Ca sequestration by the SR (Goto et al., 1972; Roulet et al., 1979; Chapman, 1979). Thus, the role of the SR and phospholamban in mediating the responses to NE or ACh is likely to be small in frog heart.

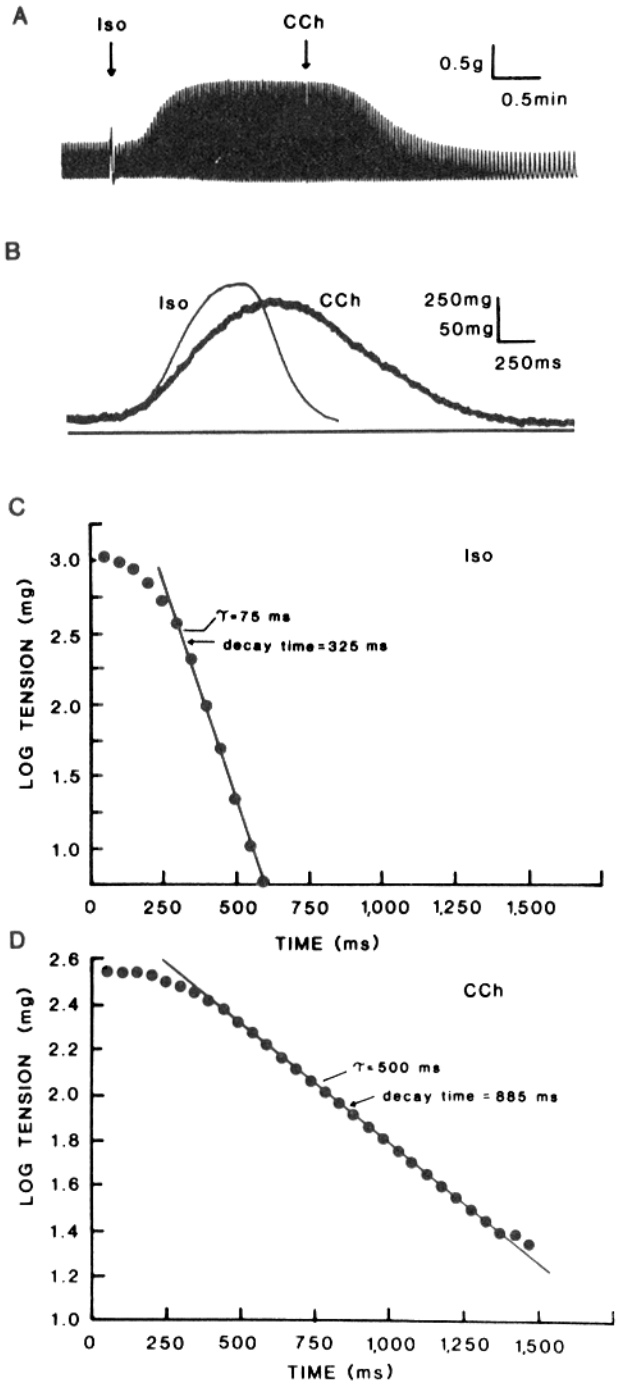
METHODS

Tension Measurements

All experiments were performed on *Xenopus laevis* hearts in Ringer solution containing 90 mM NaCl, 32 mM $NaHCO_3$, 4 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, and 20 mM glucose and gassed with 95% O_2 and 5% CO_2. In experiments involving isolated atria, tension was recorded as described previously (Hartzell and Titus, 1982). In experiments with whole hearts or isolated ventricles, the hearts were dissected, cannulated, and perfused at 0.3 ml/min. The cannula, which was 1-mm-OD polyethylene tubing with a flared end, was inserted from the sinus venosus into the right atrium and affixed by tying a thread around the sinus. Since the frog heart is three-chambered, this arrangement resulted in perfusion of the right atrium and the whole ventricle. The perfused hearts were placed in a gas-box and attached to a force transducer (FT03C; Grass Instrument Co., Quincy, MA) by a stiff wire attached to the apex of the ventricle by a stainless steel hook. The heart was stretched to give a maximal difference between the systolic and diastolic tension in the absence of drugs. Because the muscle fibers in the heart are not parallel, the tension measurement was neither isometric nor isotonic. Tension was amplified and recorded on an FM tape recorder (HP3964; Hewlett-Packard Co., Palo Alto, CA) (bandwidth = 312 Hz) and a chart recorder (Gould/Brush 220; Gould Inc., Cleveland, OH). Analog data from the tape recorder were digitized at a sampling frequency of 200 Hz and the amplitudes and decay times were analyzed by an Apple III microcomputer (Cupertino, CA). In some experiments, hearts were paced electrically by placing a cathodal platinum electrode on the sinus venosus and applying 1-ms duration, 20-V pulses between the cathodal electrode and the wire attaching the heart to the force transducer.

Measurement of ^{32}P Incorporation into C-Protein

The methods for determining the amount of phosphorylation of C-protein have been described in detail (Hartzell and Titus, 1982). Hearts were perfused with Ringer solution containing 100–200 μCi/ml of ^{32}P (orthophosphate) for 30 min–3 h. The ^{32}P was rinsed out and the hearts were perfused with drug. The atria and ventricle were separately freeze-clamped and homogenized in a Polytron (Brinkman Instruments, Westbury, NY) PT-10 homogenizer in 3 ml of 10 mM EDTA, 15.3 mM NaH_2PO_4, 135 mM Na_2HPO_4 (EDTA-PO_4) at 4°C. An aliquot was removed for determination of ATP specific activity as described previously (Hartzell and Titus, 1982) and the remainder was centrifuged at 5,000 *g* for 10 min to remove actomyosin, which becomes very viscous upon addition of sodium dodecyl sulfate (SDS). Virtually all of the C-protein was found in the supernate of this centrifugation. No phosphorylation or dephosphorylation of C-protein occurred during homogenization and centrifugation as determined by methods used previously (Hartzell and Titus, 1982). The supernate was then made to 1.3% SDS, 3% 2-mercaptoethanol and heated to 60°C for 10 min. The solubilized proteins were run on SDS gels, and ^{32}P incorporation was analyzed by autoradiography, densitometry, and scintillation counting as described previously.


A
Iso
CCh
0.5g
0.5min
B
Iso
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250mg
50mg
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C
Iso
τ=75 ms
decay time=325 ms
LOG TENSION (mg)
TIME (ms)
D
CCh
τ=500 ms
decay time = 885 ms
LOG TENSION (mg)
TIME (ms)

RESULTS

The purpose of these experiments was to evaluate the possible correlation between the time course of cardiac contraction and phosphorylation of C-protein. The first part of the results describes the effects of various drugs on the time course of contraction and the second part describes a correlation of these changes with C-protein phosphorylation.

Fig. 1 *A* illustrates a typical experiment. A frog heart was perfused with Ringer solution and attached to a force transducer. When a maximal concentration of isoproterenol (Iso) was added to the perfusing medium, the tension rapidly increased with a half-time of ~25 s. In some hearts, the response to Iso desensitized and the tension declined somewhat from the initial stimulated level. Regardless of whether desensitization occurred, the systolic tension after several minutes' exposure to Iso was usually stable and at least twofold greater than the control level. When carbamylcholine (CCh) was added to the medium containing Iso, the tension decreased in an amount proportional to the CCh concentration. In these experiments, I usually used 5×10^{-7} M CCh, which caused an average 50% decrease in systolic tension. The decrease in tension produced by CCh occurred rapidly (half-time ~30 s) and did not desensitize significantly even after 30–45 min.

