

The Role of Cyclic Adenosine 3',5'-Monophosphate and Calcium in the Regulation of Contractility and Glycogen Phosphorylase Activity in Guinea Pig Papillary Muscle

JAMES G. DOBSON, JR., PH.D., JOHN ROSS, JR., M.D., AND STEVEN E. MAYER, PH.D.

SUMMARY We studied the relationships between the positive inotropic effects of isoproterenol, increased frequency of contraction or paired electrical stimulation, and cyclic AMP concentration and phosphorylase activity in isolated guinea pig papillary muscles. The minimum concentration of isoproterenol (10 nM) that augmented isometric force development increased cyclic AMP concentration. However 100 nM isoproterenol was required to increase the phosphorylase activity ratio ($-AMP/+AMP$) from 0.15 ± 0.03 to 0.25 ± 0.03 . After addition of $1 \mu M$ isoproterenol to the bath, cyclic AMP increased within 0.5 minute from 0.58 ± 0.03 to 1.04 ± 0.13 mol/kg (wet weight), peak contractile force was elevated 2-fold at 1 minute, and the phosphorylase activity ratio rose to 0.40 ± 0.02 in 4 minutes. Although an increase in contraction frequency (6/min to 36/min) and paired stimulation produced more than a 3-fold increase in peak contractile force, there were no changes in cyclic AMP and

phosphorylase activity. The cyclic AMP concentration during diastole was 0.60 ± 0.04 and in midsystole, $0.55 \pm 0.03 \mu mol/kg$. Anoxia increased the phosphorylase activity ratio from 0.19 ± 0.02 to 0.41 ± 0.04 without elevation of cyclic AMP concentration. Removal of Ca^{2+} from the bathing medium prevented active force development and the anoxic increase in phosphorylase activity, but did not prevent the isoproterenol-induced increase in cyclic AMP and phosphorylase. These results suggest that cyclic AMP is a factor in the catecholamine-induced enhancement of inotropic state. However, it does not appear to play a role in the maintained augmentation of inotropic state produced by increased contraction frequency and paired stimulation, nor does the concentration of the cyclic nucleotide appear to vary during the contraction cycle or during anoxia. Extracellular Ca^{2+} is required for contraction, the positive inotropic action of catecholamines and phosphorylase *b* to *a* conversion by anoxia.

ADENOSINE 3',5'-monophosphate (cyclic AMP) and calcium are important mediators of catecholamine-induced cardiac glycogenolysis and increased myocardial contractility.¹⁻³ The activation of glycogenolysis induced in the myocardium by catecholamines results from the conversion of glycogen phosphorylase from the *b* to the *a* form.⁴ The sequence of reactions begins with β -adrenergic receptor-linked production of cyclic AMP through the activation of adenylate cyclase.¹⁻⁵ Cyclic AMP appears to activate protein kinase, which catalyzes the adenosine triphosphate-dependent transformation of phosphorylase kinase to its activated form.^{6, 7} Phosphorylase kinase catalyzes the phosphorylation of phosphorylase *b* and converts it to phosphorylase *a*; this is a Ca^{2+} -dependent process both with purified enzymes⁸ and in intact heart muscle.⁹ Ischemia causes phosphorylase activation via a cyclic AMP-dependent transformation of phosphorylase kinase, presumably by the release of norepinephrine from cardiac stores.^{10, 11} The activation of glycogenolysis in anoxia appears to be due to phosphorylase activation that is independent of cyclic AMP and phospho-

rylase kinase transformation. It has been proposed that anoxic phosphorylase activation is due to enhanced catalytic activity of the nonactivated form of phosphorylase kinase.¹¹ Since phosphorylase kinase is a Ca^{2+} -dependent enzyme, one possibility for augmenting its activity would be that anoxia increases the Ca^{2+} available to the enzyme.

Calcium is necessary for tension development in the myocardium.¹² The interaction between the calcium ion and cyclic AMP in influencing cardiac contractility remains unproven. However, several possible links between cyclic AMP, calcium transport, and cardiac contraction have been suggested recently. Both the uptake of Ca^{2+} by, and Ca^{2+} -activated ATPase activity of, cardiac fragmented sarcoplasmic reticulum are enhanced by cyclic AMP-dependent protein kinase and correlate with sarcoplasmic reticulum phosphorylation.¹³ Epinephrine is known to increase troponin I phosphorylation in the heart in situ.¹⁴ Phosphorylation of the cardiac contractile protein complex (possibly troponin I¹⁵) by cyclic AMP-dependent protein kinase has been postulated to be responsible for an increase in the Ca^{2+} -sensitivity and activity of the actomyosin ATPase.¹⁶ These studies suggest that the positive inotropic effect of catecholamines may be mediated through a cyclic AMP-dependent protein kinase which promotes both the phosphorylation of cardiac sarcoplasmic reticular membranes and contractile proteins and thereby (1) accelerates Ca^{2+} uptake in the sarcoplasmic reticulum and increases the rate of relaxation,¹⁸ possibly with an associated effect on Ca^{2+} release,¹⁷ and (2) increases both the Ca^{2+} sensitivity and ATPase activity of actomyosin.^{15, 16}

The purpose of this investigation was to study the relationship between increases in myocardial contractility

From the Department of Physiology, University of Massachusetts Medical Center, Worcester, Massachusetts, and the Divisions of Pharmacology and Cardiology, Department of Medicine, University of California, San Diego, La Jolla, California.

Supported by a grant-in-aid award from the American Heart Association, Massachusetts Affiliate, and Central Massachusetts Chapter Grant 1318; University of Massachusetts Medical School General Research Support Grant RRO 5712-03; a Bank of America Giannini Foundation Fellowship to J.G. Dobson, Jr.; and Program Project Grant HL 12373 from the National Heart and Lung Institute to the University of California, San Diego.

Address for reprints: Dr. James G. Dobson, Jr., Department of Physiology, University of Massachusetts Medical Center, 55 Lake Avenue, Worcester, Massachusetts 01605.

Received February 6, 1976; accepted for publication May 3, 1976.

caused by isoproterenol, frequency of contraction or paired stimulation, and cyclic AMP concentration and phosphorylase activity in isolated papillary muscles. In addition, we sought to determine whether the Ca^{2+} involved in myocardial tension development also plays a role in catecholamine- and anoxia-induced activation of glycogen phosphorylase.

