Vascular Smooth Muscle Cells Express the α1A Subunit of a P-/Q-Type Voltage-Dependent Ca2+ Channel, and It Is Functionally Important in Renal Afferent Arterioles

Pernille B. Hansen, Boye L. Jensen, Ditte Andreasen, Ulla G. Friis, Ole Skøtt

Abstract—In the present study, we tested whether the α1A subunit, which encodes a neuronal isoform of voltage-dependent Ca2+ channels (VDCCs) (P-/Q-type), was present and functional in vascular smooth muscle and renal resistance vessels. By reverse transcription–polymerase chain reaction and Southern blotting analysis, mRNA encoding the α1A subunit was detected in microdissected rat preglomerular vessels and vasa recta, in cultures of rat preglomerular vascular smooth muscle cells (VSMCs), and in cultured rat mesangial cells. With immunobLOTS, α1A subunit protein was demonstrated in rat aorta, brain, aortic smooth muscle cells (A7r5), VSMCs, and mesangial cells. Immunolabeling with an anti-α1A antibody was positive in acid-macerated, microdissected preglomerular vessels and in A7r5 cells. Patch-clamp experiments on aortic A7r5 cells showed 22±4% (n=6) inhibition of inward Ca2+ current by ω-Agatoxin IVA (10-8 mol/L), which in this concentration is a specific inhibitor of P-type VDCCs. Measurements of intracellular Ca2+ in afferent arterioles with fluorescence-imaging microscopy showed 32±9% (n=10) inhibition of the K+–induced rise in Ca2+ in the presence of 10-8 mol/L ω-Agatoxin IVA. In microperfused rabbit afferent arterioles, ω-Agatoxin IVA inhibited depolarization-mediated contraction with an EC50 of 10-17 mol/L and complete blockade at 10-14 mol/L. We conclude that the α1A subunit is expressed in VSMCs from renal preglomerular resistance vessels and aorta, as well as mesangial cells, and that P-type VDCCs contribute to Ca2+ influx in aortic and renal VSMCs and are involved in depolarization-mediated contraction in renal afferent arterioles. (Circ Res. 2000;87:896-902.)

Key Words: smooth muscle • voltage-dependent Ca2+ channel • renal • arteriole

Excitation-contraction coupling in most resistance vessels is highly dependent on Ca2+ entry through voltage-dependent Ca2+ channels (VDCCs), and inhibitors of such channels are in widespread clinical use. Voltage-gated Ca2+ influx in vascular smooth muscle cells (VSMCs) is usually ascribed to activation of high voltage–activated (L-type) and in some cases to low voltage–activated (T-type) VDCCs. The VDCC protein complex consists of up to five different subunits. The α1 subunit forms the pore of the channel, the drug binding site, and the voltage sensor.

In the kidney, L- and T-type Ca2+ currents have been identified by patch clamp in smooth muscle cells isolated from renal preglomerular vessels. Moreover, branching points along the renal resistance vessels are enriched in L-type VDCCs. Messenger RNAs encoding α1 subunits of L- and T-type VDCCs have been demonstrated in the rat kidney by Northern blotting, but in addition to this, mRNA for the α1A subunit, which encodes the neuronal P-/Q-type Ca2+ channel, has been shown to be present in kidney cortex. L- and T-type VDCCs cannot fully account for depolarization-induced Ca2+ influx in renal VSMCs. We investigated, using kidney and aorta, whether P-/Q-type VDCCs are present in vascular smooth muscle, and whether they contribute to Ca2+ fluxes and vasoreactivity.

The results demonstrate that α1A subunit mRNA and protein are expressed and that P-type VDCCs contribute to depolarization-mediated Ca2+ influx and contraction in VSMCs.

Materials and Methods

Cells

Preglomerular VSMCs were prepared as described by Dubey et al. Renal microvessels were isolated from rat kidneys after iron oxide particle infusion, enzyme-digested, separated by a magnetic field, and suspended in RPMI 1640. When cells were confluent, RNA was isolated (RN’easy kit, Qiagen). The VSMCs were characterized by immunocytochemical staining for smooth muscle α-actin (clone 1A4) (Sigma) (Figure 1). Mesangial cells were obtained by outgrowth from isolated glomeruli as previously described. Only cells from first passage were used for RNA extraction. The aortic cell line A7r5 was from American Type Culture Collection.