Time Course of Contractions

The time courses of contractions in the presence of Iso and CCh were markedly different. Fig. 1 *B* shows records of contractions from the same heart after 3 min exposure to 1 μM Iso (trace labeled Iso) and after 7 min exposure to 5×10^{-7} M CCh and 1 μM Iso together (trace labeled CCh). The peak amplitudes were normalized to facilitate comparison of the time courses. In the presence of Iso the contraction reached a peak and also relaxed much more quickly than it did in the presence of CCh. Both contractions decayed biphasically, as shown on semilog plots in Figs. 1 *C* and *D*. The initial phase of both lasted 200–250 ms. The main phase of relaxation, which was exponential, had a time constant of 75 ms in Iso and 500 ms in CCh. In the absence of any drugs, the exponential phase had a time constant of 200–250 ms (not illustrated). These time courses were

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FIGURE 1. (*opposite*) Effects of ACh and NE on contractile force. *Xenopus* heart was perfused with Ringer and attached to a force transducer as described in Methods. (*A*) A record showing the effects of Iso and CCh on tension at a slow chart speed. At the first arrow (Iso), 1 μM Iso was added to the perfusion fluid. At the second arrow (CCh), 0.5 μM CCh was added to the perfusion fluid with 1 μM Iso. (*B*) Twitch tensions shown at a faster chart speed to illustrate difference in time course. The trace labeled Iso (calibration: 250 mg) was taken from *A* ~1 min after adding Iso. The trace labeled CCh, shown at a higher gain (calibration: 50 mg), was taken from the same record but 6 min after adding CCh. (*C*) Semilog plot of the decaying phase of a twitch in the presence of Iso for 1 min. (*D*) Semilog plot of the decaying phase of the twitch in the presence of CCh for 6 min. The solid line is a least-squares fit of the linear portion of the curve and τ is the time constant of the exponential. The decay time is the time required for the twitch to decay to 25% of its peak amplitude.

virtually identical to those of frog ventricular trabeculae studied under voltage-clamp conditions (Goto et al., 1972; Roulet et al., 1979). This similarity suggested that my measurements reflected reasonably well the mechanical state of individual fibers. Throughout these studies, relaxation was expressed as "decay time,"

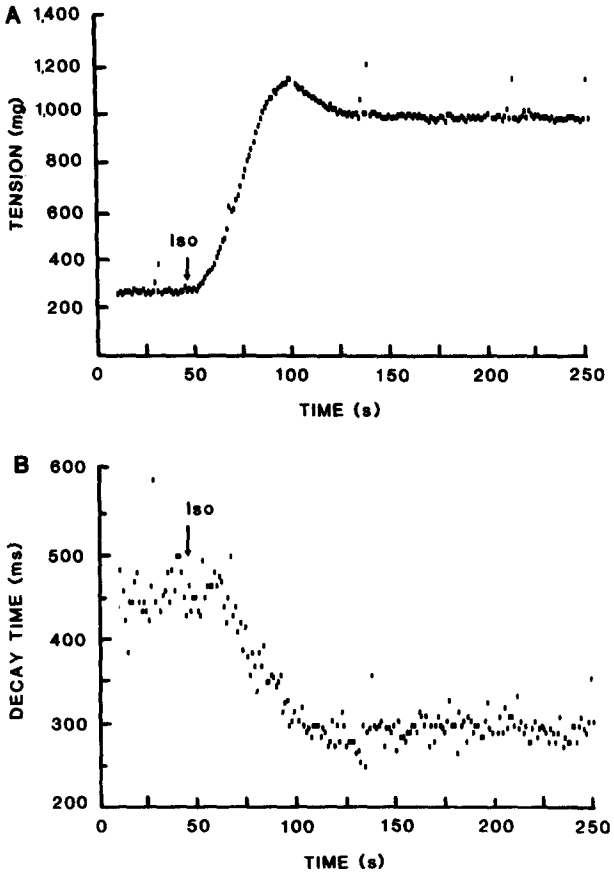


FIGURE 2. Time course of the effect of isoproterenol. (*A*) Effect of isoproterenol on peak systolic tension. (*B*) Effect of isoproterenol on decay time. Each point is a single contraction. A spontaneously beating heart was perfused with Ringer solution for 1 h without drugs and then 1 μM Iso was added at the arrow. The force of contraction of this particular heart was unusually weak in the absence of drugs; usually, unstimulated hearts produce tensions of ~600 mg. Nevertheless, the time course of the effects of Iso was the same in all hearts examined.

which was arbitrarily defined as the time required for the contraction to decay from its peak to 25% of its peak amplitude. This time is shown by the arrows in Figs. 1 *C* and *D*. This measure included both the early and the exponential phases of decay.

Time Course of Change in Decay Time and Tension

It was previously shown (Hartzell and Titus, 1982) that dephosphorylation of C-protein in response to CCh lagged behind the decrease in peak systolic tension produced by CCh. This observation raised the question of whether other features

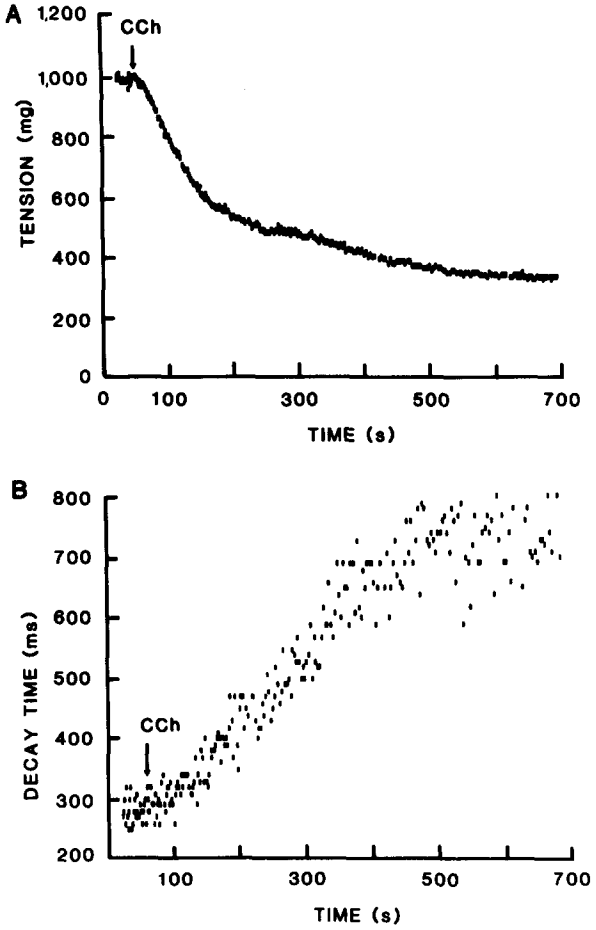


FIGURE 3. Time course of the effect of CCh. (*A*) Effect of CCh on peak systolic tension. (*B*) Effect of CCh on decay time. A spontaneously beating heart was perfused with 1 μM Iso for 3 min before the start of this record. At the arrow, 0.5 μM CCh was added in the continued presence of 1 μM Iso.