Methods

PAPILLARY MUSCLE PREPARATION

Male Hartley guinea pigs weighing 200–400 g were obtained from Camm or Elm Hill and maintained on Purina guinea pig chow ad libitum in rooms with a sequence of 12 hours of light and 12 hours of darkness. The guinea pigs were stunned by a blow to the base of the skull. A midsternal incision was made rapidly, and the hearts were excised from the chest within 5 seconds. The hearts were placed in a dissecting dish (described previously¹⁸) containing oxygenated bathing medium. Bathing medium was prepared fresh daily and contained (mmol/liter): NaCl, 118.4; KCl, 4.69; $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 2.52; NaCHO_3 , 25; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 1.18; KH_2PO_4 , 1.18; and glucose, 5. When gassed with 95% O_2 -5% CO_2 , the pH was 7.4 at 25°C.

Papillary muscles no larger than 1.0–1.2 mm in diameter were dissected from both the right and left ventricles and a 5-0 or 4-0 silk suture was tied to the chordae tendineae of each muscle. The papillary muscles were mounted vertically in methacrylate myographs (described previously¹⁸) containing 100–130 ml of the bathing medium described above which was continuously gassed with 95% O_2 -5% CO_2 (400–500 ml/min) at 25°C via a fritted glass dispersion tube submerged in the bathing medium. The lower ends of some muscles were placed in a plastic clip attached to a Statham strain gauge (Gold cell UC-3) and the upper end was tied to an adjustable lever. Other muscles were placed in a fixed plastic clip and the upper end was tied to a Grass force transducer (FT 03C). A preload of 2 g was applied, and the muscles were generally stimulated to contract isometrically at a rate of 6–12/min with either platinum or Ag-AgCl 34-gauge wire electrodes fixed to the inner surfaces of the plastic clips which made direct contact with the tissue. Square wave pulses of a voltage and duration just necessary to elicit contraction (10–50 V, 3–5 msec) were generated by either a Grass S4KR or SD9 stimulator. Paired electrical stimulation, a sustained form of post-extrasystolic potentiation,¹⁹ was produced by delivering two square wave pulses to the preparation 50–100 msec apart. With a preload of 2 g the developed force was maximal, and the lengths of the muscles ranged from 5 to 10 mm. Calculated on the basis of weight and assuming the muscle to be a cylinder, the cross-sectional areas of the preparations were 0.50–1.13 mm².

The muscles were equilibrated for 20–30 minutes and tension development was continuously recorded on either a Hewlett-Packard model 7700 or Grass model 7B polygraph. The maximum active isometric force is reported as peak contractile force. Some preparations were exposed to Ca-free bathing medium in which the Ca^{2+} was replaced with Na^+ . Anoxic conditions were introduced by gassing the bathing medium with 95% N_2 -5% CO_2 . Isoproterenol was prepared fresh daily in a 0.9% (wt/vol) sodium chloride

solution containing 0.2% (wt/vol) sodium metabisulfite and added to the bathing medium in a 10- μl volume. The preparations were generally quick-frozen in diastole while mounted in the myograph by lowering the bathing medium and immediately either submersing the muscles in dichlorodifluoromethane (Freon 12) or clamping the muscles with straight 9 inch hemostatic forceps, the clamping surfaces of which were ground smooth. Both the dichlorodifluoromethane and the hemostats were precooled to below –100°C with liquid nitrogen. The use of hemostats permitted freezing in late diastole just prior to the onset of systole and at midsystole. Proper timing of the freeze during midsystole at a time of peak tension development was facilitated by the use of an audiopulse amplifier connected to the muscle stimulator. The square waves generated by the stimulator were delivered simultaneously to both the muscle and the amplifier, and the audible signal was used to coordinate the freezing at the desired time. Polygraphic recording of tension development indicated the exact time in the contraction cycle at which the muscle was frozen.

The muscles never were stimulated to contract for a period greater than 130 minutes before freezing. Peak contractile force, phosphorylase activity, and the concentration of cyclic AMP in control muscles remained constant during this period. Resting (noncontracting) muscles were also equilibrated for 20–30 minutes and did not contract spontaneously with isoproterenol or anoxia.

ANALYTICAL PROCEDURES

Tissue Preparation and Extraction. Frozen papillary muscles were stored at –65°C in screw-cap vials and prepared for analysis in a cold chamber at –25°C. The ice that formed on the surface of the papillary muscles during freezing was scraped off with a scalpel. Wet weight of the muscles ranged from 4–10 mg. Approximately 2–3 mg of the papillary muscle was transferred to a Dual homogenizing tube (size 20, Kontes Glass) at –25°C. An ice-cold solution (50–100 vol) containing KF, 20 mM; ethylenediaminetetraacetate (EDTA), 4 mM; β -glycerophosphate, 20 mM; and β -mercaptoethanol, 20 mM (pH 6.8) was added rapidly and the mixture was homogenized at 0°C. The homogenization was performed in 1 minute with a motor-driven ground glass pestle rotating at 100–300 rpm. The homogenate was centrifuged at 3,000 g for 20 minutes at 0°C, and the supernatant fluid was assayed for phosphorylase.

Another portion (3–4 mg) of each papillary muscle was transferred to a Dual homogenizing tube; 75 μl of 10% trichloroacetic acid was added and tissue plus acid were homogenized at 0°C. The homogenate was centrifuged as described above and the supernatant fluid was extracted five times with 4 vol of H_2O -saturated diethyl ether. The extract was placed in a boiling water bath until the odor of ether no longer was detectable and was assayed for cyclic AMP. The concentration of cyclic AMP is expressed as $\mu\text{mol/kg}$, wet weight of myocardium (12.5 mg of protein/100 mg).

Biochemical Assays. Glycogen phosphorylase was measured by the production of glucose 1-phosphate in the absence and presence of AMP.²⁰ One unit of phosphorylase is defined as the amount of enzyme that produces 1 $\mu\text{mol/min}$ of glucose 1-phosphate from glycogen at 30°C.

The results are expressed as the ratio of phosphorylase activity without AMP to phosphorylase activity assayed with AMP. The concentration of cyclic AMP was measured in tissue extracts by a modification²¹ of the method of Wastila et al.,²² which is based on the activation of skeletal muscle protein kinase.