Microdissection of Rat Preglomerular Vessels and Vasa Recta

Renal vessels for RNA extraction were obtained by dissection of rat kidney tissue from 8 rats according to the protocol of Yang et al.
Bar 75 were added to all oligomers (DNA Technology): a confluent monolayer of VSMCs with anti–sucrose, 25 mmol/L imidazole, Complete, pH 7.2, and centrifuged.

Fig. 1. Immunostaining of renal VSMCs. A, Immunolabeling of a confluent monolayer of VSMCs with anti–α-actin antibody. Bar = 75 μm. B, Negative control with no primary antibody added.

Two consecutive divisions of preglomerular vessels were isolated. Vasa recta bundles were isolated from outer medulla. Of the preglomerular samples, 40 to 50 “branching points” were pooled. Vasa recta bundle length was measured with a micrometer scale built into the ocular. Total RNA was isolated according to a microadapted protocol of Chomczynski and Sacchi. B

Reverse Transcription–Polymerase Chain Reaction (RT-PCR) and Cloning

RT-PCR analysis was performed. BamHI or EcoRI restriction sites were added to all oligomers (DNA Technology):

α1A: forward: 5′-ATT ACA TCC TGA ACC-3′; reverse: 5′-CTT CAA CTT AGG CAG C-3′, covering bases 3564 to 3929, 383 bp (GenBank accession No. M64373). β-actin: copied from Yu et al. A

The cDNA used corresponded to 5 to 10 branching points (preglomerular vessels), 100 ng total RNA (smooth muscle cells and whole kidney) or 1 mm (vasa recta). PCR products were inserted in vector pSP73 (Promega) for heat-shock uptake by competent Escherichia coli (DH5a, GIBCO) using standard procedures. Plasmid DNA was extracted using the QIAGEN Plasmid Maxi kit. Inserts were sequenced using T7 and SP6 promoter primers on an ABI PRISM 350 sequencer (Perkin Elmer).

Southern Blotting

PCR products were separated by agarose gel electrophoresis and blotted to Zeta Probe GT membranes (Bio-Rad) using standard capillary blotting procedures (transfer buffer: 0.4 mol/L NaOH). Hybridization was allowed overnight to specific probe and in vitro–labeled with α-32P-dCTP, all according to Sambrook et al. A

Autoradiography was performed for 2 to 4 hours on Kodak Biomax MS film.

Western Blotting

Tissues were dissected, snap-frozen, and homogenized in 0.3 mol/L sucrose, 25 mmol/L imidazole, Complete, pH 7.2, and centrifuged 4000g for 15 minutes. Protein concentrations were determined using the Bio-Rad protein assay, with BSA as standard. Cultured cells were rinsed twice in TBS (20 mmol/L Tris-HCl, 137 mmol/L NaCl, pH 7.6), suspended in 100 μL lysis buffer (0.1% Triton-X, 1 tablet/10 mL Complete Mini [Roche Molecular Biochemicals]), and quick-frozen. SDS-PAGE and Western blotting were performed. The primary antibody was anti-α1A subunit (Alomone Labs). Secondary antibody was goat anti-rabbit IgG, HRP-labeled (NE). Proteins were detected using Renaissance Chemiluminescent Reagent Plus (NE).

Immunostaining

Renal vascular trees were microdissected from rats after HCl maceration, 1 fixed in 3.7% paraformaldehyde, and permeabilized with methanol plus 0.006% H2O2 and immunolabeled. The primary antibodies were rabbit anti-α1A antibody and rabbit anti-mouse renin antibody. The secondary antibody was goat anti-rabbit IgG, HRP-labeled. Staining was with diaminobenzidine (DAB substrate chromogen system, DAKO). Cultures of A7r5 cells were fixed, permeabilized, and immunolabeled with anti-rabbit α1A antibody in a similar way and then counterstained with hematoxylin.