of the twitch, such as decay time, change at different rates than the peak systolic tension. Figs. 2 and 3 are plots of the peak systolic tension and the decay time of the contractions recorded before and after addition of 1 μM Iso (Fig. 2) or 5×10^{-7} M CCh (Fig. 3). In response to Iso, tension and decay time changed coincidentally (Fig. 2). Within 50 s after adding Iso, both tension and decay time attained new, stable levels. In response to CCh, however, tension and decay time

changed at different rates (Fig. 3). After adding CCh, the systolic tension fell >50% within 1 min and slowly fell another 15% over the next 6 min. (In most hearts this slower phase was smaller or totally absent.) In contrast, the decay time increased linearly at a rate of 72 ms/min exposure to CCh for at least 6–7 min. After ~7 min in CCh, the decay time stabilized at a level of ~700 ms, a greater

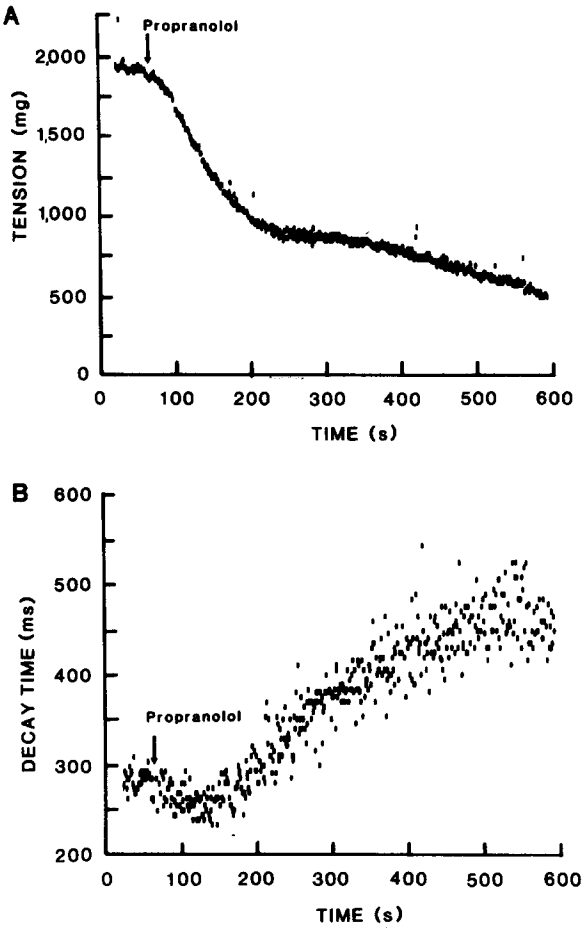


FIGURE 4. Time course of the effect of propranolol. (*A*) Effect of propranolol on peak systolic tension. (*B*) Effect of propranolol on decay time. A spontaneously beating heart was perfused with 1 μM Iso for 5 min before the start of this record. At the arrow, 50 μM propranolol was added in the continued presence of 1 μM Iso.

than twofold increase over the decay time in Iso. There was some variability from heart to heart in the rate that the decay time changed, but invariably the rate of change of decay time was slower than that of the tension in response to CCh. The average increase in decay time was 120 ms/min in CCh (n = 20). Although the data illustrated so far were gathered from whole perfused hearts, qualitatively similar data were obtained from isolated atria and ventricles.

Effects of Other Drugs

The effect of propranolol, which blocks β-adrenergic receptors, was also tested (Fig. 4). The results were similar to those observed with CCh, although peak tension decreased about twofold more slowly than it did in response to CCh.

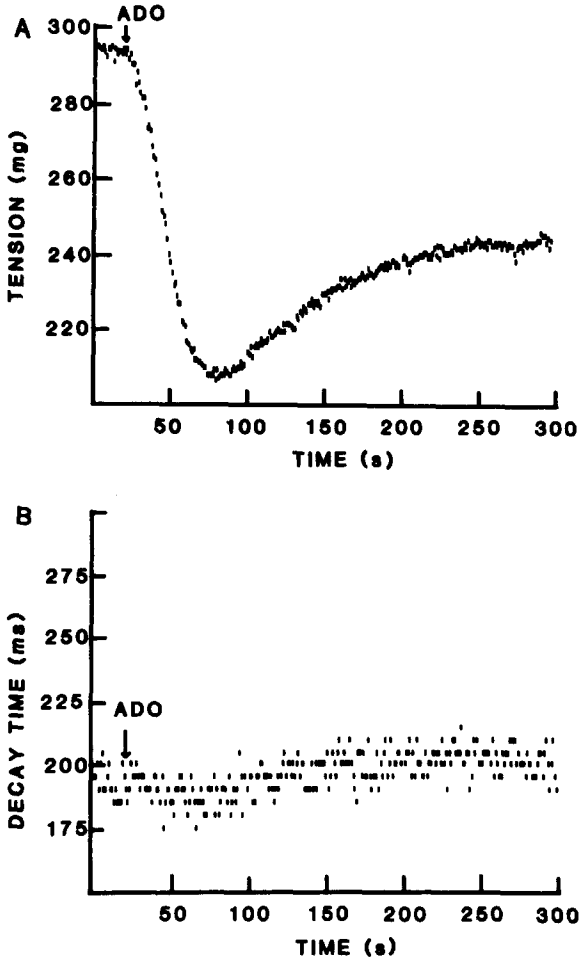


FIGURE 5. Time course of effect of adenosine. (*A*) Effect of 0.1 mM adenosine on peak systolic tension. (*B*) Effect of adenosine on decay time. A spontaneously beating atrium was exposed to 1 μM Iso for 3 min before the start of this record. At the arrow, 0.1 mM adenosine was added to the perfusion fluid in the presence of 1 μm Iso.

Previous studies have demonstrated that the effects of adenosine differ in different regions of the heart (Burnstock, 1972; Goto et al., 1977; Hartzell, 1979; Flitney and Singh, 1980). In the atrium and sinoatrial (SA) node, however, adenosine consistently decreased peak systolic tension (Hollander and Webb,

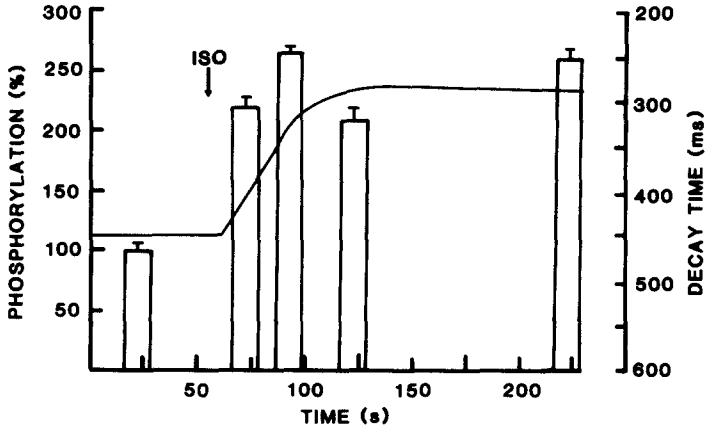


FIGURE 6. Effect of Iso on ^{32}P incorporation into C-protein. Hearts were perfused for 0.5 h with Ringer containing 100 μCi/ml ^{32}P and then perfused with Ringer for 5 min. At the arrow, 1 μM Iso was added to the perfusion fluid. Atria and ventricles were separately freeze-clamped and processed as described in Methods. Data shown are from atria only, but data from ventricles were similar. Bars show ^{32}P incorporation into C-protein relative to 100% incorporation after 4 min exposure to Ringer alone. The level of ^{32}P in C-protein in Ringer alone was constant for at least 10 min (not shown). Each bar is the mean of three to five atria. The solid line shows the decay time from Fig. 2.