Materials. Racemic isoproterenol hydrochloride (Winthrop or Sigma) was prepared in 0.2% (wt/vol) sodium metabisulfite. All salts, dextrose, and EDTA were certified grade from Fisher Scientific. Ethylene glycol bis(β -aminoethyl ether)-*N,N'*-tetraacetic acid (EGTA) was obtained from Sigma. Nucleotides were obtained from P-L Biochemicals or Boehringer-Mannheim. All enzymes used for the phosphorylase assay and the γ -³²P-ATP synthesis (a substrate for cyclic AMP assay) were from Boehringer-Mannheim. Carrier-free (³²P) inorganic phosphate was obtained from Schwarz/Mann.

Statistical Methods. Analysis of variance was performed for paired and unpaired observations.²³ A probability of <0.05 was accepted as indicating a significant difference.

Results

EFFECT OF ISOPROTERENOL ON PEAK CONTRACTILE FORCE, CYCLIC AMP, AND PHOSPHORYLASE

DL(\pm)-Isoproterenol produced dose-dependent increases in peak contractile force, cyclic AMP concentration, and activation of phosphorylase in the guinea pig papillary muscle (Fig. 1). Peak contractile force increased from a

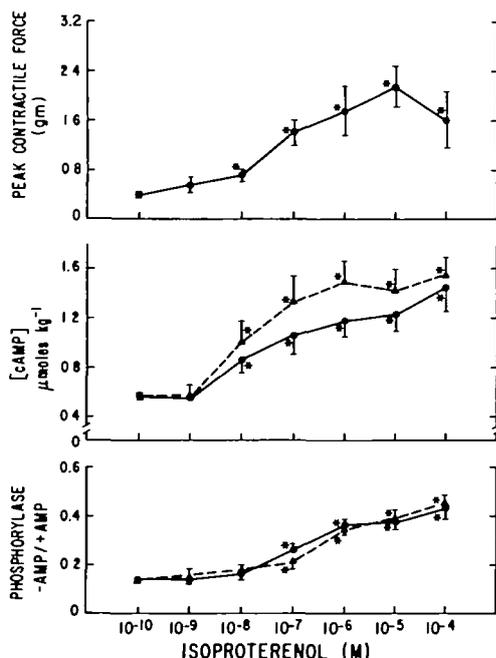


FIGURE 1 Effect of isoproterenol on papillary muscle peak contractile force, cyclic AMP concentration (per kilogram of muscle, wet weight), and phosphorylase activity ratio. The muscles were exposed to different concentrations of isoproterenol for 4 minutes. Each point represents the mean of three to five muscles for both contracting (●—●) and resting (▲----▲) muscles. The asterisks denote significant differences ($P < 0.05$) from control (no isoproterenol) values. Bars represent ± 1 SE.

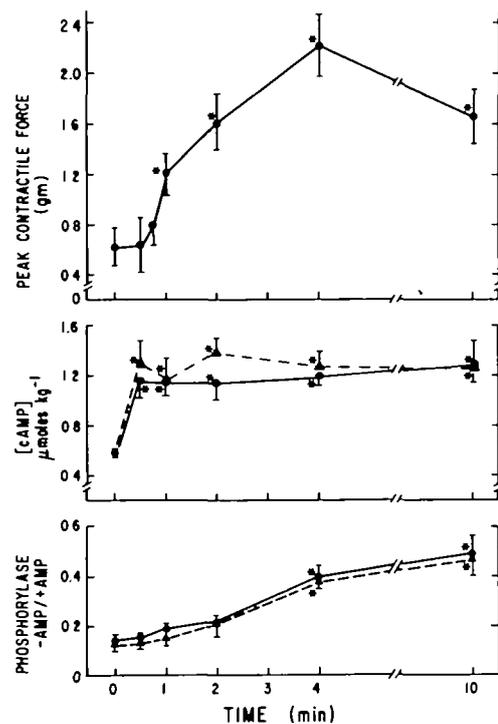


FIGURE 2 Effect of $1 \mu\text{M}$ isoproterenol on papillary muscle peak contractile force, cyclic AMP concentration, and phosphorylase activity ratio. Each point represents the mean of three to five muscles for both contracting (●—●) and resting (▲----▲) muscles. The asterisks denote significant differences ($P < 0.05$) from control (no isoproterenol) values. Bars represent ± 1 SE.

control of 0.38 ± 0.03 g to 0.67 ± 0.10 g with 10 nM isoproterenol. The increase in peak contractile force was maximal at more than 4-fold with $10 \mu\text{M}$ isoproterenol. The cyclic AMP concentration in control contracting and resting papillary muscle was $0.58 \pm 0.03 \mu\text{mol/kg}$. With 10 nM isoproterenol the cyclic AMP concentration increased to 0.88 ± 0.07 in contracting and to $1.00 \pm 0.17 \text{ mol/kg}$ in resting muscle. This difference between resting and contracting papillary muscle was not significant at any concentration of isoproterenol between 0.01 and $100 \mu\text{M}$. The control phosphorylase activity ratio was 0.13 ± 0.02 in contracting and 0.14 ± 0.02 in resting papillary muscle. It did not increase significantly with 10 nM isoproterenol, but rose to 0.26 ± 0.01 in contracting and to 0.22 ± 0.02 in resting muscle exposed to 100 nM isoproterenol. In contracting and resting muscle the activity ratio increased further, to ranges of 0.39 ± 0.02 to 0.42 ± 0.03 and 0.38 ± 0.02 to 0.43 ± 0.03 , respectively, with higher concentrations of isoproterenol (1 – $100 \mu\text{M}$). Total phosphorylase activity was $6.65 \pm 0.17 \text{ U/g}$ of muscle and did not change with any of the experimental variables introduced.

Isoproterenol ($1 \mu\text{M}$) produced an increase in papillary muscle cyclic AMP concentration before the increase in peak contractile force and phosphorylase activity ratio occurred (Fig. 2). Peak contractile force increased significantly from 0.61 ± 0.16 to 1.21 ± 0.19 g within 1 minute and to 2.23 ± 0.27 g after a 4-minute exposure to isoproterenol. Cyclic AMP concentration increased in 0.5 minute in

contracting muscle (from 0.58 ± 0.03 to 1.15 ± 0.13 $\mu\text{mol/kg}$) and in resting muscle (from 0.58 ± 0.03 to 1.27 ± 0.22 $\mu\text{mol/kg}$). There was no significant difference between contracting muscle and resting muscle cyclic AMP concentration at any time point. The phosphorylase activity ratio increased significantly only after 4 minutes of isoproterenol exposure in contracting muscle (from 0.14 ± 0.02 to 0.40 ± 0.02) and in resting muscle (from 0.13 ± 0.02 to 0.38 ± 0.02).

