Localization of Cardiac Sodium Channels in Caveolin-Rich Membrane Domains
Regulation of Sodium Current Amplitude

Tracy L. Yarborough, Tong Lu, Hon-Chi Lee, Erwin F. Shibata

Abstract—This study demonstrates that caveolae, omega-shaped membrane invaginations, are involved in cardiac sodium channel regulation by a mechanism involving the α subunit of the stimulatory heterotrimeric G-protein, Goα, via stimulation of the cell surface β-adrenergic receptor. Stimulation of β-adrenergic receptors with 10 μmol/L isoproterenol in the presence of a protein kinase A inhibitor increased the whole-cell sodium current by a “direct” cAMP-independent G-protein mechanism. The addition of antibodies against caveolin-3 to the cell’s cytoplasm via the pipette solution abrogated this direct G protein–induced increase in sodium current, whereas antibodies to caveolin-1 or caveolin-2 did not. Voltage-gated sodium channel proteins were found to associate with caveolin-rich membranes obtained by detergent-free buoyant density separation. The purity of the caveolar membrane fraction was verified by Western blot analyses, which indicated that endoplasmic/sarcoplasmic reticulum, endosomal compartments, Golgi apparatus, clathrin-coated vesicles, and sarcolemmal membranes were excluded from the caveolin-rich membrane fraction. Additionally, the sodium channel was found to colocalize with caveolar membranes by immunoprecipitation, indirect immunofluorescence, and immunogold transmission electron microscopy. These results suggest that stimulation of β-adrenergic receptors, and thereby Goα, promotes the presentation of cardiac sodium channels associated with caveolar membranes to the sarcolemma. (Circ Res. 2002;90:443-449.)

Key Words: caveolae ■ ion channel ■ signal transduction ■ cardiac ■ adrenergic

Regulation of voltage-gated ion channels at the plasma membrane of excitable cells is essential in maintaining cellular excitability and electrical impulse propagation. The amplitude and slope of the action potential upstroke are especially important in the control of cardiac conduction velocity, and the maintenance of appropriate waves of excitation through the ventricles. In the nonpacemaker cells of the heart, the voltage-gated sodium channel mediates the upstroke of the action potential. Neurohumoral regulation of this sodium current, ISNa, via β-adrenergic stimulation has been shown to increase the current by at least 2 known mechanisms: one “direct” and one “indirect”.1–7 The indirect, or protein kinase A (PKA)–dependent, mechanism regulates ISNa by phosphorylation of the channel protein at previously identified sites,6 resulting in alterations of single-channel voltage-dependent characteristics, including channel availability (inactivation).3,4,8–12 The mechanism of the direct, PKA-independent, effect is not well understood, though evidence suggests that ligand binding to plasma membrane β-adrenergic receptors (BARs) results in the activation of a signaling cascade involving the Goα protein itself. This produces an increase in ISNa without changes in single-channel characteristics or shifts in voltage-dependent current activation as demonstrated by current-voltage relationships.2,7 In the presence of inhibitors of PKA, the application of exogenous Goα to the cytoplasmic face of excised membrane patches mimics the effect of β-adrenergic receptor activation in increasing ISNa by increasing the number of functional channels at the plasma membrane.7 Thus, the direct effect of β-stimulation on ISNa appears to be the result of an increase in the number of functional sodium channels at the sarcolemma of cardiac myocytes. The source of the channels that mediate this increase in ISNa is the focus of this study.

The time course of the direct effect (generally less than 10 minutes) makes synthesis of new channel proteins an unlikely mechanism for the increased ISNa. In addition, the direct increase in ISNa is reversible by β-agonist washout and is reproducible by subsequent rounds of βAR stimulation. These 2 factors are suggestive of a store of channel proteins that can be functionally added to and removed from the plasma membrane dependent on β-adrenergic signaling events. We believe caveolae to be a prime candidate for such a storage locale. Caveolae, subsarcolemmal membrane compartments, have been implicated in cellular trafficking cas-
cles involving adrenergic receptors\textsuperscript{13,14} and endothelial nitric oxide synthase (eNOS),\textsuperscript{15} among other cell surface proteins.\textsuperscript{16,17} Caveolae are capable of effectively removing surface proteins from the plasma membrane through sequestration and endocytotic/transcytotic mechanisms on stimulation of specific receptors.\textsuperscript{18–20} Importantly, caveolae may be involved in mechanisms whereby the availability of water\textsuperscript{21} and volume-sensitive chloride channels\textsuperscript{17,22} at the plasma membrane are mediated.

