Incorporation of the Terminal Phosphate of ATP into Membranal Protein of Rabbit Cardiac Sarcoplasmic Reticulum

CORRELATION WITH ACTIVE CALCIUM TRANSPORT AND STUDY OF THE EFFECTS OF CYCLIC AMP

By Donald H. Namm, Edward L. Woods, and Joan L. Zucker

ABSTRACT

The transfer of the terminal phosphate of ATP to a material from the sarcoplasmic reticulum of cardiac muscle which can be precipitated by trichloroacetic acid was studied, and the relationship of this biochemical event to active Ca\(^{2+}\) transport was examined. A component of the phosphoryl transfer reaction was stimulated specifically by Ca\(^{2+}\). Both the phosphorylation of the reticulum and the active sequestering of Ca\(^{2+}\) were proportional to the Ca\(^{2+}\) concentration between 10\(^{-7}\) and 10\(^{-9}\)M. The time course of both phenomena was similar. These and other observations relating Ca\(^{2+}\)-dependent phosphorylation of the membrane of the sarcoplasmic reticulum with the active sequestering of Ca\(^{2+}\) suggest that the phosphoryl transfer reaction may represent the formation of a carrier system which facilitates the inward flux of Ca\(^{2+}\) against a concentration gradient. Cyclic AMP, theophylline, or the combination of these two agents did not influence the rate or extent of the phosphorylation of the sarcoplasmic reticulum. These results have led us to question the hypothesis that the cardiac sarcoplasmic reticulum is the site of action of cyclic AMP in its role as a mediator of cardiac inotropic effects.

KEY WORDS

- cyclic AMP
- protein phosphorylation
- sarcoplasmic reticulum
- protein kinase
- cardiac contractility
- calcium uptake
- phosphoryl transfer

The sarcoplasmic reticulum of cardiac and skeletal muscle is important in the regulation of calcium ion concentration within these muscle cells (1, 2), because it can concentrate Ca\(^{2+}\) within its tubular structure and thus influence the contraction-relaxation events of the cells. Recently, a considerable effort has been directed towards the elucidation of the mechanism by which Ca\(^{2+}\) is transported across the membrane of the sarcoplasmic reticulum. Hasselbach and Makinose (3) reported that the hydrolysis of adenosine triphosphate (ATP) to adenosine diphosphate (ADP) and inorganic phosphate was associated with the transport of Ca\(^{2+}\) by the sarcoplasmic reticulum of skeletal muscle. These same authors (4) and Ebashi and Lipman (5) later reported that fragments of the muscle sarcoplasmic reticulum were capable of catalyzing the exchange of phosphate from ATP to ADP. This observation led both groups to speculate that an energy-rich phosphate bond was formed in the membrane of the sarcoplasmic reticulum and that the phosphorylated site could function as a carrier substance for the transfer of Ca\(^{2+}\) from one side of this membrane to the other. This speculation has more recently received experimental support through the reports of Yamamoto and Tonomura (6, 7), Martonosi (8), Makinose (9), Inesi et al. (10), and Panet et al. (11). These workers demonstrated that the terminal phosphate of ATP was incorporated...
PREPARATION OF CARDIAC SARCOPLASMIC RETICULUM

The effects of cyclic AMP on this reaction. The phosphoryl transfer reaction in
ized the phosphoryl transfer reaction in
muscle has not been studied to determine
whether the mechanism for Ca\(^{2+}\) transport in
skeletal muscle sarcoplasmic reticulum
is involved in ATP-supported Ca\(^{2+}\) transport
in the skeletal muscle sarcoplasmic reticulum
(6, 7, 11).

The sarcoplasmic reticulum of cardiac
muscle has not been studied to determine
this phenomenon and the uptake of Ca\(^{2+}\). Thus,
this report includes a study which character-
ization the phosphoryl transfer reaction in
cardiac sarcoplasmic reticulum and a study of
the effects of cyclic AMP on this reaction.