CARDIAC CONTRACTION CYCLE AND CYCLIC AMP CONCENTRATION

The cyclic AMP concentration in papillary muscle, frozen in diastole 0.6 ± 0.2 second before delivery of the electrical stimulus to initiate a subsequent contraction, and in midsystole close to the time of peak force development ± 0.1 second, was 0.60 ± 0.04 and 0.55 ± 0.03 $\mu\text{mol/kg}$, respectively (Fig. 3). Since the duration of systole in this preparation ranged from 1.0 to 1.2 seconds, the muscle developed 75–100% of its peak contractile force when freezing occurred in midsystole. Isoproterenol ($1 \mu\text{M}$) increased muscle cyclic AMP, but there was no difference between the diastolic and systolic concentrations of cyclic AMP (Fig. 3). Papaverine ($10 \mu\text{M}$, 30 minutes) in six muscle preparations did not change peak contractile force but did increase muscle cyclic AMP from a control of 0.58 ± 0.03 to 1.05 ± 0.10 $\mu\text{mol/kg}$ in diastole and from a control of 0.59 ± 0.04 to 1.01 ± 0.14 $\mu\text{mol/kg}$ in midsystole.

THE EFFECT OF CONTRACTION FREQUENCY AND PAIRED STIMULATION

Increasing the frequency of contraction from 6/min to 18/min and 36/min enhanced peak contractile force 2.1-fold and 3.3-fold, respectively (Fig. 4). Papillary muscle phosphorylase activity ratio and cyclic AMP concentration remained unchanged over this range of frequencies.

Paired electrical stimulation produced an increase in peak contractile force from 0.36 ± 0.03 to 0.75 ± 0.13 g within

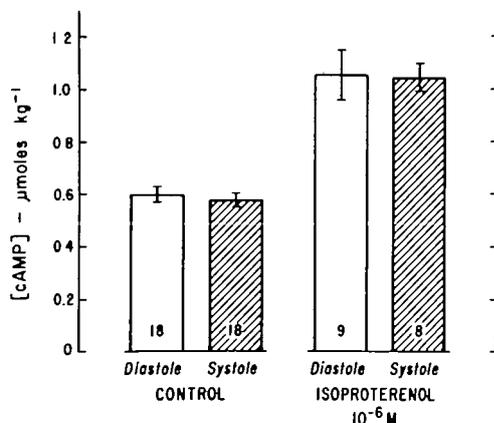


FIGURE 3 The concentration of papillary muscle cyclic AMP frozen in late diastole (open bars) and midsystole (cross-hatched bars) with and without isoproterenol. Numbers at the bottom represent the number of experiments performed to determine the mean concentration ± 1 SE. Freezing was timed to an audible signal as described in Methods, Papillary Muscle Preparation.

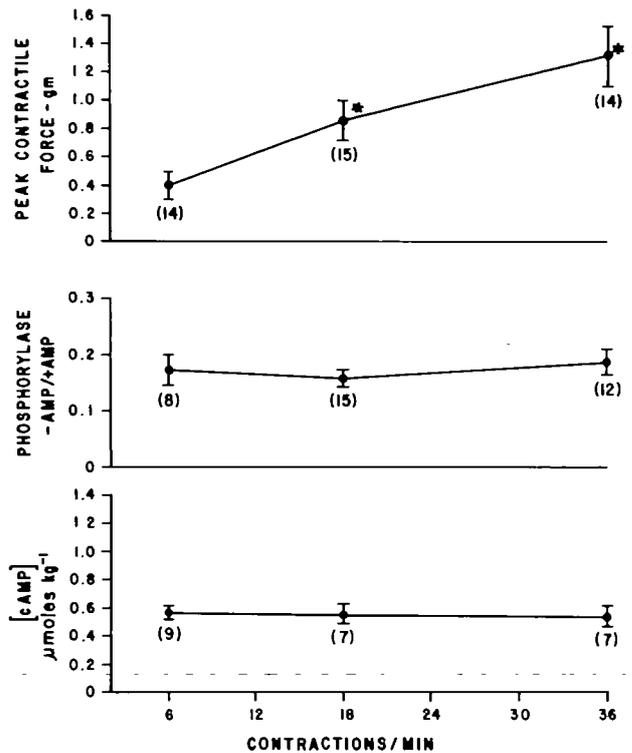


FIGURE 4 Effect of frequency of contraction on papillary muscle peak contractile force, phosphorylase activity ratio, and cyclic AMP concentration. The muscles were stimulated at a rate of 6/min during a 30-minute equilibration period, then stimulated to contract at a rate of either 6, 18, or 36/min for 10 minutes, and the above data were collected. Numbers in parentheses are shown in parentheses. The asterisks denote significant differences ($P < 0.05$) from muscles contracting at 6/min. Bars represent ± 1 SE.

0.5 minute (Fig. 5). Four minutes of paired stimulation increased peak contractile force 4.7-fold. Papillary muscle cyclic AMP concentration and phosphorylase activity ratio did not change from control over the 10-minute period of paired stimulation.

EFFECT OF CALCIUM ON PEAK CONTRACTILE FORCE, CYCLIC AMP, AND PHOSPHORYLASE

The removal of Ca^{2+} from the bathing medium of papillary muscle progressively decreased peak contractile force (Fig. 6). A 4-minute exposure to isoproterenol ($1 \mu\text{M}$) immediately before the replacement of Ca^{2+} with Na^{+} increased peak contractile force 2.6-fold (from 0.46 ± 0.07 g to 1.19 ± 0.15 g). After 10 minutes in the Ca^{2+} -free medium control peak contractile force fell to 0.03 ± 0.01 g but still increased in response to isoproterenol (0.29 ± 0.07 g). Isoproterenol had no detectable effect on peak contractile force (< 0.01 g) when the stimulated muscles were bathed with Ca^{2+} -free medium for 30 minutes. The papillary muscle phosphorylase activity ratio was not altered in Ca^{2+} -free medium; the activity ratio showed a 2-fold increase in control and in Ca^{2+} -free medium in response to isoproterenol at all times after Ca^{2+} removal. The cyclic AMP response to isoproterenol also was not altered after removal of Ca^{2+} from the medium.