Patch-Clamp Experiments

Patch-clamp experiments were performed on A7r5 cells at room temperature in the tight-seal whole-cell configuration of the patch-clamp technique 13 with heat-polished, Sylgard-coated patch pipettes with resistances of ≥ 5.5 MΩ. The pipette solution contained (mmol/L): CsCl 120, MgCl2 3, HEPES-CsOH 5, MgATP 5, and EGTA 10, pH 6.00 (24.1°C). Series resistances were 2 to 15 GΩ. High-resolution membrane currents were recorded with an EPC-9 patch-clamp amplifier (HEKA). Immediately after the whole-cell configuration was obtained, the cells were superfused with a solution that facilitated Ca2+ currents (mmol/L: tetraethylammonium acetate 148, KCl 2.8, MgCl2 1.0, BaCl2 10.8, and HEPES-CsOH 10 [pH 7.23, 21.4°C]) for 1 to 2 minutes. The cells were then superfused with the same solution supplemented with ω-Agatoxin IVA (10–4 mol/L) (Alomone Labs). A

Measurement of [Ca2+]i by Digital Fluorescence-Imaging Microscopy

Microdissected rabbit afferent arterioles were placed in a perfusion chamber on an inverted microscope, secured by holding pipettes, and loaded with 5 μmol/L fura-2/AM in physiological salt solution (PSS) for 45 minutes at room temperature. Excitation light was provided by a monochromator at 350 and 380 nm, and the output at 510 nm was detected by a charged coupled device camera and light intensifier and analyzed using Metafluor software (Universal Imaging). Measurements of fluorescence intensity were performed at a rate of 1 frame per second, and the ratio of 350:380 was used to calculate [Ca2+]i, applying fura 2/K+ calcium standards. 15 In 10 experiments, changes in [Ca2+]i were measured after addition of 100 mmol/L K+ (with phenolamine 10–5 mol/L). After a 5-minute wash in PSS, the vessel was exposed to ω-Agatoxin IVA (10–5 mol/L) for 1 minute and the response to K+ was measured again in the presence of ω-Agatoxin IVA.

PSS had the following composition (mmol/L): NaCl 115, NaHCO3 25, K,HPO4 2.5, CaCl2 1.3, MgSO4 1.2, and glucose 5.5. High potassium solution contained (mmol/L): NaHCO3 25, NaCl 20, KCl 95, MgSO4 1.2, K,HPO4 2.5, CaCl2 1.3, and glucose 5.5. Both solutions contained 0.1% BSA at pH 7.4.

Isolation of Rabbit Afferent Arterioles and Micropertusion Protocols

Afferent arterioles were microdissected from 10 rabbits and perfused with PSS. Repetitive depolarizations of each of the perfused afferent arterioles were done in the presence of increasing concentrations of ω-Agatoxin IVA (10–10 to 10–9 mol/L). After preincubation with toxin at each concentration for 1 minute, K+ (100 mmol/L) was added for 1 minute. The experiments were recorded on videotape, sequences of interest were digitized, and luminal vessel...
Results

Confirmation of Probe Sequences

The α1A PCR amplification product was cloned in vector pSP73, and the insert was sequenced. The insert was 100% identical to the published sequence. 17

Localization of α1 Subunits in Microdissected Renal Resistance Vessels and VSMCs

We performed RT-PCR analysis and subsequent Southern blotting on RNA extracted from microdissected rat preglomerular vessels and vasa recta bundles and from primary cultures of quiescent renal smooth muscle. Using cDNA from preglomerular microvessels as a template, PCR amplification revealed a significant expression of α1A (Figure 2A). Amplification products for α1A were observed when RNA from the cultured renal smooth muscle cells and mesangial cells was analyzed by RT-PCR and amplified from serial dilutions (Figure 2A). This confirms the expression of the α1A gene in contractile cells. Microdissected vasa recta bundles also expressed α1A subunit mRNA (Figure 2B). To test whether the α1A subunit is expressed in smooth muscle cells from other vascular beds, we examined α1A subunit mRNA expression in aortic smooth muscle cells and confirmed that the aortic cells (A7r5) also expressed α1A (data not shown).

Western Blotting

To verify that α1A protein, in addition to mRNA, was present in the VSMCs, we performed Western blots. In immunoblots of protein extracts, cultured renal smooth muscle cells, cultured mesangial cells, and A7r5 cells, as well as aorta and brain, gave rise to bands of the expected size (190 kDa) when labeled with an anti-α1A antibody (Figure 3). By preabsorbing the primary antibody with the peptide CNA1 (used for raising the antibody), the labeling was completely displaced, confirming the specificity.