Among the many proteins localized to caveolar membrane compartments by biochemical studies (reviewed in Okamoto et al\textsuperscript{23} and Ostrom et al\textsuperscript{24}), G\textsubscript{i} is of particular interest. Although G\textsubscript{i} can mimic the effect of BAR stimulation in increasing cardiac sodium current, G\textbeta\gamma does not appear to be involved. Therefore, the action of the G\textsubscript{i} in this process must be dependent on the protein itself and other proteins with which it might interact. Specific regions of interaction between G\textsubscript{i} and caveolin-1, the caveolar scaffolding protein, have been identified, wherein G\textsubscript{i} binds to a region contained within the caveolin-1 scaffolding domain in a GTP/GDP-dependent fashion.\textsuperscript{23,25} Although specific studies have not examined the interaction of G\textsubscript{i} with caveolin-3, the muscle-specific caveolin isoform, caveolin-3 is highly homologous to caveolin-1 in its scaffolding domain,\textsuperscript{26,27} making an analogous interaction with G\textsubscript{i} likely. In this study, we investigate the role of caveolae in the regulation of functional cardiac sodium channel number at the sarcolemmal membrane. We hypothesize that G\textsubscript{i} activation through \textbeta\textgamma stimulation results in the opening of caveolae, and thereby, the addition of functional sodium channels to the sarcolemma.

Materials and Methods

Adult rat cardiac myocytes were obtained as outlined previously\textsuperscript{7} and as approved by the Animal Care and Use Committee at the University of Iowa. Rats were procured from Harlan Sprague-Dawley (Indianapolis, Ind).

Purification of Caveolin-Rich Fraction

Isolation of caveolin-rich (CR) fraction was done using a previously described detergent-free method,\textsuperscript{20} with some modifications. Myocyte lysates were separated on a 5% to 45% discontinuous sucrose gradient, yielding a caveolin-rich membrane fraction (samples 5 and 6). For comparison, a heavy fraction (sample 11) was also collected.

Antibodies

Antibodies used were obtained as follows, and are referenced in the online data supplement. Anti-cardiac ryanodine receptor (RyR) was the kind gift of Dr Kevin P. Campbell (University of Iowa, Iowa City). Antibodies to caveolin-3, caveolin-2, clathrin, Rab 5, Rab 11, and secondary HRP-conjugated anti-mouse and anti-rabbit antibodies were obtained from Transduction Laboratories, Antibodies to caveolin-1 and G\textsubscript{i}, were obtained from Santa Cruz Biotechnology. Antibodies to cytochrome oxidase subunit 2 (COX), plasma membrane calcium ATPase (PMCA), and mannosidase II were obtained from Molecular Probes, Affinity Bioreagents, and Covance, respectively. Subtype-specific antibodies to the \( \alpha 1 \) and \( \alpha 2 \) isoforms of the Na'K' ATPase were a kind gift from Dr Kathleen J. Seward (Harvard University, Cambridge, Mass), and antibodies to both the \( \alpha 1 \) and \( \alpha 2 \) isoforms were purchased from Upstate Biotechnology. Anti-SP-19 (pan) sodium channel antibody and secondary HRP-conjugated anti-goat antibodies were obtained from Sigma. D902 polyclonal antibody was raised in goat to a 22-amino acid peptide corresponding to a sequence (DRLPKSDEGDPRALNQLSLC) located in interdomain loop I-II of the cardiac voltage-gated sodium channel as similarly described by Cohen and Levitt.\textsuperscript{28}

Immunoblotting

Samples were precipitated using the pyrogallol red-molybdate method\textsuperscript{29} and run on 7.5% to 12% polyacrylamide gel. Proteins were transferred to 0.45 \( \mu \)g/mL nitrocellulose membrane and blotted with appropriate antibodies.

Immunoprecipitation

Caveolin-rich and heavy membrane fractions were pelleted by centrifugation. Each was incubated with anti–caveolin-3 antibody (1:500), followed by Protein G-agarose. The complexes were washed and then boiled in 1X sample buffer. Supernatants were separated by SDS-PAGE for immunoblotting.

Immunofluorescence

Immunolabeled frozen sections of adult rat ventricle were visualized with a Zeiss confocal microscope. Primary antibodies were diluted as follows: anti–caveolin-3 1:50; D492 1:200. Secondary immunofluorescent conjugates were diluted at 1:200.

