Atrial Natriuretic Factor Regulates the Calcium Current in Frog Isolated Cardiac Cells

Marie-Pierre Gisbert and Rodolphe Fischmeister

The effect of external application of synthetic atrial natriuretic factor (ANF) on calcium current (I_{Ca}) was studied in single cells isolated from frog ventricle using the whole-cell patch-clamp technique. Rat atriopeptin III (APIII) and 3-28 ANF rat (rANF) had negligible effects on basal I_{Ca} at concentrations up to 200 nM. However, when I_{Ca} was increased by isoprenaline, both peptides had significant inhibitory effects. rANF (3 nM) decreased isoprenaline-elevated I_{Ca} by an average of 33% after 3–5 minutes. APIII was slightly less effective than rANF. The effects of rANF and APIII were dose-dependent in a complex manner: one stimulatory and two different inhibitory effects were observed, one being responsible for an irreversible rundown of I_{Ca}. The effects of ANF were not blocked by atropine and desensitization of the cells to isoprenaline did not play a significant role in the response to ANF. When I_{Ca} was elevated by intracellular perfusion with cyclic adenosine 3',5'-monophosphate, added to the patch electrode solution or using a perfused pipette, rANF or APIII had less inhibitory effect, and no rundown of I_{Ca} was observed. It is proposed that adenylate cyclase may be one of several mechanisms by which ANF regulates I_{Ca}. (Circulation Research 1988;62:660-667)

The atrial muscle of heart produces a family of polypeptide hormones, termed atrial natriuretic factors (ANFs), that participate in the regulation of salt and water balance and blood pressure. These peptides have been identified and characterized (see reviews in Cantin and Genest and Ballermann and Brenner) and synthetic ANFs have become available. Although the mechanism of action of ANF at the cellular level remains unclear, evidence derived from both in vitro and in vivo studies show that, in most target tissues, ANF action is generally associated with elevation in cyclic guanosine 3',5'-monophosphate (cGMP) levels and that cGMP is the likely second messenger for ANF action. For instance, ANF is a potent relaxant of a large variety of vascular and nonvascular smooth muscles that have been contracted by different agonists. The relaxing effect of ANF is associated with an elevation in intracellular cGMP levels due to activation of a particulate guanylate cyclase. Other cellular mechanisms that may be involved in the action of ANF, whether or not related to cGMP accumulation, include inhibition of adenylate cyclase (in arteries and kidney) and inhibition of agonist- but not depolarization-stimulated calcium influx in vascular smooth muscle.

Very little is known about the effect of ANF on heart. Hypotensive doses of atrial extracts were shown to transiently reduce cardiac output and depress the heart rate in anesthetized rats. A similar effect on cardiac output was found with synthetic ANF but with no modification in the heart rate, suggesting a direct inhibitory action of ANF on cardiac contractility. However, in these studies, the effects of the hormone on cardiac output were contaminated by a simultaneous reduction in total peripheral resistance. Synthetic ANF has also been shown to promote myocardial relaxation in cat and rat papillary muscle and to reduce the activity of adenylate cyclase in crude homogenates of rat cardiocytes, suggesting a participation of ANF in cardiac function. However, other studies have concluded that there are no direct effects of ANF on cardiac contractility or beat rate.

Because of the predominant role of calcium ions in the regulation of cardiovascular function, the effects of synthetic ANF on calcium current, which was recorded under voltage-clamp conditions from single isolated cells of frog ventricle, were investigated. Since frog heart, like mammalian atrium, secretes granules that contain a peptide immunologically and biochemically related to mammalian ANF and frog ANF has been shown to induce diuresis and natriuresis in mammals, frog ventricular cells are an appropriate preparation in which to study the effect of ANF. Also, we have recently shown that cGMP regulates the calcium current of these cells, so it is possible to make a direct comparison between the actions of cGMP and ANF. To our knowledge, this is the first study of the effect of ANF at the single cell level and the first report of a voltage-clamp study of a response to the polypeptide. A preliminary report has appeared elsewhere.