Figure 2. Expression of α1A subunit mRNA in vascular smooth muscle shown with RT-PCR and Southern blotting. Top, Ethidium bromide-stained agarose gel showing RT-PCR amplification products using primers specific for α1A. Middle, Southern blotting of the above gel, using an α1A-specific probe. Bottom, Ethidium bromide-stained agarose gel showing RT-PCR products for β-actin. A, Samples were as follows: dilution series of cDNA from VSMCs, cDNA from 5 separate samples of microdissected renal preglomerular resistance vessels, cDNA from cultured mesangial cells (MES), and whole kidney. Negative controls were water and tRNA. φ indicates molecular weight standard φX174/HaeIII. B, Demonstration of expression of α1A subunit mRNA in microdissected postglomerular vasa recta bundles (v. recta). For comparison, a sample of microdissected preglomerular vessels (preglom., compare panel A) is shown. Whole kidney is positive control, and tRNA serves as negative control.

Figure 3. Expression of α1A subunit protein in vascular smooth muscle demonstrated with Western blots. A, Protein isolated from an aortic smooth muscle cell line (A7r5), cultured renal VSMCs, and cultured mesangial cells (MES). Anti-α1A+CNA1 indicates negative control; the antibody was preabsorbed with the peptide it was raised against. B, Protein isolated from fresh aorta and brain.
Immunostaining
As a test of the presence of α1A protein in the isolated renal resistance vessels, we performed immunolabeling with anti-α1A antibody of HCl-macerated, microdissected renal vascular trees with anti-α1A antibody showing expression of α1A subunit protein throughout the preglomerular vasculature. This approach revealed expression of the protein throughout the preglomerular vasculature, as well as in the glomeruli (Figure 4A). Preabsorbing the primary antibody with the peptide CNA1 displaced the labeling, and no unspecific labeling was detected in the absence of primary antibody. As a control for the overall specificity of this labeling method on the acid-macerated tissue, we also used an anti-mouse renin antibody. As expected, this antibody gave a very distinct labeling of the endpoints of the afferent arterioles (Figure 4B). We then tested whether the α1A protein was present in the cultured aortic cell line A7r5. Labeling is seen in a granular pattern throughout the cells, verifying expression of the protein in smooth muscle cells (Figure 5A). In the absence of primary antibody, no labeling was seen in A7r5 cells (Figure 5B).

Patch Clamp
The presence of P-type Ca2+ channels was also tested on A7r5 cells with electrophysiology. When these cells were depolarized from a holding potential of -70 to +10 mV, a large negative current was observed (Figure 6A). Significant rundown was observed after 6 to 8 minutes. This current was sensitive to inhibition with the specific P-type VDCC blocker ω-Agatoxin IVA (10^-8 mol/L) (Figure 6A, upper trace), indicating that part of this current is an inwardly directed Ca2+ current through P-type Ca2+ channels. ω-Agatoxin IVA inhibited the current 22.1±4.2% (SE, n=6) (Figure 6B). When Na+ currents and K+ currents were eliminated by using Cs+ and tetraethylammonium in the buffers, a standard voltage-clamp protocol yielded the characteristic I-V relationship for Ca2+ currents as shown in Figure 6C. From this relationship, it can be noted that the maximal Ca2+ current is observed at +10 mV, which is the reason for the chosen pulse protocol shown in Figure 6A.

[Ca2+]i Measurements
The effect of the specific P/Q-type VDCC blocker ω-Agatoxin IVA on K+ induced increase in intracellular Ca2+ was tested on afferent arterioles. In the experiment shown, the addition of 100 nmol/L K+ rapidly increased the [Ca2+], from 65 to 205 nmol/L; it then declined slowly until K+ was removed. The response to K+ after a 5-minute resting period and 1-minute incubation with ω-Agatoxin IVA was clearly inhibited (205 versus 160 nmol/L) (Figure 7A). No tachyphylaxis was observed to repeated administration of K+.

Figure 4. Immunolabeling of α1A subunit protein in renal preglomerular vasculature. A, Immunostaining of HCl-treated, microdissected renal vascular trees with anti-α1A antibody showing expression of α1A subunit protein throughout the preglomerular vasculature. Bar=500 μm. Negative controls were displacement of antibody by preabsorbing the primary antibody with the peptide CNA1 or without primary antibody. B, Immunostaining with anti-mouse renin antibody of renin-containing cells at the ends of the afferent arterioles. Bar=75 μm.