**Methods**

**PREPARATION OF CARDIAC SARCOPLASMIC RETICULUM**

New Zealand white rabbits (3–4 kg) were killed
by a blow on the head, and their hearts were
rapidly removed and washed in isotonic saline at
0°C. Fat, atria, and large blood vessels were
removed by dissection, and the ventricles were
homogenized in a Waring blender in seven
volumes of a solution containing 0.3M sucrose and
0.02M Tris-HCl at pH 7.0. Homogenization and
all subsequent isolation procedures were carried
out at 2–4°C. The heart homogenate was
centrifuged at 1,000 g for 10 minutes in a Sorvall
RC-2B centrifuge. The supernatant fluid was
centrifuged again at 29,000 g for 30 minutes. The
supernatant fluid from the centrifugation at
29,000 g was centrifuged at 100,000 g for 90
minutes in a Beckman L3-50 centrifuge. The
sediment from this final centrifugation was
suspended in the homogenizing medium and
gently hand-homogenized with a Teflon pestle.
The suspension contained 12–25 mg protein/ml
and was always used on the day that it was
prepared. Cytochrome c oxidase activity was
measured in the various subcellular fractions
obtained by differential centrifugation and served
as a marker for mitochondria and mitochondrial
contaminants. The activity of this enzyme was
measured by the method described by Brody et
al. (14). Protein was assayed by the method of
Lowry et al. (15).

The transfer of the terminal phosphate of ATP
to cardiac sarcoplasmic reticulum was followed by
incubating the sarcoplasmic reticulum fraction
(0.75–1.0 mg/ml) in a solution containing 0.3M
sucrose and 20 mM Tris-HCl. Ethyleneglycol
bis(β-aminoethyl ether)-N, N'-tetraacetic acid
(EGTA) and CaCl\(_2\) were added in a ratio that
yielded the desired concentration of free Ca\(^{2+}\)
assuming a binding constant of 5 x 10\(^{-9}\)M\(^{-1}\) at pH
7.0 (2). Magnesium acetate was present at a
concentration equimolar to that of ATP unless
otherwise noted. \(^{32}\)P-ATP (labeled at the terminal
phosphate) was added to initiate the reaction,
and the reaction was terminated by the addition
of 5% trichloroacetic acid (TCA) containing 1
mM ATP and 1 mM P\(_2\) (at 0°C). The TCA-
insoluble precipitate was collected by centrifuga-
tion and the supernatant fluid aspirated. The
dividend was washed twice again in the TCA-
ATP-P\(_2\) solution and then twice in 2M TCA
(0°C). The washed TCA-insoluble material was
dissolved in concentrated formic acid, and a
sample of this solution was counted for \(^{32}\)P in a
Packard liquid scintillation counter. Studies of
calcium uptake were carried out using the same
reaction conditions as those described for the
phosphorylation studies with the following excep-
tions: unlabeled ATP was used, \(^{45}\)CaCl\(_2\) was
used, and the uptake reaction was terminated by
filtering the reaction medium through 0.45μ
Millipore filters. Oxalate was not added to our
takeup medium. The filtrate was counted for
$^4\text{Ca}$, and the difference between the radioactivity in a filtered zero-time control and that in the sample filtrate was calculated as uptake.

**Materials**

$^{32}\text{P}$ as inorganic orthophosphate was obtained in dilute HCl from Amersham/Searle and was incorporated into the terminal phosphate of ATP by a modification (16) of the method of Glynn and Chappell (17). $^{46}\text{CaCl}_2$ was obtained from New England Nuclear Corporation. ATP was obtained from P-L Biochemicals; enzymes used in the synthesis of $^{32}\text{P}$-ATP were obtained from Boehringer-Mannheim. Cyclic AMP was purchased from Schwarz/Mann. $^{14}\text{C}$-8-Adenosine triphosphate was purchased from Amersham/Searle.

**Results**

**Isolation of Fragments of the Sarcoplasmic Reticulum**

Since mitochondria of heart muscle can actively sequester Ca$^{2+}$ (18) and in view of their role in the metabolism of high-energy phosphates, it was imperative to remove, as extensively as possible, all traces of mitochondrial contamination from the sarcoplasmic reticulum fraction. By our fractionation procedure, the sediment obtained by centrifugation at 100,000 $g$ was essentially devoid of activity of cytochrome c oxidase, an enzyme considered to be primarily localized within mitochondria. Expressing the cytochrome c oxidase activity of the heart homogenate as 100%, the distribution of its activity was as follows: 1,000-g sediment 55.5%, 29,000-g sediment 43.8%, 100,000-g sediment 0.35%. Less than 0.2% of the total activity was unaccounted for in our analysis of the various sediments, and presumably it resided in the supernatant fractions.