Materials and Methods

The methods used for cell dissociation, whole-cell patch-clamp recording, superfusion and internal perfusion of the cells, and data analysis have been extensively described in previous papers and were used with no major modification in the present study. Briefly, for routine monitoring of I_{Ca}, the frog (Rana esculenta) ventricular cell was depolarized every 8 seconds from -80 mV holding potential to 0 mV for 200 msec. To accurately measure I_{Ca} with no contamination of other ionic currents, the cells were bathed in...
potassium-free, 20 mM Cs-Ringer's solution containing 88.4 mM NaCl, 20 mM CsCl, 22.9 mM NaHCO₃, 0.6 mM NaH₂PO₄, 1.8 mM CaCl₂, 1.8 mM MgCl₂, 5 mM d-glucose, 5 mM sodium pyruvate, and 0.3 µM tetrodotoxin (Sankyo, Japan). The standard internal solution in the patch electrode (1–3 MΩ resistance) contained CsCl 120 mM, K₂EGTA 5 mM, MgCl₂ 4 mM, Na₂CP 5 mM, Na₂ATP 3 mM, and Na₂GTP 0.4 mM, adjusted to pH 7.1 with KOH (1N). Solutions were applied to the exterior of the cell by placing the cell at the opening of 250-µm inner diameter capillary tubing (flow rate of 10 µl/min).¹⁹ Solutions were applied to the interior of the cell by the patch electrode and could be modified by a system that permitted perfusion of the patch electrode.²³ Under these conditions, Iᵦ was measured on-line as the difference between peak inward current and the current at the end of the 200-msec pulse that was determined from the current trace digitized at 5 kHz (12-bit A/D converter) by a Compaq 286 Desk-Pro computer using programs written in Pascal language. For each cell, membrane capacitance (Cₑ) was recorded to give an estimate of the total cell membrane area. When required, kinetics of Iᵦ were analyzed using the computer program EXCAL²⁶ based on the powerful method described by Yeramian and Claverie.²⁷ All external solutions were gassed with 95% O₂-5% CO₂ (pH 7.4), and experiments were done at room temperature (19.5–22.5°C).

Drugs

(±)-Isoprenaline, acetylcholine, atropine, 1-methyl-3-isobutyl xanthine, cyclic adenosine 3',5'-monophosphate (cAMP), and 8-bromo cAMP were obtained from Sigma Chemical, St. Louis, Missouri. Two different synthetic atrial natriuretic factors were used: atriopeptin III (APIII; 5-28 ANF rat, Sanofi, France) and ANF 8-33 (rANF, 3-28 ANF rat, Merck Sharp & Dohme, Teterboro, New Jersey). Quantities (50 µl) of 5-µM ANF stock solutions, made in 0.1 M acetic acid, were thawed from −27°C before each experiment and kept in ice prior to final dilution at 1–200 nM into 5 ml extracellular perfusion solution. Control solutions contained identical amounts of acetic acid as ANF-containing solutions, and pH was readjusted to 7.4 with NaOH (1N) when required.

Results

L-Type calcium currents²⁸,²⁹ (Iᵦ) were elicited in frog ventricular cells by voltage-clamp depolarizations from −80 to 0 mV. Figure 1 shows the effect of 100 nM APIII on Iᵦ, which was recorded every 8 seconds during a 200-msec depolarization to 0 mV. APIII had a weak effect on control Iᵦ in five cells exposed to 100 and 200 nM APIII or rANF, Iᵦ was 95.4 ± 7.8% and 92.7 ± 1.8% of control Iᵦ (mean ± SEM), respectively. But when Iᵦ was enhanced by superfusing the cell with 0.3 µM of the β-adrenergic agonist isoprenaline (ISO), APIII had more pronounced effects. Iᵦ was first reduced by ≈25% after 1 minute of exposure to APIII; after 3 minutes of continuous exposure, this inhibition declined (≈10%). APIII also initiated a rundown of Iᵦ that was not reversed when APIII was washed out of the cell, although ISO was still present. In fact, the washout of APIII itself induced a further rapid decrease in Iᵦ. A second exposure to APIII stimulated Iᵦ without the transient inhibition that was seen when APIII was first applied. From this experiment, it would appear that, at high concentrations, ANF had a complex pattern of actions on Iᵦ that had been elevated by β-adrenergic agonists: first, a transient inhibitory effect; then, a sustained and irreversible inhibitory action that was responsible for the rundown of Iᵦ; and finally, a small reversible stimulatory effect on Iᵦ.