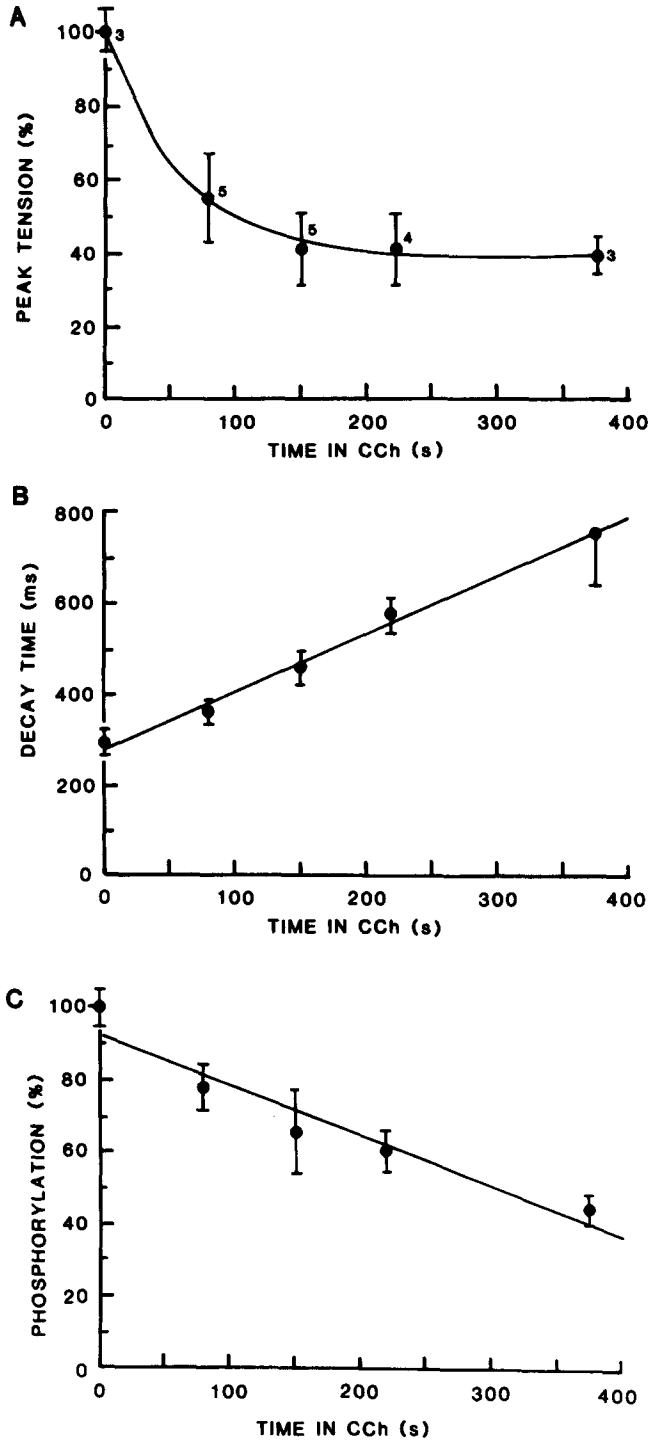
1957; Bertelli et al., 1972; Meinertz et al., 1973; Goto et al., 1977; Belardinelli et al., 1983). I therefore examined the effects of adenosine on peak tension and decay time. In Fig. 5, 10^{-4} M adenosine produced a 20–35% decrease in peak tension, but, interestingly, had no significant effect on decay time (Fig. 5*B*).

Correlations with C-Protein Phosphorylation

ISOPROTERENOL It has previously been shown that C-protein phosphorylation increased severalfold within 30 s after exposure to 1 μM Iso (Hartzell and Titus, 1982). In Fig. 6, ^{32}P incorporation (bars) and decay time (solid line from Fig. 2: note that the *x* axis is inverted in Fig. 6 compared with Fig. 2) are plotted. The increase in ^{32}P incorporation and decrease in decay time occurred with approximately the same time course. Although it may appear that the C-protein phosphorylation increased slightly more rapidly than the decay time decreased, the time resolution in the phosphorylation measurements was 10–15 s. Thus, within the resolution of the technique, the time courses were the same.

CARBAMYLCHOLINE The most rigorous test of the correlation between

FIGURE 7. (*opposite*) Effect of CCh on tension, decay time, and ^{32}P incorporation into C-protein. Hearts were treated as in Fig. 6 except that 0.5 μM CCh was added to the perfusion fluid after a 4-min exposure to 1 μM Iso. (*A*) Peak systolic tension as a percentage of the peak tension in 1 μM Iso. (*B*) Decay time. (*C*) ^{32}P incorporation into C-protein as a percentage of the incorporation after 4 min exposure to Iso alone. Data are from ventricles. Each point is the mean of three to five hearts as shown by the small numbers beside the points in *A*.


A
PEAK TENSION (%)
100
80
60
40
20
0
3
5
5
4
3
0
100
200
300
400
TIME IN CCh (s)
B
DECAY TIME (ms)
800
600
400
200
0
0
100
200
300
400
TIME IN CCh (s)
C
PHOSPHORYLATION (%)
100
80
60
40
20
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100
200
300
400
TIME IN CCh (s)

decay time and C-protein phosphorylation was made in a series of experiments involving 20 separate hearts in which tension was recorded from each heart from the time it was placed in ^{32}P until it was frozen (Figs. 7 and 8). (In the experiments of Figs. 6, 9, and 10, tension and ^{32}P incorporation were measured in separate experiments, or tension was sampled from only few of the hearts, which were analyzed biochemically.)

In this experiment, hearts were first exposed to 1 μM Iso and then exposed to 5×10^{-7} M CCh for different periods of time. The average time courses of change of tension and decay time were plotted as a function of time in CCh (Figs. 7*A* and *B*). These kinetics were very similar to those illustrated for the individual heart shown in Fig. 3. The time course of ^{32}P incorporation into C-protein (Fig. 7*C*) was similar to the time course of change in decay time. Although the effect of CCh on tension was virtually complete within 1 min, the effects of CCh on decay time and C-protein phosphorylation continued for at least 6 min.

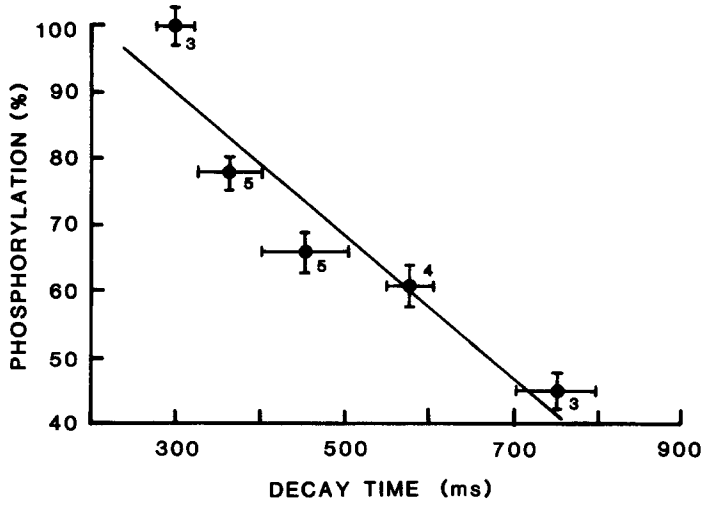

FIGURE 8. Correlation between ^{32}P incorporation into C-protein and decay time. Data from the experiment in Figs. 7*B* and *C* are replotted.