**Phosphorylation of the Sarcoplasmic Reticulum**

Preliminary studies revealed that it was necessary to employ a minimum of about 1 mg of sarcoplasmic reticulum protein to detect phosphate incorporation. Using this amount of sarcoplasmic reticulum in a reaction at 30 or 37°C caused rapid splitting of ATP and depletion of this substrate. Thus, we chose, as have others in studies of skeletal muscle sarcoplasmic reticulum phosphorylation (10, 11), to follow the reaction at 0°C since at this temperature the activity of adenosinetriphosphatase was low enough so that sampling for the intermediate could be accomplished before the ATP was extensively degraded.

Phosphate incorporation into the TCA-precipitable material of the sarcoplasmic reticulum fraction was followed for 10 minutes. When the concentration of free Ca$^{2+}$ was reduced below 10$^{-8}$M by the presence of 5 mM EGTA, there was a time-dependent incorporation of $^{32}\text{P}$ from ATP into the sarcoplasmic reticulum. This labeling could not be reduced by extensive washing with the TCA solution containing P$_i$ and ATP and, thus, represents a tight binding of the phosphate to the sarcoplasmic reticulum. We have termed this incorporation the Ca$^{2+}$-independent phosphorylation (Fig. 1). At an ATP concentration of 0.1 mM, this component of sarcoplasmic reticulum phosphorylation reached a maximum of about 0.2 $\mu$moles/g protein in about 10 minutes. When Ca$^{2+}$ was
present at a concentration of the free ion of $10^{-5}$, the pattern of phosphorylation was altered considerably. We have termed this the total phosphorylation since it is composed of both the Ca$^{2+}$-independent and Ca$^{2+}$-dependent components (Fig. 1). The Ca$^{2+}$-dependent component was calculated as the difference between total and Ca$^{2+}$-independent components. The Ca$^{2+}$-dependent phosphorylation of the sarcoplasmic reticulum material occurred very rapidly, peaking at about 30 seconds, and was apparently degraded at an exponential rate over the remaining 9.5 minutes. Like the Ca$^{2+}$-independent phosphorylation, the Ca$^{2+}$-dependent phosphorylation reached a maximum of about 0.2 μmoles/g when the initial ATP concentration was 0.1 mM. In most of our subsequent experiments we chose to measure Ca$^{2+}$-dependent phosphorylation 30 seconds after initiation of the reaction since this was the time of peak effect and of maximal difference between the two components.

Experiments were carried out to characterize the requirement of the phosphoryl transfer reaction for Mg$^{2+}$. In the absence of Mg$^{2+}$, Ca$^{2+}$-independent phosphorylation was detected at 30 seconds; the extent of this phosphorylation was somewhat decreased by the addition of Mg$^{2+}$ (Fig. 2). When Ca$^{2+}$ was present at $10^{-5}$, phosphate incorporation was augmented with no added Mg$^{2+}$. When Mg$^{2+}$ was added, phosphate incorporation was further increased with a maximum occurring at about $5 \times 10^{-6}$ Mg$^{2+}$.

Using a ratio of the concentrations of ATP and Mg$^{2+}$ of one to one in our reaction medium, we investigated the extent of phosphorylation at different initial ATP concentrations. At concentrations of ATP above 0.1 mM the Ca$^{2+}$-independent component of phosphorylation of the sarcoplasmic reticulum increased markedly, exceeding that of the Ca$^{2+}$-dependent component, and thus made quantification of the latter difficult (Fig. 3). Maximal Ca$^{2+}$-dependent phosphorylation occurred at $10^{-4}$ ATP (Fig. 3, insert).

The specificity of the Ca$^{2+}$-induced phosphoryl transfer was determined by substituting other cations for Ca$^{2+}$ in the reaction medium. Other than Ca$^{2+}$, none of the cations
tested significantly stimulated phosphate incorporation into the sarcoplasmic reticulum (Table 1).