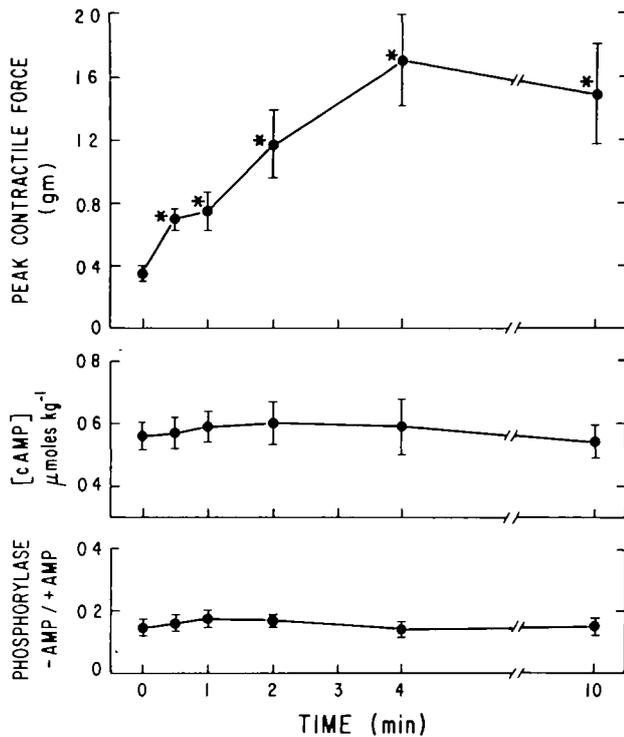


FIGURE 5 Effect of paired stimulation on contracting (6/min) papillary muscle. Paired stimulation was produced by delivering two 5-msec square wave pulses to the muscles 50–100 msec apart at a voltage 10% above threshold. The asterisks denote significant differences ($P < 0.05$) from zero time. See Figure 1 for further explanation.

There was no difference in the increase of the papillary muscle phosphorylase activity ratio in response to isoproterenol ($1 \mu\text{M}$) in Ca^{2+} -containing, Ca^{2+} -free, and EGTA- plus Ca^{2+} -containing bathing media (Fig. 7). However, after 60 minutes in a Ca^{2+} -free plus EGTA-containing medium, isoproterenol did not increase the formation of phosphorylase *a*. The control phosphorylase activity was similar in all four bathing media. Control cyclic AMP concentration was not influenced by the four different bathing media. Isoproterenol produced increases in cyclic AMP concentration in all four media that did not differ significantly from one another.

EFFECT OF CALCIUM ON CYCLIC AMP AND PHOSPHORYLASE *a* FORMATION IN ANOXIA

Anoxia produced an increase in the papillary muscle phosphorylase activity ratio in 2.5 mM Ca^{2+} bathing medium, from 0.19 ± 0.02 to 0.41 ± 0.04 , without producing an increase in cyclic AMP concentration (Table 1). In the Ca^{2+} -free bathing medium this response in phosphorylase activity to anoxia was abolished. The phosphorylase activity ratio was not increased beyond that observed with either isoproterenol or anoxia alone when both stimuli were present together in the Ca^{2+} -containing medium. The increase in the phosphorylase activity ratio produced by isoproterenol in Ca^{2+} -free medium was prevented in anoxic muscles; the elevation in cyclic AMP concentration was not. Lowering the Ca^{2+} content of the medium also increased the

control value for cyclic AMP, as has been observed previously in isolated rat heart.⁹

Discussion

ISOPROTERENOL

Isoproterenol produced an elevation in cyclic AMP concentration which preceded in time the increase in peak contractile force in papillary muscle (Fig. 2). Both responses were observed with concentrations of the catecholamine as low as 10 nM (Fig. 1). However, 100 nM isoproterenol was required for phosphorylase activation which lagged in time behind the increases in cyclic AMP concentration and peak contractile force (Figs. 1 and 2). These results suggest that low concentrations of catecholamines are capable of augmenting contractility without a concomitant formation of phosphorylase *a*. Similar results have been reported previously in the dog heart in situ.²⁴ The temporal relationship between the formation of cyclic AMP, the increase in contractility, and the formation of phosphorylase *a* in response to isoproterenol observed in this study has been well documented in various preparations of cardiac muscle (for references see Mayer³). The data are consistent with the

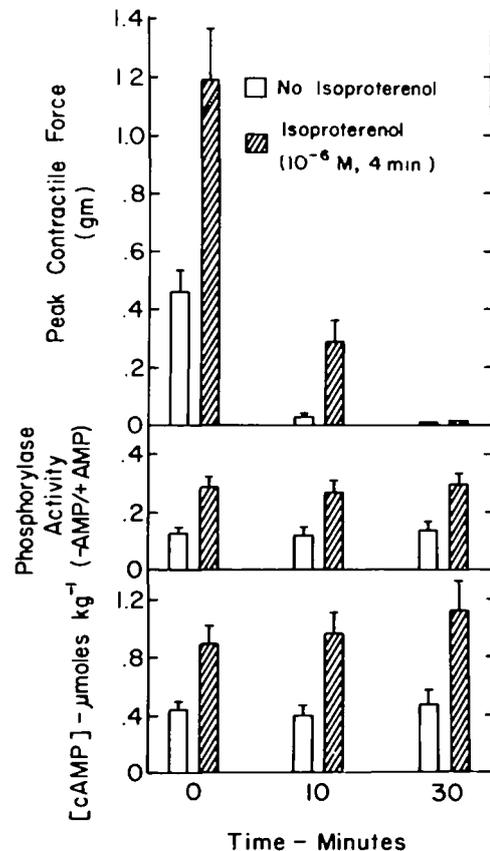


FIGURE 6 Effect of exposure to Ca^{2+} -free bathing medium on papillary muscle peak contractile force, phosphorylase activity ratio, and cyclic AMP concentration in the absence and presence of isoproterenol ($1 \mu\text{M}$). Ca^{2+} was removed at zero time. Isoproterenol was added for 4 minutes before the medium was changed at zero time (0) and at time points 10 and 30 minutes later. Each bar represents the mean $\pm 1 \text{ SE}$ for three to five experiments.