The correlation between decay time and C-protein phosphorylation for this experiment is shown quantitatively in Fig. 8. In this graph, the C-protein phosphorylation for each time point in Fig. 7*C* was plotted vs. the decay time for the corresponding times in Fig. 7*B*. The correlation in this and two similar experiments was very good (the correlation coefficient of Fig. 8 is 0.94).

OTHER DRUGS The time course of the effect of propranolol also correlated well with C-protein phosphorylation (Fig. 9). Adenosine (Fig. 10), which had no effect on decay time (Fig. 5), also had no effect on C-protein phosphorylation in isolated atria.

Effect of Beat Frequency

In the experiments described above, hearts were beating spontaneously and were not electrically paced for several reasons. Frog hearts normally beat sponta-

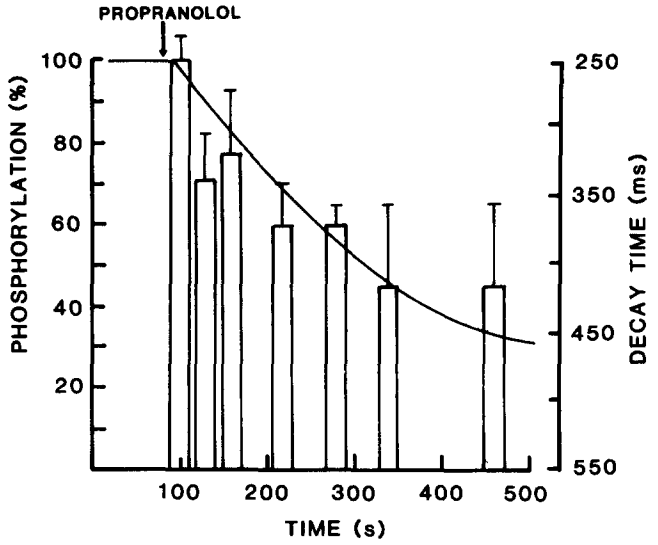

FIGURE 9. Effect of propranolol on ^{32}P incorporation into C-protein. Hearts were treated as in Fig. 6 except 50 μM propranolol was added after 4 min exposure to 1 μM Iso. Bars are ^{32}P incorporation into C-protein as a percentage of incorporation after 4 min exposure to Iso alone. Data are from atria. Each bar is the mean of three to six atria. The solid line shows the decay time from Fig. 4.

neously at a frequency of 0.8–1 Hz. It was not possible to entrain them to beat at frequencies lower than 1–1.5 Hz in the presence of Iso, and at 1–1.5 Hz in the presence of CCh the contractions often ran together so that the hearts did

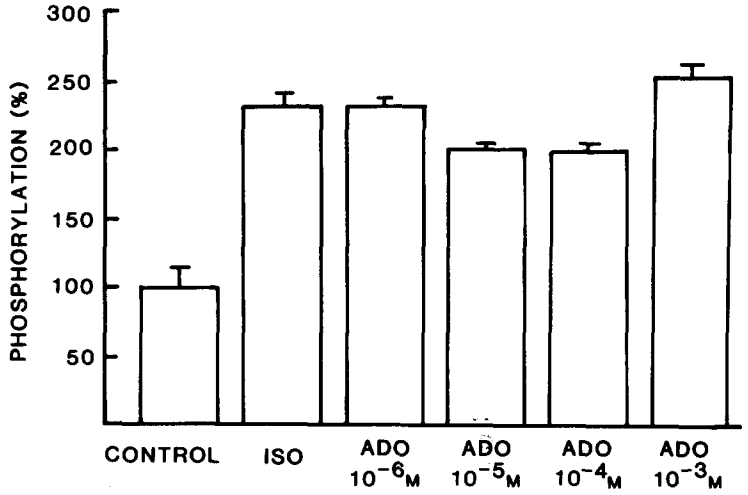

FIGURE 10. Effect of adenosine on ^{32}P incorporation into C-protein. Hearts were treated as in Fig. 6 except that after exposure to Iso for 4 min, hearts were exposed to different concentrations of adenosine for 15 min. Data are from atria. Each bar is the mean of three atria.

not relax fully between beats. Although ventricle strips can be prepared that do not beat spontaneously, I was concerned that such extensive dissection might alter the biochemical results. Thus, intact hearts were used for these initial experiments.

Since CCh usually caused a decrease in spontaneous beat frequency, this raised the question of whether the changes in decay time and C-protein phosphorylation occurred secondarily to changes in beat frequency. To examine this possibility, the effects of beat frequency on decay time and C-protein phosphorylation were examined (Fig. 11) by perfusing isolated ventricles with Iso while pacing them to beat at 1 Hz and then shifting to another pacing frequency when CCh was added. The ventricles were frozen 7 min after adding CCh. The pacing frequency

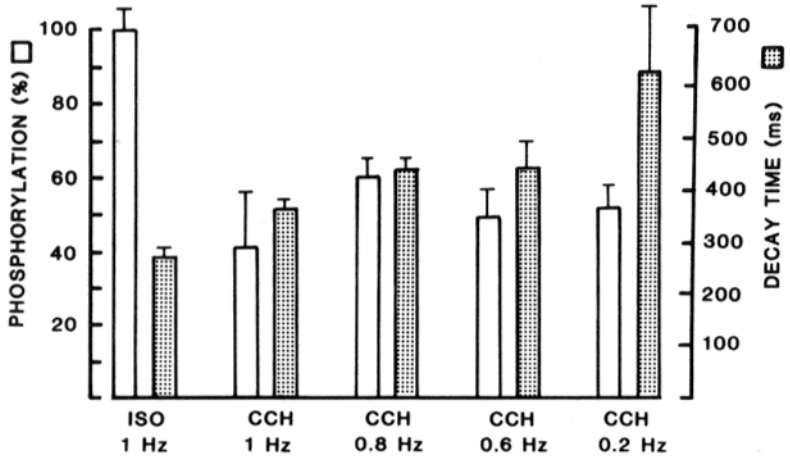

FIGURE 11. Effect of beat rate on ^{32}P incorporation into C-protein and decay time. Solid bars are ^{32}P incorporation into C-protein in ventricles expressed as a percentage of incorporation after 4 min exposure to 1 μM Iso. Stippled bars are decay time. Each bar is the mean of three ventricles. Ventricles were treated as described in Fig. 6 except that the ventricles were paced electrically at 1 Hz during exposure to Iso and were then paced at the frequency shown upon addition of CCh. The data shown are taken after 7 min exposure to 0.5 μM CCh.

affected the maximum decay time that was attained after a 7-min exposure to CCh (Fig. 11, stippled bars). Hearts paced at 0.2 Hz attained an average decay time of 620 ms, whereas hearts paced at 1 Hz had an average decay time of 385 ms. Thus, high pacing frequencies attenuated the effect of CCh on decay time. Nevertheless, the decay time of hearts paced at 1 Hz was significantly greater in the presence of CCh than in Iso. In contrast to the effect on decay time, pacing had no significant effect on C-protein phosphorylation (Fig. 11, open bars). These data demonstrated that C-protein phosphorylation did not correlate with beat frequency. Further, they showed that although the absolute magnitude of the decay time did not correlate with C-protein phosphorylation, increases in decay time produced by CCh were always accompanied by decreases in C-protein phosphorylation. These results were interpreted to mean that two separate

processes control decay time, one process related to C-protein phosphorylation and another related to beat frequency.

