Localize of Pacemaker Channels in Lipid Rafts Regulates Channel Kinetics

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Abstract—Lipid rafts are discrete membrane subdomains rich in sphingolipids and cholesterol. In ventricular myocytes a function of caveolae, a type of lipid rafts, is to concentrate in close proximity several proteins of the β-adrenergic transduction pathway. We have investigated the subcellular localization of HCN4 channels expressed in HEK cells and studied the effects of such localization on the properties of pacemaker channels in HEK and rabbit sinoatrial (SAN) cells. We used a discontinuous sucrose gradient and Western blot analysis to detect HCN4 proteins in HEK and in SAN cells, and found that HCN4 proteins localize to low-density membrane fractions together with flotillin (HEK) or caveolin-3 (SAN), structural proteins of caveolae. Lipid raft disruption by cell incubation with methyl-β-cyclodextrin (MβCD) impaired specific HCN4 localization. It also shifted the midpoint of activation of the HCN4 current in HEK cells and of Ii in SAN cells to the positive direction by 11.9 and 10.4 mV, respectively. These latter effects were not due to elevation of basal cyclic nucleotide levels because the cholesterol-depletion treatment did not alter the current response to cyclic nucleotides. In accordance with an increased Ii, MβCD-treated SAN cells showed large increases of diastolic depolarization slope (87%) and rate (58%). We also found that the kinetics of HCN4- and native f-channel deactivation were slower after lipid raft disorganization. In conclusion, our work indicates that pacemaker channels localize to lipid rafts and that disruption of lipid rafts causes channels to redistribute within the membrane and modifies their kinetic properties. (Circ Res. 2004;94:1325-1331.)

Key Words: HCN channels ■ pacemaker current ■ lipid rafts ■ caveolin ■ sinoatrial node

The sinoatrial node (SAN) is the region of the heart from which spontaneous action potentials originate and propagate to determine cardiac rhythm. This particular anatomical district is composed by specialized myocytes (pacemaker cells) whose activity is characterized by a slow diastolic depolarization phase at the end of the action potential. The pacemaker (Ii) current plays a key role in the generation of diastolic depolarization. f-Channels open toward the end of the action potential repolarization process and carry an inward current that depolarizes the membrane and drives the membrane potential up to threshold for initiating a new action potential. Although spontaneous activity is an intrinsic property of the heart, independent of innervation, fine modulation of heart rate is achieved through the release of the neurotransmitters norepinephrine (NE) and acetylcholine (ACh) by sympathetic and parasympathetic branches of the autonomic nervous system. It is well established that in cardiac cells NE and ACh, through specific β-adrenergic (β-AR) and muscarinic (mAChR) receptors, modulate intracellular level of cAMP. Direct binding of cAMP to f-channels shifts their activation curve toward more depolarized potentials, thus increasing the steepness of the diastolic depolarization.1 cAMP is a widespread second messenger, which affects a number of intracellular processes. For this reason, a broad, generalized increase or decrease in cytoplasmic cAMP concentration would be wasteful for the cell. One way to avoid an indiscriminate increase in cAMP concentration is through localization of the factors involved in its synthesis and degradation in discrete membrane subdomains such as the lipid rafts. The lipidic composition of these structures is different from the rest of the membrane: lipid rafts2 are rich in sphingolipids and cholesterol packed together, which results in a less permeable and less fluid environment.3 Cholesterol-binding agents like cyclodextrins can deplete cells of membrane cholesterol and thus disrupt lipid rafts and reorganize the spatial distribution of associated proteins.4–6 Caveolae represent a morphologically identifiable type of lipid rafts and are described as stable flask-like membrane invaginations.2 It has been recently shown that several elements of the β-AR pathway are localized to caveolae in cardiac myocytes, cardiac fibroblasts, and HEK293 cells.5 Because SAN myocytes are rich in caveolae,7,8 and because f-channels, and their molecular correlates HCN channels, are finely regulated by β-AR and mACHR activation, we asked...
whether these channels, too, are localized to discrete membrane microenvironments such as the lipid rafts. To this aim, we investigated the subcellular localization of HCN channels heterologously expressed in HEK293 cells and studied the functional effects of disrupting lipid rafts on the properties of HCN channels in HEK293 cells and of native f-channels in isolated SAN myocytes.

**Materials and Methods**

**Isolation of Rabbit SAN Cells**

All the procedures adopted in this work conformed to guidelines for the care and use of laboratory animals as established by State (D.L. 116/1992) and European directives (86/609/CEE). We used a standard protocol to isolate rabbit SAN cells. Briefly, animals weighing 0.8 to 1 kg were anesthetized by intramuscular injection of xylazine 4.6 mg/kg and ketamine 60 mg/kg and euthanized by cervical dislocation and exsanguination. Hearts were removed and kept at 37°C in Tyrode solution (in mmol/L: 140 NaCl, 5.4 KCl, 1.8 CaCl₂, 5.5 d-glucose, and 5 HEPES-NaOH; pH 7.4). After dissection, myocytes were digested and isolated by enzymatic and mechanical Trituration.