Immunogold Labeling and Electron Microscopy

Ultrathin sections of CR fractions were fixed and labeled prior to embedding, followed by silver-enhancement and counterstaining for visualization with a H-7000 transmission electron microscope. Primary antibodies were diluted as follows: anti–caveolin-3 1:20; D492 1:100. Secondary gold-conjugates were diluted at 1:40.

Electrophysiological Protocol

Whole-cell voltage clamp data were generated by stepping membrane potential from –100 mV to –30 mV for 20 ms. In the experimental groups, \( 100 \mu \)g/mL anti–caveolin-1, -2, or -3 antibody was added to the pipette solution. In the control group, no antibody was added. Raw data are plotted as \( I_{\text{Na}} \) relative to baseline \( I_{\text{Na}} \) (current prior to bath addition of isoproterenol [ISO]). Histogram data are presented as the mean±SEM. One-way ANOVA was used to compare the percent change in \( I_{\text{Na}} \) among the control and experimental groups. Statistical significance is defined as \( P<0.05 \), with \( n \) number of experiments as indicated.

An expanded Materials and Methods section can be found in the online data supplement available at http://www.circresaha.org.

Results

Implication of Caveolin-3 in Adrenergically Mediated Increases in Sodium Current

Previous work\textsuperscript{2,7} suggests that the direct G-protein effect of \( \beta \)-adrenergic stimulation on cardiac sodium current is the result of an increase in the number of functional channels present at the sarcolemma. Increasing functional channel number could be accomplished by modifications of nonfunctional channels in the membrane that make them conductive, or by the recruitment of channels from a previously unrecognized intracellular storage location. We therefore sought to determine the role of caveolae in the mechanism of channel recruitment. Adult myocytes were clamped in the whole-cell configuration with an intracellular pipette solution containing 0.022 mg/mL protein kinase A inhibitor (PKI), a concentration at which the effects of PKA on sodium current have been shown to be completely inhibited;\textsuperscript{3} that is, the addition of 500 \( \mu \)mol/L 8-CPT-cAMP or 10 \( \mu \)mol/L forskolin could not induce an increase in \( I_{\text{Na}} \) in the presence of 0.022 mg/mL PKI.

Representative traces of the resulting currents obtained by whole-cell patch clamp are shown in Figure 1. \( I_{\text{Na}} \) was recorded before (baseline, BSLN) and after (ISO) the addition
The addition of ISO induces an increase in the voltage-gated sodium current in control cells wherein no antibody was added. The addition of ISO traces were obtained during superfusion with isoproterenol. ISOS test wherein significance is defined as *(experimental groups versus control using a Dunnett comparisons test wherein significance is defined as P<0.05). Figure 2 demonstrates that neither caveolin-1 nor caveolin-2 is detectable in the whole-cell lysate of isolated ventricular cells, whereas caveolin-3 is detectable; rather, caveolins-1 and -2 can be detected in rat brain lysate. Furthermore, caveolin-3 protein is detectable in caveolin-rich membrane fractions obtained by sucrose gradient fraction, whereas caveolins-1 and -2 are not. Taken together, the data presented in Figures 1 and 2 suggest that caveolin-3 plays a specific functional role in the direct Gαs-mediated increase in cardiac sodium current. To further examine this hypothesis, it was necessary to demonstrate that sodium channels localize to the caveolar membrane compartment.

Isolation of Caveolin-Rich Membrane Fraction From Adult Rat Cardiac Myocytes

The isolation of caveolin-rich membranes through a detergent-free method that exploits the buoyant density of caveolae by separation on sucrose density gradient has been previously demonstrated. Myocyte lysates were separated using this discontinuous sucrose density gradient fractionation method, yielding 12 fractions (numbered 1 through 12, with sample 1 being the lightest [top] fraction), with distinct protein compartmentalization. As shown in Figure 3, a caveolin-rich fraction (samples 5 and 6, CR fraction), occurred at the 25% sucrose cushion of the gradient. Caveolin-3 could also be detected in heavier samples (samples 7 to 12), though enrichment for caveolin-3 occurred only in samples 5 and 6 when samples were compared with the whole-cell lysate. Electron microscopic examination of this CR fraction demonstrated a relatively uniform population of vesicles ranging in size from 75 to 150 nm (data not shown), by 10 μmol/L ISO (relative percent change=71.9±16.3%, n=7, P>0.05; and 76.7±25.2%, n=5, P>0.05, respectively), as was anti–caveolin-3 antibody that had been preincubated with its antigenic peptide (data not shown) (relative percent change=149.7±76.7%, n=6, P>0.05). As shown in Figure 1E, the mean percent change in I_Na in the caveolin-1 and caveolin-2 experimental groups is not significantly different from control, whereas the mean of the caveolin-3 group is (experimental groups versus control using a Dunnett comparisons test wherein significance is defined as P<0.05). Figure 2 demonstrates that neither caveolin-1 nor caveolin-2 is detectable in the whole-cell lysate of isolated ventricular cells, whereas caveolin-3 is detectable; rather, caveolins-1 and -2 can be detected in rat brain lysate. Furthermore, caveolin-3 protein is detectable in caveolin-rich membrane fractions obtained by sucrose gradient fraction, whereas caveolins-1 and -2 are not. Taken together, the data presented in Figures 1 and 2 suggest that caveolin-3 plays a specific functional role in the direct Gαs-mediated increase in cardiac sodium current. To further examine this hypothesis, it was necessary to demonstrate that sodium channels localize to the caveolar membrane compartment.
consistent with previous estimations of caveolar size in adult rat myocytes. 21