In an attempt to clarify the action of ANF, lower and more physiological doses were used to separate these multiple effects of the hormone. In Figure 2, 3 nM APIII strongly reduced ISO-enhanced Iᵦ and induced a rundown process. When the concentration of APIII was increased to 10 nM, a transient plateau in this decline was observed that was followed by a further acceleration in the rate of rundown of Iᵦ, suggesting competition between two opposite effects. This competition was confirmed when the concentration of APIII was increased to 30 nM: there was a clear transient increase in Iᵦ and a reduction in the rate of the rundown process. Further increasing the concentration to 100 nM had no additional effect on Iᵦ. When
APIII was washed from the cell, I\(_a\) did not show any tendency to recover. The dose-dependent effects of rANF are shown in Figure 3. While 5 minutes' successive exposures to 10 and 30 nM rANF depressed ISO-elevated I\(_c\), by 17% and 60%, respectively, 100- and 300-nM concentrations were less effective. Again, the effects were not reversed when rANF was washed away from the cell.

Desensitization to isoproterenol has been shown to occur relatively quickly in cultured myocytes of chick ventricle.\(^{30,31}\) A possible contribution of this process to the inhibitory effect of ANF on I\(_a\) elevated by ISO has been checked. Figure 4 shows an experiment where 3 nM rANF was applied to a cell after more than 40 minutes of continuous exposure to 2 \(\mu\)M ISO. While I\(_a\) did not decrease during the long exposure to ISO, ANF induced a slow and irreversible inhibition of I\(_a\) after an initial transient stimulatory period. Thus, electrophysiological desensitization to ISO was slower in our experiments than inotropic desensitization in chick heart\(^{19}\) and is unlikely to significantly contribute to the response to ANF. The effects of ANF on I\(_a\) were not voltage-dependent because the current-voltage relation of I\(_a\) was not affected by the polypeptide (data not shown). However, the time constants of inactivation of I\(_a\) were slightly reduced by the hormone (Figure 4).