**Cell Culture and Transfection**

Modified HEK293 cells (Phoenix cells, referred to as HEK cells) were kept in a culture medium (D-MEM, Gibco) containing FBS 10%, (Gibco) and PenStrep solution 1% (Sigma) at 37°C in a 5% CO₂ incubator. Cells were transiently transfected according to a standard calcium phosphate protocol. For biochemistry, 24 µg of rabbit HCN4 (rbHCN4) cDNA was used for each 100-mm Petri dish. For electrophysiology, 4 µg of rbHCN4 cDNA and 2 µg of CD8 cDNA were cotransfected in 35-mm Petri dishes. Two to 3 days after transfection cells were selected by immunoreactivity with CD8 antibody-covered beads (Dynabeads).

**Cholesterol Depletion**

Membrane cholesterol depletion was achieved by methyl-β-cyclodextrin (MβCD, Sigma) treatment (see the expanded Materials and Methods section in the online data supplement at http://circres.ahajournals.org).

**Lipid Raft Isolation and Western Blot**

Isolation of lipid raft–enriched membranes from either HEK cells or SAN tissue was performed according to a detergent-free method, as described previously. Samples were solubilized in 1× SDS-PAGE buffer, separated by 10% acrylamide SDS-PAGE (12% for SAN tissue), and transferred overnight to nitrocellulose. Nitrocellulose was cut transversely for incubation with anti-HCN4 primary antibody for caveolin-free HEK cells (1:250, Transduction Labs) and transferred overnight to nitrocellulose. Nitrocellulose buffer, separated by 10% acrylamide SDS-PAGE (12% for SAN preparations) was used for each 100-mm Petri dish. For electrophysiology, 4 µg of rbHCN4 cDNA and 2 µg of CD8 cDNA were cotransfected in 35-mm Petri dishes. Two to 3 days after transfection cells were selected by immunoreactivity with CD8 antibody-covered beads (Dynabeads).

**Electrophysiology and Data Analysis**

Methods for electrophysiology and data analysis are described as in the expanded Materials and Methods. Statistical analysis was performed by t test comparison of paired or unpaired data as appropriate. Significance level was set to P=0.05.
By its cholesterol-depleting action, M\(\beta\)CD (squares). Individual curves were curves of cholesterol-depleted (circles) and control cells (bar), scaled to maximal amplitude. A, right, Current activation

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significantly: at mean density of HCN4 current expression did not change with 1% M\(\beta\)CD treatment. Statistical analysis of individual cell data yielded significant differences for \(V_{1/2}\) values in A and for deactivation time constants in B (see text for details).

In Figure 1B, HCN4-expressing HEK cells were treated with 1% M\(\beta\)CD before running the sucrose gradient protocol. By its cholesterol-depleting action, M\(\beta\)CD disorganizes lipid rafts and associated proteins. As a confirmation that this procedure effectively led to disruption of lipid rafts, the flotillin signal in Figure 1B was detected in all the membrane fractions. Under these conditions, the HCN4 signal, too, was present in the high- as well as in the low-density fractions, confirming the hypothesis that HCN4 proteins are compartmentalized in lipid rafts.

We next asked if the specific localization of HCN4 channels could affect their properties. To address this question, we recorded the current induced by HCN4 expression in HEK cells before and after treatment with 1% M\(\beta\)CD. The mean density of HCN4 current expression did not change significantly: at \(-135\) mV, it was 44.5±14.5 and 37.1±6.7 pA/pF in control (n=6) and M\(\beta\)CD-treated cells (n=4), respectively (not significantly different). In Figure 2A, mean activation curves for HCN4 current were measured (see online data supplement) and plotted for untreated (squares, n=7) and for treated cells (circles, n=4).

Treatment with the cholesterol-depleting agent shifted the current activation curve to more positive voltages. Mean values of half-activation voltage and inverse slope factor were \(V_{1/2}=-93.1\pm2.5\) and \(-81.2\pm3.6\) mV (significantly different), and \(s=9.9\pm0.7\) and 12.0±0.4 mV for untreated (n=7) and M\(\beta\)CD-treated cells (n=4), respectively (not significantly different). We also found that the M\(\beta\)CD-treatment altered channel kinetics because current deactivation was strongly slowed, as shown in Figure 2B (open symbols; see also deactivation records at \(-55\) mV in the left panels in Figure 2B). At voltages in the range \(-75/-35\) mV, the deactivation time constants of M\(\beta\)CD-treated cells increased 1.9- to 2.6-fold relative to untreated cells (significantly different). Time constants of activation were slightly modified at negative potentials (Figure 2B, filled symbols), but changes were not statistically significant.

These results suggest that heterologously expressed channels are located in lipid rafts and that this location affects their kinetic properties. This prompted us to investigate whether a similar mechanism could also be operating for native f-channels in SAN cells.