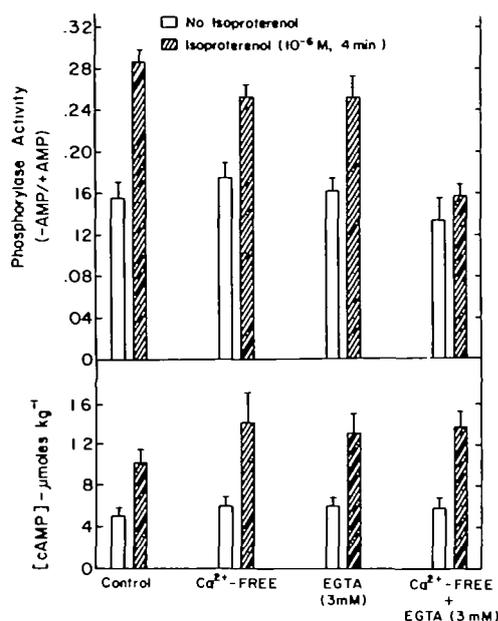


FIGURE 7 Effect of Ca^{2+} -containing, Ca^{2+} -free, EGTA plus Ca^{2+} and Ca^{2+} -free plus EGTA bathing media on phosphorylase activity ratio and cyclic AMP concentration in papillary muscle. The calcium concentration was 2.5 mM. The muscles were first equilibrated for 30–60 minutes then placed in the various media for 60 minutes while continuously stimulated electrically at 6–12 pulses/min and gassed with 9.5% O_2 -5% CO_2 . See legend of Figure 6 for further explanation.

hypothesis that cyclic AMP not only initiates the sequence of events that lead to glycogenolysis via phosphorylase activation but enhances cardiac inotropic state as well.

The increase in cyclic AMP concentration in response to isoproterenol at 0.5 minute remained elevated at a constant value while there was a gradual increase in peak contractile force between 1 minute and 4 minutes (Fig. 2). These observations suggest that, whatever action the cyclic nucleotide is exerting on the mechanisms controlling contractility, it takes time to be manifested and become fully expressed. Although the cyclic AMP concentration may have appeared to increase to a slightly greater extent in resting as compared to contracting cardiac muscle in response to isoproterenol (Fig. 1) the difference was not significant.

If the muscle could have been stimulated to contract at a rate similar to that in the intact guinea pig heart, approximately 180/min, a greater difference might have been observed. However, we have previously reported¹⁸ that the guinea pig papillary muscle preparation, if stimulated to contract at rates greater than 36/min, becomes hypoxic as reflected by a decrease in the concentration of creatine phosphate and ATP, and by the activation of phosphorylase.

CARDIAC CONTRACTION CYCLE

No difference between diastolic and midsystolic cyclic AMP concentration was observed in either the absence or presence of isoproterenol (Fig. 3). However, resolution obtained in these experiments may not have been adequate to demonstrate rapid changes in cyclic AMP concentration that may occur at the onset of systole, as has been reported to occur in the frog ventricle.^{25, 26} The duration of systole in the amphibian heart is 2–3 times longer than in the mammal, and extremely rapid freezing techniques would be needed to fix mammalian cardiac tissue at several time points during the contraction cycle. In addition, species difference may account for the apparent disparity between our results and those of others using amphibian myocardium.

Papaverine, a potent cardiac cyclic nucleotide phosphodiesterase inhibitor, did not reveal a difference between diastolic and midsystolic cyclic AMP concentrations which were increased equally. This suggests that either there was no elevation of cyclic AMP with the onset of systole or that a small change was masked by the increase produced by papaverine both in late diastole and midsystole. Previously, Henry et al.²⁷ demonstrated that papaverine produced an increase in myocardial cyclic AMP concentration and phosphorylase *a* formation in the isolated isovolumic, perfused guinea pig heart but was without effect on contractility. However, with such experiments it is difficult to ascertain whether the papaverine did not prevent an increase in contractility by some as yet undefined mechanism.

CONTRACTION FREQUENCY AND PAIRED ELECTRICAL STIMULATION

Cyclic AMP concentration and phosphorylase activity were not influenced by increasing the frequency of contraction or by paired electrical stimulation (Figs. 4 and 5). The

TABLE 1 The Effect of Anoxia on Cyclic AMP and the Phosphorylase Activity Ratio in Resting Papillary Muscle

	Isoproterenol (1 μM)	Cyclic AMP (μmol/kg)		Phosphorylase activity ratio	
		2.5 mM Ca^{2+}	0 Ca^{2+}	2.5 mM Ca^{2+}	0 Ca^{2+}
Control	-	0.58 ± 0.03	0.66 ± 0.03*	0.19 ± 0.02	0.19 ± 0.04
	+	1.11 ± 0.15*	1.15 ± 0.20*	0.42 ± 0.02*	0.38 ± 0.03*
Anoxia	-	0.55 ± 0.04	0.65 ± 0.04*	0.41 ± 0.04*	0.24 ± 0.04
	+	1.02 ± 0.11*	1.10 ± 0.21*	0.40 ± 0.04*	0.22 ± 0.03

Values represent the mean ± 1 SE for four to six experiments. Control muscles were gassed with 95% O_2 -5% CO_2 , and anoxia was introduced by gassing with 95% N_2 -5% CO_2 for 20 minutes. Muscles were bathed with Ca^{2+} -free medium for 30 minutes. Isoproterenol exposure was 10 minutes. * Significantly different from control without isoproterenol in bathing medium containing 2.5 mM calcium

results suggest that these two positive inotropic interventions augment the cardiac contractile state via cyclic AMP-independent mechanisms, probably by increasing Ca^{2+} available to the myofilaments. It has been shown in hearts from a variety of species that an increase in the frequency of contraction has a positive inotropic effect²⁸ and also increases calcium influx.²⁹ Increased frequency of contraction also appears to stimulate Ca^{2+} -sequestration mechanisms.³⁰ Therefore, the augmentation in cardiac contractility resulting from an increase in contraction frequency is probably due to an enhanced Ca^{2+} influx which in turn increases both the availability of the ion to the myofilaments and sarcoplasmic reticulum Ca^{2+} loading. However, it is thought that a greater release from a more fully loaded sarcoplasmic reticulum is most important for augmented tension development with each subsequent contraction.¹² Paired electrical stimulation probably utilizes a similar Ca^{2+} -dependent mechanism to produce an increase in contractility.³¹ Our results further indicate that the frequency and timing of electrical depolarizations which may be associated with sarcolemmal Ca^{2+} influx,³² as well as sarcoplasmic reticulum Ca^{2+} release, do not increase phosphorylase activity or cyclic AMP concentration.