The observation that low doses of ANF only inhibited I\(_a\) that had been elevated by \(\beta\)-adrenergic stimulation raises questions about the mechanism(s) of action of the peptide. \(\beta\)-Adrenergic agonists stimulate the activity of adenylate cyclase and increase the level of intracellular cAMP. The increase in cAMP is responsible for the \(\beta\)-adrenergic stimulation of I\(_c\) by increasing the degree of phosphorylation of the calcium channels because a similar action is seen when cAMP is perfused into the cell.\(^9\) In an attempt to clarify whether the action of ANF occurred before or after cAMP synthesis, the effects of ANF on calcium current that was elevated by direct application of intracellular cAMP were studied. Figure 5 shows a typical experiment where the patch electrode contained 5 \(\mu\)M cAMP in addition to the regular intracellular solution. Immediately after rupture of the patch membrane (time 0), I\(_c\) began to increase as cAMP permeated the cell and stabilized in about 5 minutes. The application of 3 nM rANF then had a biphasic effect: an initial transient stimulation (\(= +7\%\)) of I\(_c\) was followed by a sustained inhibition (\(= -12\%\)). In each cell perfused with cAMP, ANF never induced a rundown of I\(_a\) as had generally been seen when ANF was applied to ISO-elevated I\(_a\). The effects of rANF on ISO- and cAMP-elevated I\(_a\) were compared in the same cell in one experiment (Figure 6). This cell was particularly suitable for this type of comparison because the strong inhibitory effect of 10 nM rANF during first exposure on I\(_a\) elevated by ISO (\(=60\%\) inhibition after 5 minutes' exposure) was mostly reversible (92%). This provided an opportunity to reexamine the effect of ANF on control I\(_a\) after all ISO was washed out of the cell and I\(_a\) returned to control levels and, finally, on I\(_a\) elevated by perfusion of the patch electrode with 5 \(\mu\)M cAMP. rANF had no significant effect on control I\(_a\). Intracellular perfusion with 5 \(\mu\)M cAMP elevated I\(_a\) less than external application of 0.3 \(\mu\)M ISO, although both manipulations have been shown previously to have similar effects on the amplitude of I\(_a\).\(^{12,23,24}\) This may suggest that rANF had exerted a sustained irreversible inhibitory action on I\(_a\) after first application. Exposure of the cell to 10 nM rANF under these conditions had a much weaker effect on cAMP-elevated I\(_a\) than previous application of the peptide on ISO-elevated I\(_a\). Unfortunately, the seal between the patch electrode and the cell was suddenly lost during the development of the ANF response, which, thus, was recorded only during 4.5 minutes. However, after a
but also from cell to cell. Moreover, it should be mentioned that the response to ANF not only varied with the dose of the peptide used when ANF was added to ISO alone (Figure 7). Thus, experiments conducted with ANF in the presence of internally applied cAMP (5 μM). As shown, the effects of ANF did not seem to have muscarinic agonist activity atropine, muscarinic ACh-receptors were blocked by internally perfused atropine, muscarinic ACh-receptors were blocked by internally perfused ACh was sufficient to completely inhibit the stimulatory action of acetylcholine (ACh) on calcium current seen in the same cells. For this reason, a possible participation of muscarinic ACh-receptors in the regulation of I_{Ca} by ANF was investigated. Figure 7 shows an experiment where the cell was exposed simultaneously to ISO (2 μM), ACh (1 μM), and atropine (2 μM). A concentration of 1 μM ACh was sufficient to completely inhibit the stimulatory action of 2 μM ISO. In the presence of 2 μM atropine, muscarinic ACh-receptors were blocked because ACh did not prevent the stimulatory action of ISO on I_{Ca}. The addition of 3 nM rANF to the solution induced an inhibitory response similar to those seen when ANF was added to ISO alone (Figure 7). Thus, ANF did not seem to have muscarinic agonist activity in our preparation (see also Ackermann et al). Table 1 tentatively summarizes the results of the experiments conducted with ANF in the presence of maximal doses of externally applied ISO (0.3 μM) or internally applied cAMP (5 μM). As shown, the effects of ANF not only varied with the dose of the peptide used but also from cell to cell. Moreover, it should be mentioned that the response to ANF seemed to be strongly affected by the dissociation procedure because the cells obtained from certain dissociations did not show any response to ANF (rANF) either on control or after β-adrenergic stimulation (see “Discussion”). For this reason, the summary values in Table 1, which includes either all experiments or only those experiments showing ANF responses (for rANF) that were >5%, should be regarded as indicative. ISO (0.3 μM) increased I_{Ca} by an average of 639.5 ± 62.6% (n = 35). While 1 nM rANF had negligible effects, 3 and 10 nM rANF significantly reduced I_{Ca} in the presence of ISO. Low doses of APIII seemed to be somewhat less effective than equivalent concentrations of rANF. At 3- and 10-nM concentrations, the effects of rANF on I_{Ca} elevated by the β-adrenergic agonist ISO were larger than those observed on I_{Ca} directly elevated by intracellular perfusion with cAMP.

Discussion

Although ANF has been shown to antagonize both nonreceptor-induced (e.g., potassium depolarization) and receptor-induced (e.g., angiotensin II, norepinephrine, and histamine) contractions in vascular smooth muscle, the effects are more pronounced on the latter. Calcium channels may participate in this differential action of the hormone. Indeed, both 45 Ca influx and contraction of isolated rabbit aorta were inhibited by rat atriopeptin II (APII) when prestimulated by norepinephrine but were much less affected by the peptide when prestimulated by high potassium concentration: this suggests a different action of ANF compounds on voltage-operated and receptor-operated calcium channels. However, in rabbit and guinea pig renal arteries and in rat juxtaglomerular cells, α-human ANF had no effect on calcium influx. Rather than involving sarcolemmal calcium channels, the relaxing effect of ANF on vascular smooth muscle has often been attributed to a reduction in cytosolic free calcium concentration caused by an inhibition of the Ca channel.
FIGURE 6. Effects of 3-28 ANF rat (rANF) on calcium current (I_{Ca}). At time zero, perfusion of the cell with control intracellular medium was begun and continued until the solution in the patch electrode was changed to an intracellular solution containing 5 mM cyclic AMP during the period indicated (cAMP). The cell was exposed externally to 0.3 nM isoprenaline (ISO) and/or 10 nM rANF during the periods indicated. (Cell No. 87031104; C_m = 63 pF.)