Figure 5. Immunolabeling of α1A subunit protein in cultured VSMCs (A7r5). A, Immunostaining of A7r5 cells with anti-α1A antibody showing expression of the protein. Bar=50 μm. B, Negative control without primary antibody.
In Figure 8A, in the absence of 

with increasing concentrations of 

Isolated, Perfused Arteriole Studies

To test the functional significance of the P/Q-type Ca\(^{2+}\) channel for K\(^+\)-induced contraction in microperfused afferent arterioles, we used \(\omega\)-Agatoxin IVA. A test stimulus of 100 mmol/L K\(^+\) was given initially to ensure viability of the vessel. The addition of \(\omega\)-Agatoxin IVA for 1 minute to afferent arterioles perfused at physiological pressures (60 to 80 mm Hg) did not change the basal diameter of the arterioles. The time course of changes in vessel inner diameter was directed current was large and stable (in this experiment at 40 ms; see pulse protocol above the traces). When the inwardly directed response was fully restored. All vessels were treated with this, Ca\(^{2+}\) influx in response to depolarization was significantly reduced by the specific P/Q-type Ca\(^{2+}\) channel blocker \(\omega\)-Agatoxin IVA in isolated rabbit renal afferent arterioles. In agreement with this, Ca\(^{2+}\) influx in response to depolarization was significantly reduced by the specific P/Q-type Ca\(^{2+}\) channel blocker \(\omega\)-Agatoxin IVA in isolated rabbit renal afferent arterioles (diameter \(\leq 20 \mu\)m at physiological pressure). This was reflected by an extremely potent inhibition of K\(^+\)-induced contraction of perfused afferent arterioles by \(\omega\)-Agatoxin IVA. Together these sets of functional data
Hansen et al  A Neuronal Ca\(^{2+}\) Channel in Smooth Muscle Cells  901

Figure 8. Inhibition of depolarization-induced contraction in microperfused rabbit afferent arterioles by \(\omega\)-Agatoxin IVA. A, Blocking effect of \(\omega\)-Agatoxin IVA (\(\omega\)-agatx) on K\(^+\) action in a single experiment. The sensitivity to K\(^+\) was tested before addition of \(\omega\)-Agatoxin IVA, in the presence of 10\(^{-12}\) mol/L \(\omega\)-Agatoxin IVA (after 1-minute preincubation), and after a resting period without \(\omega\)-Agatoxin IVA. B, Dose-response curve for the effect of \(\omega\)-Agatoxin IVA on K\(^+\)-induced contraction (n=13). Basal diameter and response to K\(^+\) were tested before exposure to \(\omega\)-Agatoxin IVA (squares). In individual arterioles, the sensitivity to repetitive 1-minute depolarizations was tested at increasing concentrations of \(\omega\)-Agatoxin IVA (diamonds). After a resting period, the sensitivity to depolarization was tested again (triangle).

Demonstrate that Ca\(^{2+}\) influx and intracellular Ca\(^{2+}\) increases mediated by P/Q-type Ca\(^{2+}\) channels are necessary to elicit contraction in response to depolarization, at least in renal resistance vessels. At present, we cannot explain the extreme sensitivity by which \(\omega\)-Agatoxin IVA blocks contraction in afferent arterioles. The high sensitivity might reflect voltage-dependent binding of the blocker at depolarized potentials as seen during pressurization or specific modulation of the channel by auxiliary subunits expressed in afferent arterioles.

Alternative splicing of mRNA originating from the \(\alpha_{1A}\) subunit gene results in generation of two different gene products considered responsible for P/Q-type Ca\(^{2+}\) currents, respectively. \(\omega\)-Agatoxin IVA blocks P-type channels at concentrations <10 nmol/L, whereas the Q channels are inhibited at higher concentrations, in the range of 0.1 to 1 \(\mu\)mol/L. We did not perform a full dose response for the effect of \(\omega\)-Agatoxin IVA on Ca\(^{2+}\) and [Ca\(^{2+}\)].