These results are not in agreement with previous studies performed in the isolated rat heart, where an increase in contraction frequency and paired stimulation produced a transient increase in phosphorylase *a* formation.³³ Perhaps the type of preparation and species differences account for the discrepancy.

CALCIUM, CONTRACTILITY, CYCLIC AMP, AND PHOSPHORYLASE

Removal of Ca^{2+} from the bathing medium of papillary muscles produced, as expected, a gradual decline in peak contractile force to undetectable levels and attenuation of the inotropic effect of isoproterenol. However, removal of Ca^{2+} was without effect on the catecholamine-induced increase in cyclic AMP concentration and formation of phosphorylase *a* (Fig. 6). This finding suggests that Ca^{2+} in the external medium is essential for tension development and for maximum positive inotropic effects of catecholamines. While the removal of Ca^{2+} from the bathing medium for 30 minutes presumably depleted extracellular Ca^{2+} in the papillary muscle, it apparently did not influence the intracellular Ca^{2+} necessary for catecholamine-induced phosphorylase *a* formation. However, treatment of papillary muscles for 60 minutes with a Ca^{2+} -free bathing medium containing EGTA prevented phosphorylase conversion in response to isoproterenol (Fig. 7). Therefore, it appears possible to reduce the intracellular pool of Ca^{2+} that is required for phosphorylase kinase activity. Previously Namm et al.⁹ reported that in the perfused rat heart omission of Ca^{2+} resulted in a rapid loss of catecholamine-induced conversion of phosphorylase ($t_{1/2} \approx 10$ seconds). However, in our present experiments on the guinea pig papillary muscle it was possible to deplete a rapidly exchangeable pool of Ca^{2+} involved in excitation-contraction coupling long before the intracellular pool of Ca^{2+} required for phosphorylase kinase activity was influenced.

There are several possible explanations for these differ-

ences observed in the responses to removal of Ca^{2+} : (1) The ultrastructure of the rat heart and guinea pig papillary muscle are different so that the Ca^{2+} required for phosphorylase kinase activity is mobilized more readily in the rat myocardium. (2) Capillary perfusion in the rat preparation is more effective than diffusion into the muscle bath in depleting intracellular Ca^{2+} . (3) Mobilization of the pool of Ca^{2+} required for phosphorylase kinase activity is dependent on contraction frequency. In regards to the latter point, the perfused rat heart becomes asystolic within 1–5 seconds after perfusion with a Ca^{2+} -free medium, whereas the papillary muscle requires 10–15 minutes. Therefore, contraction at a faster rate may result in a more rapid exchange between Ca^{2+} in the sarcoplasmic reticulum and Ca^{2+} that moves across the sarcolemma. In the papillary muscle contracting at a low rate (12/min) either the sarcoplasmic reticulum is depleted at a slower rate, or there is sufficient time for equilibration with other intracellular Ca^{2+} pools, e.g., mitochondrial or other compartments. A similar dissociation to the one observed here has been reported previously in the isolated rat heart perfused with lanthanum, in which contractile activity and isoproterenol-induced phosphorylase activation were inactivated at different rates.³⁴

ANOXIA, CALCIUM, AND PHOSPHORYLASE

External Ca^{2+} was required for the activation of phosphorylase in the anoxic papillary muscle but there was no elevation of cyclic AMP (Table 1). Isoproterenol did not cause a further conversion of phosphorylase beyond that elicited by the anoxia itself. However, exposure of papillary muscle to a Ca^{2+} -free bathing medium for a period (30 minutes), known not to prevent isoproterenol-induced phosphorylase *a* formation, prevented both the anoxic and the isoproterenol-induced activation of phosphorylase, but not the cyclic AMP elevation induced by the catecholamine (Table 1). Prolonged hypoxia probably causes an effect on the phosphorylase activating system similar to the effect we have previously observed as the immediate (10–60 seconds) response to anoxia.¹¹ The latter consisted of a rapid stimulation of phosphorylase *a* formation that did not require cyclic AMP-dependent conversion of phosphorylase kinase to its activated form. These observations have led us to postulate^{3, 11} that Ca^{2+} , Mg^{2+} , increased pH, or creatine phosphate depletion augmented the activity of the nonactivated form of the kinase. During this phase epinephrine could still stimulate cyclic AMP formation and contraction. However, in the papillary muscle exposed to 30 minutes of anoxia and a Ca^{2+} -free medium both the anoxic and isoproterenol-induced formation of phosphorylase *a* are blocked, perhaps because of low energy charge, acidosis, or more rapid depletion of intracellular Ca^{2+} .

Acknowledgments

The expert technical assistance of George Schwab is gratefully acknowledged. Philip Breckel, a California Heart Association Summer Trainee, conducted some of the anoxia experiments. The papaverine hydrochloride used in this study was a gift from Eli Lilly Laboratories.

References

1. Robison GA, Butcher RW, Øye I, Morgan HE, Sutherland EW: The effect of epinephrine on adenosine 3',5'-phosphate levels in the isolated