Besides demonstrating for the first time a direct action of ANF on calcium influx in heart, our results also suggest that ANF acts preferentially on agonist-stimulated calcium influx. In frog ventricular cells, APIII and rANF were much less effective on control I_{Ca} than on isoprenaline-elevated I_{Ca}. In that respect, the effects of ANF on I_{Ca} resemble those of acetylcholine or internal cGMP.23-24 In cardiac cells, the stimulatory effect of beta-adrenergic agonists on I_{Ca} involves an increased activity of the adenylate cyclase. Anand-Srivastava and Cantin15 have demonstrated that adenylate cyclase activity was reduced by ANF in rat cardiocytes. Again, the inhibitory effect of the peptide was more pronounced when the activity of the enzyme had been enhanced by various agonists, including isoproterenol, forskolin, prostaglandins, and others.13 Interestingly, that the inhibition of adenylate cyclase by ANF was similar when its activity was enhanced by beta-adrenergic agonists or forskolin indicates that ANF is unlikely to exert beta-adrenergic antagonist activity.15 In our experiments, adenylate cyclase would clearly seem to be involved in the action of ANF because ANF was more effective when I_{Ca} was enhanced indirectly by beta-adrenergic stimulation than when I_{Ca} was stimulated directly by intracellular cAMP. A similar observation was obtained in the effects of acetylcholine on I_{Ca}.19

The accurate analysis of the effect of ANF is limited by the rapid degradation of the peptide when bound to its receptor. Using radiolabeled ANF ([125I]-ANF) in cultured Leydig tumor cells or in isolated mesenteric artery,38 it was shown that after the binding of ANF to the cell surface receptor, the ligand-receptor complex was rapidly degraded (95% within 3 minutes at 37°C) either by a membrane-bound enzyme or after internalization of the complex.37-38 This could be partly responsible for the transient effects of ANF on I_{Ca} seen in some of our experiments (Figure 1) and makes it even more difficult to understand the complex multiphasic effects of the hormone on I_{Ca}. Degradation of ANF in physiological solutions (room temperature) should be relatively small: although some of our cells did not show any response to ANF, other cells exposed up to 2 hours later to the same ANF solutions gave clear effects of the peptide.

Whether the absence of ANF response is an intrinsic property of some cardiac cells or is due to damage of the cells or downregulation of the receptors during the dissociation procedure remains unknown. However, one possible explanation is that cardiac cells secrete a significant amount of ANF during the dissociation procedure and/or in the cell suspension, which would downregulate the receptors and prevent a subsequent response to the hormone. Preliminary radioimmunoassay measurements of ANF levels, using radiolabeled [125I]ANF-(Ser'-Tyr') and anti-rat ANF antibody,16 were performed in the recirculating enzymatic dissociation medium (20 ml) collected at the end (45 minutes) of seven cell dissociations (see Fischmeister and Hartzell19 for composition). ANF content varied from 3 pg/ml to 22 ng/ml, although the cell dissociations were performed under controlled identical conditions. Since purified or synthesized frog ANF is not yet available, it was necessary to assume a complete cross-immunoreactivity of frog ANF with rat ANF, so the measurements of ANF levels were most likely

FIGURE 7. Effects of 3-28 ANF rat (rANF) on calcium current (I_{Ca}) elevated by isoprenaline (ISO) in the presence of acetylcholine and atropine. After 12 minutes exposure of the cell to ISO (2 mM) + acetylcholine (ACH, 1 mM) + atropine (ATROP, 2 mM), 3 nM rANF was added to solution during period indicated. (Cell No. 87072206, C_m = 102 pF.)
TABLE 1. Effect of Rat Atriopeptin III and 3-28 ANF Rat on Calcium Current