Once the conditions which optimized Ca$^{2+}$-dependent phosphorylation of the sarcoplasmic reticulum were established, we attempted to determine if a direct phosphoryl transfer reaction was involved or if the $^{32}$P incorporation occurred through other mechanisms. One possible alternative mechanism was that unhydrolyzed ATP was bound to the sarcoplasmic reticulum thus allowing $^{32}$P incorporation without phosphoryl transfer. We tested this possibility by substituting $^{14}$C-8-adenosine triphosphate for the $^{32}$P-ATP in our reaction medium. No $^{14}$C labeling of the washed TCA-insoluble precipitate was detected in either the presence or the absence of Ca$^{2+}$. Next we carried out studies to determine whether the $^{32}$P incorporated into the sarcoplasmic reticulum in our experiments came from inorganic $^{32}$P in the medium after hydrolysis from $^{32}$P-ATP by the action of adenosinetriphosphatase. Studies were carried out using unlabeled ATP and adding $^{32}$P-orthophosphate to our usual reaction medium. Both ATP and P$_i$ were present at $10^{-4}$M and the specific activity of the P$_i$ exceeded that of the $^{32}$P-ATP used in previous experiments. In these experiments there was no detectable $^{32}$P incorporation in the TCA-insoluble precipitate when Ca$^{2+}$ was absent or present up to concentrations of $10^{-4}$M. At $10^{-4}$M, a tenfold higher concentration than that usually used in our other experiments, there was a small incorporation of $^{32}$P. The magnitude of this incorporation from P$_i$ never exceeded 0.02 μmoles/g protein, which was less than 10% of that seen when $^{32}$P-ATP was used as substrate at a tenth the Ca$^{2+}$ concentration.

**RELATIONSHIP BETWEEN THE CALCIUM-DEPENDENT PHOSPHORYL TRANSFER REACTION AND CALCIUM UPTAKE BY THE SARCOPLASMIC RETICULUM**

The time courses of Ca$^{2+}$-dependent phosphate incorporation into cardiac sarcoplasmic reticulum and ATP-dependent Ca$^{2+}$-binding are shown in Figure 4. ATP-dependent binding was calculated as the net difference between the amount of Ca$^{2+}$ bound in the presence of ATP (0.1 mM) and that bound when no ATP was added to the reagent. The ATP-independent binding (not shown) was rapid, reaching a maximum of 4.4 μmoles Ca$^{2+}$ bound/g at 30 seconds and was unchanged for at least 5 minutes. As seen in previous experiments, phosphate incorporation was rapid, i.e., demonstrable at 30 seconds. Likewise, most of the Ca$^{2+}$ that was sequestered by cardiac sarcoplasmic reticulum

### TABLE 1

**Effects of a Variety of Cations on Phosphorylation of the Sarcoplasmic Reticulum**

<table>
<thead>
<tr>
<th>Cation added</th>
<th>Phosphate incorporation (μmoles/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>0.047 ± 0.024</td>
</tr>
<tr>
<td>Ca$^{2+}$</td>
<td>0.188 ± 0.034</td>
</tr>
<tr>
<td>Na$^+$</td>
<td>0.042 ± 0.027</td>
</tr>
<tr>
<td>K$^+$</td>
<td>0.050 ± 0.020</td>
</tr>
<tr>
<td>Ba$^{2+}$</td>
<td>0.077 ± 0.021</td>
</tr>
<tr>
<td>NH$_4^+$</td>
<td>0.041 ± 0.021</td>
</tr>
</tbody>
</table>

All ions were added as chloride salts at a concentration of $10^{-4}$M. [ATP] = [Mg$^{2+}$] = $10^{-4}$M. The control reaction medium contained 5 mM EGTA. Reaction time was 30 seconds. Specific activity of ATP was 1.1 × 10$^5$ counts/min μmole$^{-1}$.
was bound within that time period. The degradation of the membrane-phosphate complex was not associated with any marked loss of Ca\(^{2+}\) from the sarcoplasmic reticulum. We analyzed the relationship between the concentration of Ca\(^{2+}\) in the medium and both Ca\(^{2+}\) uptake and phosphoryl transfer. Both phenomena were similarly dependent on the Ca\(^{2+}\) concentration from 10\(^{-7}\) to 10\(^{-5}\)M (Table 2). Half-maximum effects for both phenomena occurred at Ca\(^{2+}\) concentrations of 2–4 \(\times\) 10\(^{-5}\)M. There appears to be a proportionality between the two phenomena which is approximately linear between 0.005 and 0.025 \(\mu\)moles phosphate incorporated/g protein.