During various states of cell growth and adaptation, caveolin and channel proteins are localized to intracellular compartments including endosomes and the Golgi apparatus. Additionally, the process of lysate preparation by sonication is likely to produce vesiculation of subcellular organelle membranes. Work by other labs18,31,32 has suggested that caveolin-rich fractions obtained exclusively by the detergent-free method may contain membrane derivatives (and, therefore, membrane components) not specific to caveolae. Therefore, it was essential to confirm that our detergent-free preparation efficiently excluded potentially contaminating membranes, and selectively enriched for caveolin-rich membranes. To examine the membrane purity of the CR fraction, we performed Western blot analysis on the fractionation samples using membrane markers for plasmalemmal and subcellular organelle membranes.

 Whereas caveolin-3 enriched membranes float in samples 5 and 6 (25% sucrose) on the gradient (see Figure 3), clathrin-enriched membranes appear to settle at predominantly heavier levels of the gradient (mainly in fractions 9 through 12), suggesting the exclusion of cytoplasmic clathrin-coated pits from the CR fraction and sequestration of these vesicles (as well as those pits not yet fully dissociated from the sarcolemma) at greater sucrose concentrations corresponding to denser membrane fragments. Western analysis of our CR fraction for the presence of several other organelle and membrane compartments is shown in Figure 4. We probed CR membrane fractions and were not able to detect the following protein markers: Na\(^+/\)H\(^+\) ATPase, plasma membrane calcium ATPase (PMCA), ryanodine receptor (RyR), mannosidase II, cytochrome oxidase subunit IV (COX), Rab 5, and Rab 11. This suggests that, overall, we have been successful in obtaining an enriched caveolar fraction, as determined by Western analysis, that is not significantly contaminated by sarcolemma or subcellular organelle membranes and vesicles, including SR (RyR), Golgi apparatus (mannosidase II), mitochondria (COX), endosome (Rab 5 and Rab 11), and clathrin-coated pits. In contrast, both caveolin-3 and sodium channels are detected in the caveolar membrane fraction.

Localization of Sodium Channel to Caveolar Membrane Domains

In support of our hypothesis, we sought to localize sodium channel and G\(_\alpha\) proteins to gradient fractions rich in caveolin-3. Samples obtained by sucrose density-gradient fractionation were analyzed by Western blot and probed with anti-caveolin, SP-19 (sodium channel), and anti-G\(_\alpha\) antibodies. As shown in Figure 5, sodium channel proteins are detected in fractions 5 and 6, the caveolin-rich membrane fractions, and in fractions at the bottom of the gradient where 5, and Rab 11. This suggests that, overall, we have been successful in obtaining an enriched caveolar fraction, as determined by Western analysis, that is not significantly contaminated by sarcolemma or subcellular organelle membranes and vesicles, including SR (RyR), Golgi apparatus (mannosidase II), mitochondria (COX), endosome (Rab 5 and Rab 11), and clathrin-coated pits. In contrast, both caveolin-3 and sodium channels are detected in the caveolar membrane fraction.

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plasma membrane and T-tubule membranes rest (Figure 4). Diffuse or double bands obtained on Western blot for sodium channel proteins using SP-19 antibody reflects subtleties of channel glycosylation. Additionally, Figure 5 demonstrates that Goα proteins are distributed in the CR fraction and in fractions at the bottom of the gradient. Caveolin-3 labeling of the gradient is provided as a reference.