<table>
<thead>
<tr>
<th>ANF (nM)</th>
<th>ISO/control (%)</th>
<th>ISO + ANF/control (%)</th>
<th>cAMP/control (%)</th>
<th>cAMP + ANF/control (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>rANF All</td>
<td>638.2 ± 64.6 (12)</td>
<td>614.3 ± 63.4 (12)</td>
<td>659.0 ± 71.8 (5)</td>
</tr>
<tr>
<td>3</td>
<td>APIII Variations &gt;5%</td>
<td>563.8 ± 102.6 (3)</td>
<td>458.8 ± 26.6 (3)</td>
<td>623.8 ± 80.6 (4)</td>
</tr>
<tr>
<td>3</td>
<td>rANF All</td>
<td>674.6 ± 124.1 (11)</td>
<td>455.1 ± 57.2 (11)</td>
<td>593.0 ± 65.9 (15)</td>
</tr>
<tr>
<td>10</td>
<td>APIII Variations &gt;5%</td>
<td>789.6 ± 209.2 (6)</td>
<td>391.3 ± 79.6 (6)</td>
<td>526.2 ± 57.0 (10)</td>
</tr>
<tr>
<td>10</td>
<td>rANF All</td>
<td>642.7 ± 141.5 (3)</td>
<td>470.1 ± 114.3 (3)</td>
<td></td>
</tr>
<tr>
<td>30</td>
<td>APIII Variations &gt;5%</td>
<td>672.2 ± 201.7 (7)</td>
<td>399.9 ± 85.8 (7)</td>
<td>991.5 ± 394.4 (6)</td>
</tr>
<tr>
<td>30</td>
<td>rANF</td>
<td>642.7 ± 141.5 (3)</td>
<td>486.9 ± 87.4 (3)</td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>APIII</td>
<td>510.1 ± 150.5 (3)</td>
<td>300.6 ± 42.9 (3)</td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>rANF</td>
<td>678.0 ± 96.5 (3)</td>
<td>491.0 ± 55.9 (3)</td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>rANF</td>
<td>628.2 ± 80.9 (3)</td>
<td>447.2 ± 67.0 (3)</td>
<td></td>
</tr>
</tbody>
</table>

ANF, atrial natriuretic factor; ISO, isoprenaline; cAMP, cyclic adenosine 3',5'-monophosphate; rANF, 3-28 ANF rat; APIII, rat atriopeptin III; ICa, calcium current.

ICa was elevated by either 0.3 μM external ISO or 5 or 20 μM internal cAMP. For each experiment, currents were expressed with respect to control ICa (i.e., prior to ISO or cAMP application). Values are expressed as mean ± SEM (n). ICa values in ANF were measured at the end of ANF application (i.e., after 3–6 minutes) and do not reflect additional transient effects of the peptide. When ICa was elevated by ISO, exposure of the cell of ANF generally induced a rundown of ICa, thus, the values of ICa measured at the end of the exposure to ANF are necessarily underestimated. For experiments using rANF, certain cells showed no response to the peptide at any concentration tested. These experiments were either included (all) or discarded (variations >5%) in the summary data; in the latter, the data included only those experiments where ANF responses were >5% of ICa elevated by either ISO or cAMP. In the former, *indicates a statistically significant difference from the data in the absence of ANF; **p<0.1, ***p<0.05. (For further details, see text.)

**Note added in proof:** Cramb et al (Biochem Biophys Res Comm 1987;148:962–970) have recently shown a stimulation of cGMP production by ANF in isolated rabbit ventricular myocytes.
way to parasympathetic effectors such as acetylcholine, although through different receptors. This could imply an interaction between acetylcholine and ANF on calcium channels in heart. We are currently investigating this possibility.

In conclusion, our findings demonstrate an inhibitory action of nanomolar concentrations of ANF on cardiac calcium current, which suggests that ANF may exert a negative inotropic effect that could be an additional mechanism by which ANF regulates blood pressure.

Acknowledgments

We thank Dr. Guy Vassort for permanent support, Drs. Ian Findlay and H. Criss Hartzell for critical reading of the manuscript, Patrick Lechene for skillful computer programming, and Adama Diarra and Michel Puciat for preparing the cells. ANFg_33 was a generous gift of Dr. Daniel F. Veber, Merck Sharp & Dohme, and APIII was generously given by Dr. D. Nisato, Sanofi, France. Radioimmunoassay measurements of ANF were done at the Institut Henri Beaufour, Les Ulis, France (Dr. P.E. Chabrier).

References


Key Words • atrial natriuretic factor • cardiac isolated cells • calcium current • isoprenaline • adenylate cyclase • cyclic AMP • voltage clamp
Atrial natriuretic factor regulates the calcium current in frog isolated cardiac cells.
M P Gisbert and R Fischmeister

doi: 10.1161/01.RES.62.4.660

Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 1988 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/62/4/660

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/