**EFFECTS OF CYCLIC AMP AND THEOPHYLLINE ON THE CALCIUM-DEPENDENT PHOSPHORYLATION OF CARDIAC SARCOPLASMIC RETICULUM**

We tested the effects of cyclic AMP alone and in the presence of an inhibitor of its degradation (theophylline) on the transfer of the terminal phosphate of ATP to the sarcoplasmic reticulum fragments. Theophylline alone had no effect on phosphate incorporation in either the presence or the absence of Ca\(^{2+}\) (Fig. 5). Cyclic AMP at 10\(^{-6}\)M, a concentration 100 times that which produces maximal stimulation of protein kinases (16), also was without effect on the extent of sarcoplasmic reticulum phosphorylation. Finally, the combination of cyclic AMP and theophylline was also ineffective in altering the degree of phosphate incorporation. Studies were carried out sampling the sarcoplasmic reticulum at earlier (10, 15, and 20 seconds) time periods to determine whether the nucleotide affected the rate of phosphorylation. Again, no effect of the nucleotide was detectable. Likewise, preincubation of the sarcoplasmic reticulum with 10\(^{-5}\)M cyclic AMP for up to 10 minutes did not affect the phosphoryl transfer reaction.

**Discussion**

Specific cationic stimulation of the formation of a membranal protein-phosphate complex has been demonstrated in association with the ATP-supported transport of Na\(^{+}\) and K\(^{+}\) by kidney membranes (19) and of Ca\(^{2+}\) by sarcoplasmic reticulum of skeletal muscle (6–11). Extensive kinetic analysis of the formation and degradation of the skeletal muscle membranal protein-phosphate complex has suggested that the phosphoryl transfer reaction results in the formation of an energetic carrier molecule which is capable of translocating Ca\(^{2+}\) from the outer to the inner surface of the sarcoplasmic reticulum membrane (6–11, 20). Our in vitro experiments indicate that a similar reaction mechanism may be operative in association with Ca\(^{2+}\) transport across the membrane of the sarcoplasmic reticulum of heart muscle. We demonstrated a specific Ca\(^{2+}\) stimulation of

### Table 2

Effects of Variations in the Calcium Concentration on the Binding of Calcium to Cardiac Sarcoplasmic Reticulum and Its Phosphorylation by ATP

<table>
<thead>
<tr>
<th>(P_{ca})</th>
<th>Original radioactivity (counts/min ml(^{-1}))</th>
<th>Final radioactivity</th>
<th>ATP (counts/min ml(^{-1}))</th>
<th>ATP-dependent calcium binding ((\mu)moles/g)</th>
<th>Calcium-dependent phosphorylation ((\mu)moles/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>7.4</td>
<td>5,052</td>
<td>3,938</td>
<td>3,093</td>
<td>1.4</td>
<td>0.006</td>
</tr>
<tr>
<td>6.7</td>
<td>20,090</td>
<td>18,690</td>
<td>16,853</td>
<td>8.1</td>
<td>0.011</td>
</tr>
<tr>
<td>6.3</td>
<td>53,411</td>
<td>51,971</td>
<td>48,108</td>
<td>6.3</td>
<td>0.019</td>
</tr>
<tr>
<td>6.0</td>
<td>84,144</td>
<td>82,403</td>
<td>75,994</td>
<td>10.5</td>
<td>0.049</td>
</tr>
<tr>
<td>5.7</td>
<td>136,108</td>
<td>134,319</td>
<td>123,452</td>
<td>17.8</td>
<td>0.049</td>
</tr>
<tr>
<td>5.4</td>
<td>200,481</td>
<td>188,640</td>
<td>174,040</td>
<td>24.0</td>
<td>0.153</td>
</tr>
<tr>
<td>5.1</td>
<td>217,504</td>
<td>215,043</td>
<td>197,003</td>
<td>28.6</td>
<td>0.153</td>
</tr>
</tbody>
</table>

Experimental conditions: \([Mg^{2+}] = [ATP] = 0.1\) mm, EGTA 0.5 mm, \(4\)CaCl\(_2\) specific activity 544,000 counts/min \(\mu\)mole\(^{-1}\). Uptake and phosphorylation studies were terminated at 30 seconds. All values represent means of triplicate observations using one preparation of the sarcoplasmic reticulum. \(P_{ca} = – \log [Ca^{2+}]\).
Effects of cyclic AMP, theophylline, or their combination on phosphate incorporation into the sarcoplasmic reticulum. Cyclic AMP or theophylline were added to the medium 5 minutes prior to the initiation of the phosphoryl transfer reaction by addition of $^{32}$P-ATP. Each bar represents the mean of four to six observations and the SE is indicated.