To confirm that the caveolin-3 and sodium channel proteins reside in the same membrane compartment, we subjected the CR fraction to immunoprecipitation with anti-caveolin-3 antibodies. As shown in Figure 6, immunoblots suggest that sodium channels localize to CR fraction membranes immunoprecipitated with anti-caveolin-3 antibody. This was not true for samples immunoprecipitated with caveolin-2 or no primary antibody. Additionally, sodium channels were coimmunoprecipitated when "heavy" membrane fractions were immunoprecipitated with antibodies to caveolin-3. This is expected in that some caveolin-3 rich membrane remains in the denser regions of the gradient following centrifugation (see Figure 5) and is presumably made up of caveolar membranes that do not shear completely from the sarcolemma during myocyte sonication. Because the PKA-independent ISO-mediated increase in Is always appears to be a result of direct Goα actions, we also examined the presence of Goα in the samples obtained by immunoprecipitation with anti-caveolin antibodies. Positive detection of Goα in these samples (Figure 6) suggests that Goα may have a physical role in mediating the ISO-induced increase in Is in rat cardiac myocytes.

As shown in Figure 7, we examined the localization of sodium channel proteins by indirect immunofluorescence. Labeling of frozen sections of rat ventricle with D492 Na+ channel antibody demonstrates the presence of sodium channel proteins throughout the cardiac sarcolemma (Figure 7A). Colabeling with antibodies to caveolin-3 demonstrates punctate areas of colocalization of the 2 proteins along the plasma membrane (Figure 7B), with the merged image shown in panel C. Concurrent experiments performed in the absence of primary antibody were negative (data not shown). With the limitations on resolution inherent in fluorescent microscopy, we further endeavored to colocalize sodium channel and caveolin-3 proteins by immunogold labeling of our CR fraction. As demonstrated in Figure 8, CR fraction membranes obtained by density gradient separation colabel with sodium channel (large, 10 nm gold particle) and caveolin-3 (small, Ag+ enhanced ultra-small gold particle) antibodies, with examples of clustering indicated by arrowheads and boxes. Sections labeled with primary antibodies preincubated with their antigenic peptide or with secondary gold-conjugated antibodies alone were negative (data not shown). This supports our hypothesis that sodium channels are localized to caveola membrane compartments where they can be presented to the plasma membrane on receptor stimulation.

Discussion
Because the direct Goα effect appears to result in an increase in the number of functional channels at the sarcolemma, we hypothesized that functional channels were being recruited from an intracellular pool, allowing for the rapid and controlled presentation of channels to the cell membrane on stimulation of the βAR. Previous work by other labs supports the localization of channel proteins to distinct membrane lipid raft compartments, including caveolae. In addition, recent work by Zhou et al demonstrates that a blocker of vesicular membrane trafficking can eliminate a PKA-mediated increase in Is in Xenopus laevis oocytes expressing the human heart sodium channel protein, hH1, by blocking the stimulated increase in plasma membrane channel number.
Western analysis of these density gradient fractions yielded vesicles cross-reactive with antibodies to caveolin-3. The detergent-free buoyant density method of caveolar membrane isolation routinely involved in the receptor-mediated presentation of ion channels at the cell membrane. Use of the detergent-free buoyant density of caveolar membranes obtained by sucrose gradient fractionation demonstrates clustering of caveolin-3 (corresponding to small beads) and sodium channel (corresponding to large beads), examples of which are indicated by arrows and boxes. Boxes indicate enlarged regions A, B, and C. Bar=0.1 μm.

In the present study, we have been able to implicate the Go protein and caveolae in the direct β-adrenergic effect on the cardiac sodium current. We demonstrate, with both biochemical and functional evidence, that caveolae may be involved in the receptor-mediated presentation of ion channels at the cell membrane. Use of the detergent-free buoyant density method of caveolar membrane isolation routinely yielded vesicles cross-reactive with antibodies to caveolin-3. Western analysis of these density gradient fractions demonstrates that both the sodium channel and Go, are associated with caveolar membranes. In contrast, membrane markers for several membrane compartments were not detectable in our caveolin-rich fraction by Western analysis, suggesting that the sodium channels present within the caveolar membrane are functionally capable of mediating the PKA-independent isoproterenol-induced increase in sodium current in the heart.

Evidence by Couet et al. has suggested a physical association between the G-protein α-subunit and caveolin-1, wherein Go proteins negatively regulate the function of caveolin-1. At the neuronal cell membrane, β-adrenergic stimulation and attendant Go activation results in a decrease in the voltage-gated sodium current (reviewed in Catterall).