Support for this interpretation was provided by the finding that these two processes occurred with different kinetics (Figs. 12 and 13). Changes in decay time produced by CCh that were presumably related to C-protein dephosphorylation required minutes to develop (Fig. 12). Fig. 12 shows the effect of CCh on the decay time of a typical heart, which was either beating spontaneously or paced at 0.33 or 0.85 Hz. The changes in decay time occurred with similar time courses regardless of whether the heart was beating spontaneously or paced at different frequencies: the decay time approached a maximum with a half-time of ~120 s. The change of decay time of this heart was somewhat faster than the heart shown in Fig. 3.

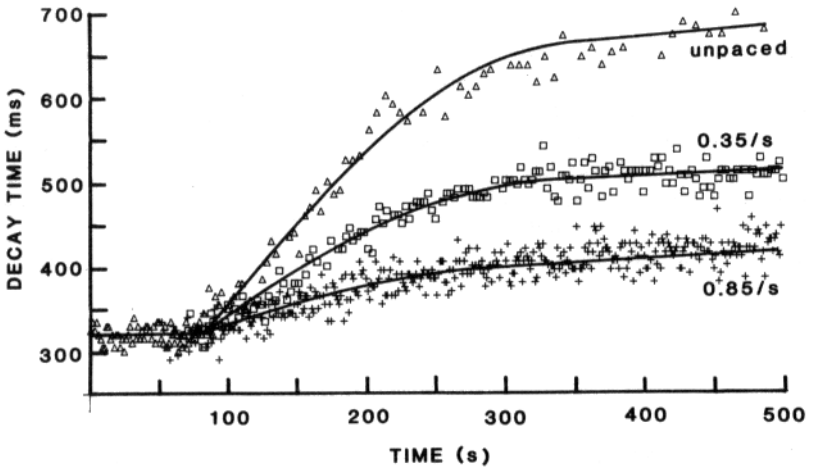

FIGURE 12. Effect of pacing frequency on change of decay time. Conditions were the same as in Fig. 3 except the heart was either not paced (triangles) or paced to beat at 0.85 (crosses) or 0.35 Hz (squares).

Changes in decay time produced by changing beat frequency occurred much more quickly (Fig. 13). In the experiments of Figs. 11 and 12, hearts were paced at a constant frequency from the time CCh was added to the perfusion fluid. In Fig. 13, however, CCh was added to the perfusate and pacing frequency was altered. If pacing frequency was reduced, the decay time jumped quickly to the value it would have been if the heart had been paced at the lower frequency from the onset of CCh application. Within 10 s after changing the stimulation frequency, the decay time increased to a new stable level (Fig. 13). Often the change was even faster. Decreases or increases in stimulation frequency produced equally rapid changes in decay time. These results supported the idea that two separate processes with different kinetics control decay time (see Discussion).

After equilibrating hearts in CCh (7 min) or Iso (3 min), the decay time could be rapidly and reversibly modulated over a wide range by changing the beat frequency. Fig. 14 shows the decay time of contractions as a function of stimu-

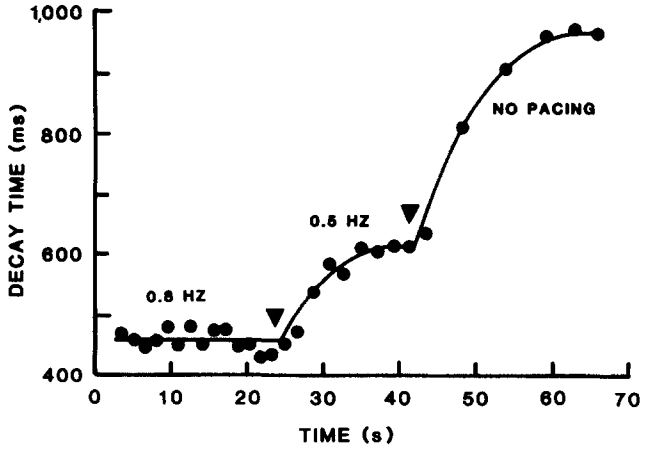

FIGURE 13. Time course of change of decay time after changing pacing frequency. Hearts were paced at 0.8 Hz and perfused with Ringer solution containing 1 μM Iso for 5 min and then 1 μM Iso and 0.5 μM CCh for an additional 4 min. After exposure to CCh, decay time increased from 295 to 460 ms and stabilized at this level. The record shown begins after 4 min in CCh and Iso. At the first arrowhead, pacing frequency was decreased to 0.5 Hz, and within 10 s decay time increased to a new level. At the second arrowhead, the stimulator was turned off so that the heart beat spontaneously and the decay time again increased within 10–15 s to a new level.

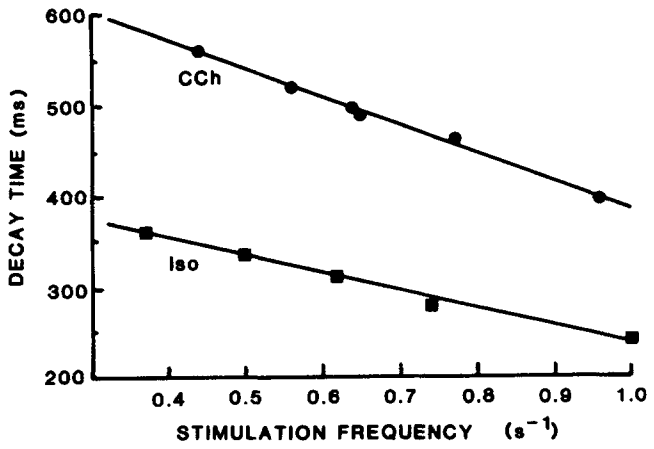

FIGURE 14. Effect of stimulation frequency on decay time. An isolated ventricle was perfused with Ringer solution containing 0.5 μM CCh and 1 μM Iso (CCh, circles) or 1 μM Iso (Iso, squares) for 7 min and was then stimulated to beat at the frequency shown for at least 1 min. Each point is the mean of at least five contractions. Solid lines are least-squares fit to the points.

lation frequency. The ventricle was stimulated to beat at each frequency shown for 1 min and the average decay time was calculated. The decay time decreased as pacing frequency was increased. In the presence of 5×10^{-7} M CCh and 10^{-6} M Iso, there was a 28% decrease (154 ms) in decay time as the pacing frequency was increased from 0.5 to 1 Hz. In the presence of Iso, the decay times at all pacing frequencies were faster, but the effect of pacing frequency on decay time was similar to that in CCh: increases in pacing frequency from 0.5 to 1 Hz decreased the decay time 29% (99 ms). It should be noted that the increment in decay time produced by CCh is greater at lower frequencies of beating than at higher frequencies.

DISCUSSION

The main finding of this study is that phosphorylation of C-protein correlates extremely well with the relaxation rate of the twitch tension. When C-protein is phosphorylated, decay time is short, but when C-protein is dephosphorylated, decay time is long. I favor the hypothesis that C-protein is involved in regulating relaxation of cardiac muscle for several reasons. (*a*) The correlations described are strong and reproducible. (*b*) The effects of neurotransmitters on intracellular Ca transients and the affinity of troponin for Ca (Tsien, 1977; Stull, 1980; Solaro et al., 1981; Tada and Katz, 1982; Creazzo et al., 1983) seem inadequate to explain all the effects of NE and ACh in frog heart. (*c*) C-protein has a number of very important and interesting properties that make it a suitable and likely candidate for a myofibrillar regulatory protein. These points are discussed in more detail below.