- perfused rat heart. *Mol Pharmacol* 1: 168-177, 1965
2. Rasmussen H, Goodman DBP, Tenenhouse A: The role of cyclic AMP and calcium in cell activation. *CRC Crit Rev Biochem* 1: 95-148, 1972
 3. Mayer SE: Effect of catecholamines on cardiac metabolism. *Circ Res* 35 (suppl III): 129-137, 1974
 4. Hess ME, Haugaard N: The effect of epinephrine and aminophylline on the phosphorylase activity of perfused contracting heart muscle. *J Pharmacol Exp Ther* 122: 169-175, 1958
 5. Murad F, Chi Y-M, Rall TW, Sutherland EW: Adenyl cyclase. III. The effect of catecholamines and choline esters on the formation of adenosine 3',5'-monophosphate by preparations from cardiac muscle and liver. *J Biol Chem* 237: 1233-1238, 1962
 6. Walsh DA, Perkins JP, Brostrom CO, Ho ES, Krebs EG: Catalysis of the phosphorylase kinase activation reaction. *J Biol Chem* 246: 1968-1976, 1971
 7. Namm DH, Mayer SE: Effects of epinephrine on cardiac cyclic 3',5'-AMP, phosphorylase kinase, and phosphorylase. *Mol Pharmacol* 4: 61-69, 1968
 8. Krebs EG, Huston RB, Hunkeler FL: Properties of phosphorylase kinase and its control in skeletal muscle. *Adv Enzyme Regul* 6: 245-255, 1968
 9. Namm DH, Mayer SE, Maltbie M: The role of potassium and calcium on the effect of epinephrine on cardiac cyclic adenosine 3',5'-monophosphate, phosphorylase kinase and phosphorylase. *Mol Pharmacol* 4: 522-530, 1968
 10. Wollenberger A, Krause E-G, Heier G: Stimulation of 3',5'-cyclic AMP formation in dog myocardium following arrest of blood flow. *Biochem Biophys Res Commun* 36: 664-670, 1969
 11. Dobson JG Jr, Mayer SE: Mechanisms of activation of cardiac glycogen phosphorylase in ischemia and anoxia. *Circ Res* 33: 412-420, 1973
 12. Morad M, Goldman Y: Excitation-contraction coupling in heart muscle: membrane control of development of tension. *Prog Biophys Mol Biol* 27: 259-313, 1973
 13. Tada M, Kirchberger MA, Katz AM: Phosphorylation of a 22,000-Dalton component of the cardiac sarcoplasmic reticulum by adenosine 3',5'-monophosphate-dependent protein kinase. *J Biol Chem* 250: 2640-2647, 1975
 14. England PJ: Correlation between contraction and phosphorylation of the inhibitory subunit of troponin in perfused rat heart. *FEBS Lett* 50: 57-60, 1975
 15. Stull JT, Brostrom CO, Krebs EG: Phosphorylation of the inhibitor component of troponin by phosphorylase kinase. *J Biol Chem* 247: 5272-5274, 1972
 16. Rubio R, Bailey C, Villar-Palasi C: Effects of cyclic AMP dependent protein kinase on cardiac actomyosin: increase in Ca²⁺ sensitivity and possible phosphorylation of troponin I. *J Cyclic Nucleotide Res* 1: 143-150, 1975
 17. Kaufmann R, Bayer R, Furniss T, Krause H, Tritthart H: Calcium-movement controlling cardiac contractility. II. Analog computation of cardiac excitation-contraction coupling on the basis of calcium kinetics in a multi-compartment model. *J Mol Cell Cardiol* 6: 543-559, 1974
 18. Dobson JG Jr, Schwab GE, Ross J Jr, Mayer SE: Comparison of the biochemical composition of four preparations of contracting cardiac muscle. *Am J Physiol* 227: 1452-1457, 1974
 19. Sonnenblick EH, Ross J Jr, Covell JW, Braunwald E: Alterations in resting length-tension relations of cardiac muscle induced by changes in contractile force. *Circ Res* 11: 980-988, 1966
 20. Hardman JG, Mayer SE, Clark B: Cocaine potentiation of the cardiac inotropic and phosphorylase response to catecholamines as related to the uptake of H³-catecholamines. *J Pharmacol Exp Ther* 150: 341-348, 1965
 21. Mayer SE, Stull JT, Wastila WB, Thompson B: Assay of cyclic AMP by protein kinase activation. *Meth Enzymol* 38: 66-73, 1974
 22. Wastila WB, Stull JT, Mayer SE, Walsh DA: Measurement of cyclic 3',5'-adenosine monophosphate by the activation of skeletal muscle protein kinase. *J Biol Chem* 246: 1996-2003, 1971
 23. Sokal RR, Rohlf FJ. *Biometry*. San Francisco, Freeman, 1969, pp 175-203
 24. Mayer SE, Cotton M DeV, Moran NC: Dissociation of the augmentation of cardiac contractile force and the activation of myocardial phosphorylase by catecholamines. *J Pharmacol Exp Ther* 139: 275-282, 1963
 25. Brooker G: Oscillation of cyclic adenosine monophosphate concentration during the myocardial contraction cycle. *Science* 182: 933-934, 1973
 26. Wollenberger A, Babskii EB, Krause E-G, Genz S, Blohm D, Bogdanova EV: Cyclic changes in levels of cyclic AMP and cyclic GMP in frog myocardium during the cardiac cycle. *Biochem Biophys Res Commun* 55: 446-452, 1973
 27. Henry PD, Dobson JG Jr, Sobel BE: Dissociation between changes in myocardial cyclic adenosine monophosphate and contractility. *Circ Res* 36: 392-400, 1975
 28. Koch Weser J, Blinks JR: The influence of the interval between beats on myocardial contractility. *Pharmacol Rev* 15: 601-652, 1963
 29. Langer GA: Ion fluxes in cardiac excitation and contraction and their relation to myocardial contractility. *Physiol Rev* 48: 708-757, 1968
 30. Blinks JR, Olson CB, Jewell BR, Braveny P: Influence of caffeine and other methylxanthines on mechanical properties of isolated mammalian heart muscle. *Circ Res* 30: 367-392, 1972
 31. Forester GV, Mainwood GW: Interval dependent inotropic effect in the rat myocardium and effects of calcium. *Pflugers Arch* 352: 189-196, 1974
 32. Reuter H: Exchange of calcium ions in the mammalian myocardium: mechanisms and physiological significance. *Circ Res* 34: 599-605, 1974
 33. Friesen AJD, Oliver N, Allen G: Activation of cardiac glycogen phosphorylase by calcium. *Am J Physiol* 217: 445-450, 1969
 34. Brockman EL, Rubio R, Berne RM: Effect of lanthanum on isoproterenol-induced activation of myocardial phosphorylase. *Am J Physiol* 225: 438-443, 1973

Circulation Research

JOURNAL OF THE AMERICAN HEART ASSOCIATION



The role of cyclic adenosine 3', 5'-monophosphate and calcium in the regulation of contractility and glycogen phosphorylase activity in guinea pig papillary muscle.

J G Dobson, Jr, J Ross, Jr and S E Mayer

Circ Res. 1976;39:388-395

doi: 10.1161/01.RES.39.3.388

Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231

Copyright © 1976 American Heart Association, Inc. All rights reserved.

Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:

<http://circres.ahajournals.org/content/39/3/388>

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in *Circulation Research* can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the [Permissions and Rights Question and Answer](#) document.

Reprints: Information about reprints can be found online at:
<http://www.lww.com/reprints>

Subscriptions: Information about subscribing to *Circulation Research* is online at:
<http://circres.ahajournals.org/subscriptions/>