In contrast, previous work by our laboratory and others has shown that stimulation of β-adrenergic receptors with ISO results in an increase in I Na in cardiac myocytes. We have demonstrated that this increase in I Na in cardiac myocytes has 2 sources: a PKA-dependent component that results in changes in both current amplitude and channel-gating (inactivation) kinetics, and a PKA-independent component that increases current amplitude without changing single-channel voltage-dependent parameters. When blocking concentrations of PKI are superfused across the cytoplasmic face of the myocyte membrane, the addition of the cAMP analog CPT-cAMP or forskolin, an adenylyl cyclase activator, fails to elicit an increase in I Na suggesting that the second component involves Go directly. This direct effect elicited by β-adrenergic stimulation with ISO can be mimicked by the addition of short Go peptide sequences and is blocked by the addition of antibodies to Go, or propranolol, but not by the addition of α-adrenergic antagonists (data not shown).

The results of the present study demonstrate that the ISO effect on I Na is also blocked by the addition of anti–caveolin-3 antibodies to the intracellular milieu. Thus, our model of caveolar function entails direct action by Go, on caveolae, resulting in the presentation of caveolar membrane components to the sarcolemma.

This work should be examined in light of its applicability to other cell types and signaling pathways. It should be noted that, although we focus on the mechanism whereby β-adrenergic stimulation regulates caveolar protein presentation at the sarcolemma, we expect that other signaling cascades via different receptors may involve separate, although similar, mechanisms. Further experiments to determine which proteins are involved in caveolar kinesis and their role, if any, in regulating receptor-mediated protein presentation at the cell membrane, are necessary. Some vesicle transport molecules, including NSF and VAMP, have already been localized to caveolae derived from endothelium. The role of these proteins or homologous proteins in muscle cells expressing caveolin-3 remains to be studied.

Finally, we employed cell isolation techniques that do not account for in vivo regional cell origins (endocardium versus myocardium versus epicardium or apex versus base). It is likely that some variability in caveolar and sodium channel density exists throughout the heart, and future studies in this field are expected to further elucidate the subtleties of caveolar regulation in different cell types and regions of the heart. In spite of these limitations, this work does provide interesting and important insight into the role of caveolae in mediating rapid, reversible, and reproducible membrane events that can affect overall cell excitability by regulating plasma membrane protein presentation.
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**References**


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Expanded Methods

Myocyte Isolation. Adult rat cardiac myocytes were obtained as outlined previously\(^1\), and as approved by the Animal Care and Use Committee at the University of Iowa. Briefly, thoracotomy was performed on 50-70 day-old Sprague-Dawley male rats anesthetized with methoxyflurane or isoflurane. The heart was excised and retrogradely perfused for 4 minutes via the aorta on a modified Langendorff apparatus. The perfusing solution was nominally calcium-free modified Tyrode’s solution that contained, in mmol/L: 138 NaCl, 5.4 KCl, 0.5 MgCl\(_2\), 0.33 Na\(_2\)HPO\(_4\), 5.5 HEPES, 5.5 dextrose, and 1mg/ml albumin; pH 7.38 with NaOH. Following this washout, the heart was then perfused for 7-9 minutes at 37\(^\circ\)C with nominally calcium-free Tyrode’s solution to which 0.25-0.3 mg/ml collagenase was added. Perfusion time and collagenase concentrations were a function of the size and age of the animal. After perfusion, hearts were mechanically dissociated by mincing the tissue with scissors, and cells were centrifuged and washed in KB solution containing, in mmol/L: 70 KOH, 40 KCl, 1 K\(_2\)HPO\(_4\), 0.5 MgCl\(_2\), 50 L-glutamic acid, 20 taurine, 0.5 EGTA, 10 HEPES, 5 creatine, 5 Na\(_2\)ATP, 10 dextrose, and 5 pyruvic acid; pH 7.38 with KOH.