Correlation Between C-Protein Phosphorylation and Decay Time

In this paper, I have shown that C-protein phosphorylation parallels in time the onset of rapid relaxant effects of Iso and also the slower reversal of this effect produced by CCh or propranolol. Furthermore, the concentration dependence of the effects of Iso on peak tension and decay time and C-protein phosphorylation are correlated (Hartzell and Titus, 1982; H. C. Hartzell, unpublished data). In a previous study (Hartzell and Titus, 1982), dephosphorylation of C-protein in isolated atria required a slightly higher concentration of CCh than did the change in peak tension.

Although C-protein phosphorylation correlates well with decay time, the possibility remains that phosphorylation of other proteins, such as troponin, also correlates with decay time. In addition to C-protein, only two other proteins in purified frog heart myofibrils are phosphorylated by exogenous cyclic AMP-dependent protein kinase (H. C. Hartzell, unpublished data). These proteins have molecular weights of 33,000 and 18,000 (rabbit heart tropomyosin [34,000], troponin I [28,000], and troponin C [19,000] were used as molecular weight standards). I have not unambiguously identified these proteins, but the 33,000-dalton protein may be troponin I. Phosphorylation of this protein in intact cells, however, is variable and does not appear to correlate with inotropic state or decay time. Additional experiments are required to resolve this question. It is interesting to note, however, that adenosine, which does not affect decay

time (Fig. 5), has been reported to decrease troponin I phosphorylation without affecting C-protein phosphorylation in isolated rat heart (Dobson and Fenton, 1983).

The interpretation of the experiments with unpaced hearts is partially complicated by changes in beat frequency that occur in response to CCh in particular. It is unlikely, however, that the effects of inotropic agents on C-protein phosphorylation are secondary to changes in beat frequency for several reasons. (*a*) C-protein phosphorylation does not correlate with beat frequency (Fig. 11). (*b*) Iso usually produces only very small changes in beat frequency but consistently decreases decay time and increases C-protein phosphorylation (Figs. 1*A*, 2, and 6). Similarly, propranolol has little or no effect on beat frequency but consistently increases decay time and reduces C-protein phosphorylation (Figs. 4 and 9). (*c*) CCh produces statistically significant increases in decay time even when hearts are paced at a constant rate of 1 Hz (Fig. 11). (*d*) C-protein phosphorylation and dephosphorylation occur even in hearts that have been arrested (Hartzell and Titus, 1982).

Although C-protein phosphorylation does not occur secondarily to changes in beat frequency, beat frequency does affect decay time (Fig. 14) and thus alters the relationship between C-protein phosphorylation and decay time. This suggests that two processes regulate relaxation. This suggestion is supported by the results of Figs. 12 and 13, which show that these two processes occur with different kinetics. Upon addition of CCh, decay time changes slowly over a period of several minutes, regardless of the pacing frequency (Fig. 12). However, if the stimulator is turned off after the heart has been paced in CCh for 6 min, the decay time rapidly increases (within 10–15 s) to the value that the decay time would have attained if the heart had not been paced (Fig. 13). Thus, it appears that the state of C-protein phosphorylation and dephosphorylation may set the range of decay times that can be rapidly modulated by beat frequency. This rapid modulation probably involves changes in intracellular free Ca transients.

In this study, C-protein phosphorylation was determined by isotopic labeling with ^{32}P, but, as has been discussed previously (Manning et al., 1980; Rudolph et al., 1978), ^{32}P incorporation may not reflect changes in protein-bound phosphate. Changes in C-protein labeling caused by changes in ATP-specific activity have been eliminated by correcting C-protein phosphorylation for differences in ATP-specific activity of each heart, which varied by a factor of <2. Furthermore, different drug treatments did not produce consistent changes in ATP-specific activities, even over the long times examined. It seems unlikely that changes in ^{32}P incorporation reflect only changes in phosphate turnover since changes in the labeling of C-protein occurred quickly and were reversible.

Regulation of Cardiac Contraction and Relaxation

The events leading to contraction of cardiac muscle are very similar to those of skeletal muscle (Mannherz and Goody, 1976; England, 1980). Contractile force is produced as the result of the formation of tension-generating links between myosin heads of the thick filament and the actin thin filament. The formation of these links is inhibited in relaxed muscle by the troponin-tropomyosin system.

Force-generating actin-myosin bonds form only when intracellular free [Ca] rises during excitation and Ca binds to troponin. The conformation of troponin changes when Ca binds and this results in removal of the inhibition of actin-myosin interaction. The muscle relaxes as intracellular free [Ca] is reduced by Ca transport systems and Ca dissociates from troponin.

Although this general scheme is similar in skeletal and cardiac muscle, the source of Ca that activates the contractile apparatus ("activating Ca") and the mechanisms that remove Ca from the sarcoplasm to effect relaxation differ (Langer, 1973; Van Winkle and Schwartz, 1976; Ebashi, 1976; Fozzard, 1977; Fabiato and Fabiato, 1979; Chapman, 1979). In cardiac muscle, much of the activating Ca comes from the extracellular space. Depolarization of the sarcolemma by the action potential opens voltage-sensitive Ca channels that permit the influx of Ca into the cell (Reuter, 1973, 1974*a*, 1979, 1983; Reuter and Scholz, 1977; Chapman, 1979; Reuter et al., 1982). In frog heart, a variety of evidence supports the idea that Ca influx is the major, if not the sole source of activating Ca (Morad and Orkand, 1971; Anderson et al., 1977; Fabiato and Fabiato, 1979; Chapman, 1979; Winegrad, 1982; Morad et al., 1983). In contrast, in mammalian heart additional Ca is released from intracellular stores, mainly the SR (Van Winkle and Schwartz, 1976; Fozzard, 1977; Fabiato and Fabiato, 1979; Chapman, 1979; Winegrad, 1982). It has been proposed that the influx of extracellular Ca stimulates the release of additional Ca from the SR (Fabiato and Fabiato, 1978). The minor role of the SR as a source of activating Ca in frog heart is supported by the finding that SR is sparse and T-tubules are largely absent in frog heart (Sommer and Johnson, 1968, 1969; Staley and Benson, 1968; Page and Niedergerke, 1972), that Ca-induced Ca release from frog SR does not occur (Fabiato and Fabiato, 1978), that contractile force is rapidly and reversibly affected by changes in extracellular [Ca] (Weidmann, 1959; Kavaler, 1974; Anderson et al., 1977), and that rapid inactivation of Ca channel blockers by light flashes produces rapid increases in tension (Morad et al., 1983).

Relaxation of cardiac muscle involves reduction of intracellular [Ca] by extrusion into the extracellular space via Na/Ca exchange (Reuter, 1973; Langer, 1982) and possibly an ATP-dependent Ca pump (Busselen and Van Kerkhove, 1978; Caroni and Carafoli, 1980), and by sequestration of Ca into the SR. In the frog, Na/Ca exchange alone seems to be sufficient to account for relaxation (Goto et al., 1972; Roulet et al., 1979; Chapman, 1979). When Na/Ca exchange is attenuated by decreasing extracellular Na or by adding La, the relaxation of frog heart muscle is greatly prolonged (Roulet et al., 1979). Furthermore, the limited content of the SR in frog heart and the slow rate of Ca accumulation by frog heart SR (Fabiato and Fabiato, 1978) further support the idea that the SR plays a minor role in regulation of relaxation in frog cardiac muscle.