In the present study, \(\alpha_{1A}\) subunit mRNA and protein were detected both in vascular cell cultures and in freshly isolated cell and tissue samples. Together, the data imply constitutive expression of P-type Ca\(^{2+}\) channels in vascular tissue. Data were recently presented that showed the presence of a nifedipine-insensitive high voltage-activated Ca\(^{2+}\) channel in guinea pig mesenteric resistance arterioles (diameter 40 to 100 \(\mu\)m) with some biophysical similarities to the P-type Ca\(^{2+}\) channels whereas the pharmacological profile was markedly different from P- or Q-type channels. It is conceivable that true arteriolar resistance segments contain as-yet unrecognized subsets of Ca\(^{2+}\) channels.

As to the functional role in excitation-contraction coupling, the distinguishing features of P-type Ca\(^{2+}\) currents compared with L-type Ca\(^{2+}\) currents are their very slow and Ca\(^{2+}\)-independent rate of inactivation and the strong modulation by G proteins. The last feature is involved in hormonal regulation of channel properties. The presence of P-type currents could significantly expand the time for active Ca\(^{2+}\) influx and allow hormonal influence on vascular reactivity. Mutant mice lacking the \(\alpha_{1A}\) subunit gene display a phenotype with pronounced neurological deficits with ataxia and dystonia, and the mice die 3 to 4 weeks after birth. No major abnormalities of cardiovascular control were reported. In humans, mutations in the gene encoding the \(\alpha_{1A}\) subunit are associated with neurological disorders such as episodic ataxia type 2 (EA2) and spinocerebellar ataxia type 6 (SCA6), as well as familial hemiplegic migraine, which is a disorder with significant vascular component. Future studies should clarify whether mutations in the \(\alpha_{1A}\) subunit gene are involved in the pathogenesis of vascular disorders.

An important observation of the present study is that specific pharmacological inhibition of a single Ca\(^{2+}\) channel subtype abolished K\(^{+}\)-induced contraction. This suggests that several Ca\(^{2+}\) channels are present in the smooth muscle and...
that equal contribution is required for full responsiveness of renal afferent arterioles to depolarization. The present demonstration of P-type Ca\(^{2+}\) channels in the renal vasculature raises the question to what degree previous data on the control of renal blood flow by Ca\(^{2+}\) channels reflect interaction of pharmacological blockers with the P-type channel. In this context, it is interesting to note that L-type Ca\(^{2+}\) channel blockers, such as the dihydropyridines, verapamil, and diltiazem, have been reported to significantly inhibit P-type Ca\(^{2+}\) currents when used in concentrations that are not maximal for the L-type blockade.\(^{34,35}\) It is therefore likely that the role of L-type Ca\(^{2+}\) channels has been overestimated and that the response to therapeutic concentrations of these compounds includes the sum of inhibiting at least L- and P-type Ca\(^{2+}\) channels. We conclude that mRNA and protein encoding an \(\alpha_{1A}\) subunit for a P-/Q-type Ca\(^{2+}\) current is expressed in VSMCs and mesangial cells, and we provide evidence that P-type VDCCs play a significant functional role in A7r5 cells and in renal afferent arterioles.

Acknowledgments

This work was supported by grants from the Danish Health Research Council (9903058, 9601829, and 9902742), the NOVO Nordisk Foundation, The Danish Heart Association (98-1-2-8-22283, 99-2-2-36-22743), the Foundation for the Advancement of Medical Sciences, the Ruth König Petersens Foundation, The Danish Medical Association Research Fund, Alfred Andersens Foundation, the Foundation of 23-9-1909, and Overlægerådets Legatudvalgs Fond. We thank Anthony M. Carter for language revision, Peter D. Ottosen for immunostaining photographs, and Mette Fredenslund and Inge Andersen for skillful technical assistance.

References

19. Deleted in proof.
Vascular Smooth Muscle Cells Express the α1A Subunit of a P-/Q-Type Voltage-Dependent Ca2+ Channel, and It Is Functionally Important in Renal Afferent Arterioles

Pernille B. Hansen, Boye L. Jensen, Ditte Andreasen, Ulla G. Friis and Ole Skøtt

Circ Res. 2000;87:896-902
doi: 10.1161/01.RES.87.10.896

Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2000 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/87/10/896

Data Supplement (unedited) at:
http://circres.ahajournals.org/content/suppl/2000/11/03/87.10.896.DC1

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/