Many similarities between the phosphoryl transfer reaction in skeletal and cardiac sarcoplasmic reticulum are indicated by our results. First, Ca$^{2+}$, but not Mg$^{2+}$, appears to be absolutely required for substantial phosphate incorporation (8, 10, 11). Mg$^{2+}$ augments the Ca$^{2+}$ stimulatory effect and appears to have its maximal effect when employed in a concentration about equimolar to that of ATP. In both heart and skeletal muscle, the sarcoplasmic reticulum is phosphorylated rapidly with maximal incorporation occurring at 5-30 seconds. The phosphorylation is a function of the concentration of Ca$^{2+}$ between $10^{-7}$ and $10^{-5}$M in both heart and skeletal muscle sarcoplasmic reticulum fragments (7, 9, 11). Thus, it is probable that the transport systems for Ca$^{2+}$ in these membrane fragments are qualitatively similar. However, quantitative differences are obvious. Skeletal muscle sarcoplasmic reticulum is able to accept considerably more phosphate (ranging from 0.4 to 3 $\mu$moles/g) than is cardiac sarcoplasmic reticulum (maximum of 0.2 $\mu$moles/g). Assuming similar degrees of purity of the preparations, our results indicate that the number of transport sites on the cardiac sarcoplasmic reticulum is considerably smaller than on the skeletal muscle.
reticulum membranes may be less than that on the skeletal muscle membranes. This observation may relate directly to the fact that cardiac sarcoplasmic reticulum is known to possess a Ca\textsuperscript{2+} transport system considerably inferior to that of muscle in terms of both ion flux and storage capacity (21).

Our experiments provided no indication that cyclic AMP was able to modify the phosphoryl transfer reaction in cardiac sarcoplasmic reticulum. The ineffectiveness of cyclic AMP cannot be due to its inability to cross membranes since the phosphorylation must take place on the outer surface of the sarcoplasmic reticulum, a site readily accessible to ATP in the medium. Thus, we can conclude that, if cyclic AMP does act to modify the Ca\textsuperscript{2+} pump of cardiac sarcoplasmic reticulum, it does so by means other than activation of sarcoplasmic reticulum phosphorylation by ATP. That cyclic AMP acts on the Ca\textsuperscript{2+} uptake process of cardiac sarcoplasmic reticulum is itself open to serious question. Shinbourne and White (13) were only able to show definitive effects of the nucleotide on Ca\textsuperscript{2+} uptake by cardiac sarcoplasmic reticulum at 10^{-3}M cyclic AMP and did not attempt to determine whether these effects of the nucleotide, at 10,000 times its physiological concentration, were specific. The report by Entman et al. (12) that cyclic AMP stimulates Ca\textsuperscript{2+} uptake by cardiac sarcoplasmic reticulum when employed at very low concentrations has been recently contradicted by the work of Sulakhe and Dhalla (22): the latter workers were unable to find any effects of cyclic AMP on ion movements in canine cardiac sarcoplasmic reticulum. We have also been unable to detect a specific effect of cyclic AMP on Ca\textsuperscript{2+} uptake into or efflux from dog or rabbit cardiac sarcoplasmic reticulum (unpublished observations). Until these disparities in the literature are resolved, the concept that the mediation of cardiac inotropic effects by cyclic AMP results from the effects of cyclic AMP on the Ca\textsuperscript{2+} pump of the sarcoplasmic reticulum should be considered with reservation.

References


Incorporation of the Terminal Phosphate of ATP into Membranal Protein of Rabbit Cardiac Sarcoplasmic Reticulum: CORRELATION WITH ACTIVE CALCIUM TRANSPORT AND STUDY OF THE EFFECTS OF CYCLIC AMP

Donald H. Namm, Edward L. Woods and Joan L. Zucker

Circ Res. 1972;31:308-316
doi: 10.1161/01.RES.31.3.308

Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 1972 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/31/3/308

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/