In adult mammalian cardiac muscle, Ca uptake by the SR does play a major role in relaxation. Although the quantity of SR (Page et al., 1971; Peachey, 1965), as well as the concentration of Ca-ATPase in the SR (Shigekawa et al., 1976), is lower in cardiac than in skeletal muscle, cardiac SR seems to have the Ca pumping capacity to account for relaxation (Solaro and Briggs, 1974; Kita-

zawa, 1976; Will et al., 1976; Chapman, 1979). Moreover, the rate of relaxation of skinned fibers lacking a plasma membrane and intact muscle is similar (Fabiato and Fabiato, 1978), which suggests that extrusion of Ca into the extracellular space is not necessary for relaxation.

Neurotransmitters are thought to modulate cardiac contraction largely by affecting the processes which control the intracellular Ca transient (the inward Ca channels [Reuter and Scholtz, 1977; Reuter et al., 1982] and the sarcoplasmic reticulum) and by altering the affinity of troponin for Ca (Tsien, 1977; Stull, 1980; Tada and Katz, 1982; Creazzo et al., 1983). There are a variety of reasons, however, to suspect that other processes (which could include C-protein as a component) are involved as well. These reasons are discussed below.

(*a*) Recently, Winegrad (Weisberg et al., 1983; Winegrad et al., 1983) has proposed that two separate processes are involved in regulating the maximum Ca-activated tension and the Ca sensitivity of the contractile apparatus. Evidence in support of this view comes from studies on cardiac muscle preparations which have their plasma membranes permeabilized in various ways. In these preparations, cAMP can produce increases in peak tension and decreases in Ca sensitivity. Under certain circumstances these two effects can be dissociated (Weisberg et al., 1983; Winegrad et al., 1983; McClellan and Winegrad, 1980). Changes in Ca sensitivity correlate with the level of troponin I phosphorylation (Mope et al., 1980) but the molecules responsible for regulation of maximum tension in these permeabilized cells have not been identified (Weisberg et al., 1983). The molecules are presumed to be regulatory proteins of the contractile apparatus because the sarcoplasmic reticulum and sarcolemma have been effectively inactivated by the permeabilization procedure and the Ca buffers employed. Troponin I and myosin light chain phosphorylation, however, do not correlate with changes in contractility (Weisberg et al., 1983; Mope et al., 1980; McClellan and Winegrad, 1980). This also seems to be true of other contractile proteins including C-protein. These studies, therefore, suggest that the ionotropic state of the myofibril can be regulated by cAMP acting through unidentified target proteins and that the Ca sensitivity can be regulated at least in part by troponin I phosphorylation.

Other evidence that the force of contraction can be modulated by factors other than the Ca transient is suggested by the finding that shortening muscle length dramatically increases the peak tension and increases the duration of the twitch without greatly affecting the Ca transient (Allen and Kurihara, 1982).

The relationship of Ca sensitivity to the physiological characteristics of the heartbeat is unknown, but it seems likely that the relaxation rate is at least partly determined by the Ca sensitivity of the contractile apparatus. If intracellular free Ca is always reduced at the same rate, the contractile apparatus will turn off more quickly after peak contraction when Ca sensitivity is high than when Ca sensitivity is low. Our studies suggest that C-protein phosphorylation may also be involved in regulating the Ca sensitivity of the myofibril or may regulate relaxation by another mechanism separate from the processes that determine the Ca sensitivity of the contractile apparatus (see below).

(*b*) Another reason other processes are likely to be involved in regulating

cardiac contraction is that the sarcoplasmic reticulum in frog heart is poorly developed and seems to play only a minor role in relaxation in this species (see above). Thus, it is unlikely that phospholamban phosphorylation is important in mediating the effects of NE in frog heart. In addition, net Ca efflux is not affected by NE (Porzig et al., 1975). Thus, changes in relaxation rate produced by NE probably are not mediated exclusively by changes in the removal of Ca from the sarcoplasm. Moreover, recent studies by Lindemann et al. (1983) in guinea pig ventricle show that phosphorylation of phospholamban does not correlate well with either inotropic state or relaxation rate. For example, the rate of dephosphorylation of phospholamban is slower than the return of the relaxation rate to control levels after washing out Iso. Also, dibutyryl cyclic AMP increases phospholamban phosphorylation without significantly affecting the relaxation rate.

(*c*) The dissociation rate of Ca from troponin is sufficiently slow ($k_{off} = 20/s^{-1}$) to contribute to the relaxation rate of mammalian hearts (Robertson et al., 1981, 1982) and thus it is plausible that changes in dissociation rate due to troponin phosphorylation will affect relaxation. If the dissociation rate of Ca from frog troponin is similar to that of mammalian troponin, however, the dissociation rate seems to be much too fast to be the rate-limiting step in relaxation, which is much slower in frog than mammal.

(*d*) Several other proteins become phosphorylated in heart in response to Iso. These include myosin light chain (Frearson et al., 1976) and C-protein (Jeacocke and England, 1980; Hartzell and Titus, 1982). The possible role of myosin light chain phosphorylation and, indeed, the question of whether the phosphate content is physiologically altered remain controversial even in skeletal muscle (see Stull, 1980, for review; Butler et al., 1983; Crow and Kushmerick, 1982).

Role of C-Protein in Regulation of Contractility

For the reasons discussed above, it seems likely that several mechanisms are involved in regulating cardiac contractility. It seems likely that C-protein is involved, because C-protein from skeletal muscle has a number of very interesting properties that suggest that it could be a regulatory protein of the myofibril. (*a*) C-protein is an integral component of the thick filament (Offer et al., 1973). (*b*) C-protein binds with high affinity to actin and to myosin in solution (Moos et al., 1975, 1978; Yamamoto and Moos, 1983). (*c*) The binding of C-protein to actin is competed by myosin subfragment-1 (Moos et al., 1978). (*d*) At low ionic strength, C-protein from skeletal and cardiac muscle inhibits skeletal muscle actomyosin ATPase activity but has no effect on basal myosin ATPase (Offer et al., 1973; Moos et al., 1978; Moos and Feng, 1980; Yamamoto and Moos, 1983). (*e*) Myosin binding to native (regulated) thin filaments in myofibrils requires Ca concentrations similar to those that activate actomyosin ATPase in the myofibril (Moos, 1981). This observation suggests that C-protein binding to the thin filament may be controlled by the troponin-tropomyosin system.

These observations suggest two hypotheses for the mechanism by which C-protein might regulate relaxation rate in cardiac muscle. (*a*) Termination of contraction by phospho-C-protein: In this mechanism, phosphorylated C-protein

has the potential for binding to thin filament and inhibiting actin-activated myosin ATPase. The binding of phospho-C-protein to the thin filament, however, occurs only after intracellular Ca rises to a level that "turns on" the thin filament. Thus, when C-protein is phosphorylated, the thin filament would be turned off prematurely. When C-protein is dephosphorylated, it cannot bind to the thin filament under any circumstances, and relaxation of the muscle would be determined by the rate of calcium removal from the sarcoplasm by Na/Ca exchange and sequestration by the SR and other Ca sinks. (*b*) Prolongation of contraction by dephospho-C-protein: Alternatively, dephospho-C-protein would bind to the thin filament in the presence of Ca and prevent or slow the return of tropomyosin to its blocking position as intracellular Ca declines and thus would prolong the active state.

These hypotheses have the advantage that they can be directly tested experimentally. Both of these hypotheses predict that the binding of purified C-protein to native, regulated thin filaments will depend upon the level of C-protein phosphorylation and [Ca]. These studies are presently underway.

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