Isolation of Caveolin-rich Fraction. Isolation of caveolin-rich fraction was done using a detergent-free method largely as described previously\(^2\), with some modifications. All procedures were carried out at 4\(^\circ\)C. After a final wash in KB solution, the rat cardiac
myocyte pellet was resuspended in 2.0 ml of 500 mmol/L Na₂CO₃ plus 0.25 protease inhibitor cocktail tablet (Boehringer Mannheim). Approximately 500 x 10³ myocytes were sonicated (3 times for 20 s using a Fisher Scientific 550 Sonic Dismembrator), and the lysate was transferred to a centrifuge tube for discontinuous sucrose density gradient separation using sucrose cushions of 5-45% (5%, 15%, 25%, 35%, and 45% w/v). The gradient was centrifuged in a Beckman SW41 rotor at 39,000 rpm for 20 hours, and twelve 1 ml-samples were collected, starting from the top of the gradient. We routinely obtained a “caveolin-rich fraction” (samples 5 and 6, constituting the 25% sucrose cushion in the density gradient). For comparison, a “heavy fraction” (sample 11) was collected. To removed soluble proteins and pellet membranes, the caveolin-rich fraction (and, similarly, the heavy fraction) was then washed in MBS (containing, in mmol/L: 25 Mes, 150 NaCl; pH 6.5) and centrifuged in the SW41 rotor at 40,000 rpm for 2 hours. The pellet was then solubilized in Thorner buffer (4%SDS; 50mmol/L Tris; 8 mol/L urea; pH 6.8 with NaOH) for use in protein assays and Western blotting. Protein determinations were done using the copper/bicinchoninic acid assay (Pierce assay) from Sigma.

**Antibodies.** Antibodies used were obtained as follows. Anti-cardiac ryanodine receptor (RyR)³ was the kind gift of Dr. Kevin P. Campbell. Monoclonal antibodies to caveolin-3⁴, caveolin-2⁵, clathrin⁶, Rab 5⁷, and Rab 11⁸, and secondary HRP-conjugated anti-mouse and anti-rabbit antibodies were obtained from Transduction Laboratories. Polyclonal antibodies to caveolin-1⁹ and Go₁₀ were obtained from Santa Cruz Biotechnology. Antibodies to subunit 2 of cytochrome oxidase (COX)¹¹, the plasma
membrane calcium ATPase (PMCA)\textsuperscript{12}, and mannosidase II\textsuperscript{13} were obtained from Molecular Probes, Affinity Bioreagents, and Covance, respectively. Subtype-specific antibodies\textsuperscript{14,15} to the $\alpha_1$ and $\alpha_2$ isoforms of the Na$^+$/K$^+$ ATPase were a kind gift from Dr. Kathleen J. Sweadner, and antibodies to both the $\alpha_1$ and $\alpha_2$ isoforms were purchased from Upstate Biotechnology. Anti-SP-19 (pan) sodium channel antibody\textsuperscript{16} used for Western blot analysis and secondary HRP-conjugated anti-goat antibodies were obtained from Sigma. D492 polyclonal antibody used for immunofluorescence and immunogold electron microscopy was raised in goat to a 22-amino acid peptide corresponding to a sequence (DRLPKSDSEDGPRALNQLSLC) located in interdomain loop I-II of the cardiac voltage-gated sodium channel as similarly described by Cohen and Levitt (1993)\textsuperscript{17}.

**Immunoblotting.** Samples were precipitated using the pyrogallol red-molybdate method as described previously for SDS-PAGE\textsuperscript{18}. Precipitated samples were heated for six minutes at 100°C in SDS-polyacrylamide electrophoresis buffer containing $\beta$-mercaptoethanol. Samples were then run on 7.5-12% polyacrylamide gel at 200 V for 45 minutes, and proteins were transferred to 0.45 $\mu$M nitrocellulose membrane by application of 0.3 mA current for 2 hours. Membranes were blocked for 25 minutes in TBS-T containing, in mmol/L: 50 Tris, 150 NaCl, 3.8 HCl, 0.05% Tween-20, pH 7.6; with 5% nonfat dry milk. The membranes were incubated with primary antibody at 4°C overnight, washed twice in TBS-T, and incubated for 2 hours at room temperature in the presence of HRP-conjugated secondary antibody. To remove excess antibody,
membranes were then washed 5 times in TBS-T. Detection was performed using enhanced chemiluminescence substrates from Amersham Pharmacia Biotech.

**Immunoprecipitation.** Enzymatically isolated adult rat ventricular cells were lysed by sonication and membranes were separated on sucrose gradient cushion as described in *Isolation of caveolin-rich fraction* above. Caveolin-rich fractions (gradient fractions 5 and 6, hereafter called “CR fraction”) and “heavy” fractions (gradient fraction 11) obtained in this way were resuspended in buffer consisting of, in mmol/L: 150 NaCl, 5 EDTA, 1 phenylmethylsulfonyl fluoride (PMSF), 25 Mes; pH 6.7 with NaOH. To this was added anti-caveolin-3 monoclonal antibody (1:500) for incubation at 4°C overnight, followed by incubation with Protein G-agarose beads overnight (16 hours) at 4°C. For negative controls, fractions were incubated with anti-caveolin-2 (1:500) or no primary antibody overnight. Samples were then treated similarly to those incubated with anti-caveolin-3. Following incubation, the membrane-antibody-bead complexes were washed five times with Buffer A containing, in mmol/L: 150 NaCl, 50 Tris-HCl, 1 EDTA, 0.5% (w/v) Triton X-100. Following the final wash, the fraction was centrifuged and the supernatant was collected. 1X Sample Buffer was added to the bead complexes, which were then boiled, and the supernatant run on polyacrylamide gel for Western blotting.

**Immunofluorescence.** Adult rat ventricles were fixed in 4% paraformaldehyde solution, infused with 30% sucrose solution for 2 hours, then frozen in liquid N₂, and sectioned using a cryostat. Sections collected on glass slides were incubated with primary antibodies for one hour at room temperature, washed with PBS, and then incubated with
fluorescent-conjugate secondary antibodies for one hour at room temperature. Sections were then mounted and visualized on a Zeiss Laser Scanning Confocal Microscope. Primary antibodies were diluted as follows: anti-caveolin-3 1:50; D492 1:200. Immunofluorescent conjugates were diluted at 1:200.

**Immunogold labeling and electron microscopy.** CR fractions were fixed in 2% paraformaldehyde/0.5% glutaraldehyde solution and labeled prior to embedment in Eponate 12. Embedded capsules were sectioned with an ultramicrotome, with sections collected on copper grids. Grids were incubated overnight at 4°C in primary antibodies to the cardiac sodium channel (D492) and caveolin-3. After buffer washes, grids were then incubated in secondary gold-conjugate for 2 hours at room temperature. Samples were washed, fixed in 2.5% glutaraldehyde, and silver-enhanced. Finally, counterstaining with uranyl acetate and lead citrate allowed for visualization with an H-7000 transmission electron microscope. Primary antibodies were diluted as follows: anti-caveolin-3 1:20; D492 1:100. Secondary gold-conjugates were diluted as follows: goat anti-mouse ultra-small gold conjugate 1:40; rabbit anti-goat 10nm gold-conjugate 1:40.

**Electrophysiological protocols and data analysis.** Voltage pulse protocols were generated and data was recorded using pCLAMP6.04 software and a personal computer interfaced with an Axopatch 200 integrating patch-clamp amplifier (software and amplifier from Axon Instruments). Solution-filled (for composition see below) pipette resistances ranged from 0.5 to 1 MΩ and seal resistances were 20-50 GΩ. The signal
was filtered at 5 kHz, and digitized at a sampling rate of 50 kHz. Whole-cell series resistance was compensated to 80-85% of the uncompensated value. Due to spontaneous shifts in the current inactivation-voltage relationship after establishing the whole-cell configuration, $I_{Na}$ data were recorded only after the currents became stable (usually within 5 minutes). All experiments were performed at 21-23°C to help maintain voltage-clamp control.

Whole-cell voltage clamp data was generated using test pulses wherein cell membrane potential was held at –100 mV and then stepped to –40 mV for 20 ms, after which it was returned to its resting level of –100 mV, with an interepisode interval of 1 s. Pipette solutions contained, in mmol/L: 130 CsCl, 0.5 CaCl$_2$, 2 MgCl$_2$, 5 Na$_2$ATP, 0.5 GTP, 5 EGTA, 10 HEPES, 0.022 mg/ml protein kinase A inhibitor (PKI); pH 7.25 with KOH/HCl. Bath solution contained, in mmol/L: 130 choline chloride, 20 NaCl, 0.5 KCl, 1 CaCl$_2$, 2 CoCl$_2$, 10 HEPES, 5.5 dextrose; pH 7.38 with CsOH. In experiments with anti-caveolin antibody, 100 µg/mL anti-caveolin –1, -2 or -3 antibody was added to the pipette solution. In the corresponding control experiments, no antibody was added to the pipette solution. Additional experiments were also performed in which 100 µg/ml anti-caveolin-3 antibody + 100 µg/ml caveolin-3 antigenic peptide were added to the pipette solution. Raw data are plotted as $I_{Na}$ relative to baseline $I_{Na}$ (sodium current prior to bath addition of isoproterenol). Histogram data are presented as the mean ± S.E.M. One-way analysis of variance (ANOVA) was used to compare the ISO-stimulated percent change in $I_{Na}$ among the control and experimental groups (populations in which antibodies against caveolins -1, -2, or -3 were added to the pipette solution). Statistical significance is defined as p<0.05, with n=number of experiments as indicated. In addition, Dunnett
multiple comparisons test was performed to evaluate difference between each experimental group versus control.
References