We first applied the discontinuous sucrose gradient protocol to SAN membranes to verify if pacemaker channels and lipid rafts colocalize. SAN myocytes are rich in caveolae, a type of lipid raft containing caveolin and characterized by flask-shaped membrane invaginations. We therefore choose caveolin-3 to identify lipid raft–enriched membranes. In Figure 3, Western blots of SAN proteins precipitated from the various fractions of the gradient show that both HCN4 and caveolin-3 localize to the lipid raft–enriched membrane fractions 4-5 and 6-7.

We then measured the \(I_{f}\) current from isolated SAN myocytes before and after treatment with M\(\beta\)CD (2%). M\(\beta\)CD treatment did not modify the \(I_{f}\) current density, which was \(-23.0\pm1.4\) and \(-26.1\pm3.8\) pA/pF at \(-115\) mV in untreated (n=14) and M\(\beta\)CD-treated cells (n=13), respectively (not significantly different).

In Figure 4, mean activation curves of \(I_{f}\) current measured in untreated (squares) and treated SAN cells (circles) are shown. In cholesterol-depleted cells, the activation curve was shifted toward more depolarized voltages relative to untreated cells, as found in HEK cells (Figure 2A). The mean half-activation voltage (\(V_{1/2}\)) was \(-70.1\pm1.0\) mV (n=24) in untreated cells and \(-59.7\pm1.7\) mV (n=21) in M\(\beta\)CD-treated cells (statistically significant); the inverse slope factor(s) did
Disruption of caveolae is known to alter the activity of adenylate-cyclase and guanylate-cyclase. In endothelial cells, for example, interaction between caveolin-1 and eNOS (endothelial nitric oxide synthase) is inhibitory on the activity of the enzyme and disruption of caveolae has been reported to increase basal levels of NO, which in turn activates guanylate cyclase. The subsequent increase in cytosolic cGMP concentration could therefore modulate f-channel by binding to its cyclic nucleotide-binding domain and/or indirectly through inhibition of phosphodiesterases. Disorganization of caveolae by 2-hydroxipropyl-Î±-cyclodextrin has also been reported to lead to an increase in isoproterenol-, zinterol-, and forskolin-induced cAMP elevation in cardiac myocytes.

We therefore checked whether the positive shift of the activation curve could not result from an increased concentration of intracellular nucleotides. To this aim, we recorded I_f from SAN cells and analyzed the action on I_f of forskolin and of SNP, which are known to stimulate adenylate cyclase and guanylate-cyclase. A positive shift of activation curve due to elevation of intracellular cAMP and cGMP, respectively.

In Figure 5A, hyperpolarizing steps were applied to near the midactivation voltage and I_f recorded before and during perfusion with either forskolin (50 µmol/L, left) or SNP (100 µmol/L, right) or SNP (100 µmol/L, right) in untreated (top) and 2% MβCD-treated cells (bottom). Shifts of the I_f activation curve causing the current increase were measured as previously reported (see Materials and Methods), averaged, and plotted in the bar graphs of Figure 5B. In neither case did the cholesterol-depleting treatment cause a significant change in responsiveness to the activating agents. Mean shifts caused by forskolin were 6.4±0.7 (n=6) and 6.1±0.1 mV (n=3), and those caused by SNP were 6±1.3 (n=4) and 6.2±0.9 mV (n=6) in untreated and MβCD-treated cells, respectively. These data rule against the view that the cholesterol-depleting maneuver induces a positive shift of the I_f activation curve by increasing basal levels of cyclic nucleotides.

Increase of I_f, such as the one induced by ßAR-mediated depolarization shift of the activation curve, leads to acceleration of spontaneous rate. To see if the I_f changes caused by the cholesterol-depleting procedure were accompanied by significant changes in rate, we measured activity from free-beating SAN myocytes. Figure 6A shows representative action potentials recorded from a control myocyte (top) and from a cell treated with MβCD (2%) (bottom). The rate was about 58% faster after exposure to MβCD (277±25 bpm, n=5) than in untreated cells (175±16 bpm, n=7, significantly different) (Figure 6C). The rate acceleration was due mostly to a steeper slope of the diastolic depolarization, although the action potential dura-
In control cells to 1336 ms in treated cells. It should be noted that this change is opposite to that caused by treatment with MβCD alone (see Figures 2B and 7). Treatment with MβCD/cholesterol did not affect spontaneous activity; spontaneous rate was 189±31 and 170±12 bpm, and diastolic depolarization rate was 110.0±13.7 and 87.6±13.4 V/s in day-matched control (n=3) and treated cells (n=3), respectively (not significantly different). These data agree with the hypothesis that treatment with MβCD causes membrane cholesterol depletion and consequent disassembly of lipid rafts, and that this effect is responsible for modification of I₁ kinetics.

In SAN myocytes, I₁ activation during the last fraction of action potential repolarization is a key process in the generation of the diastolic depolarization and spontaneous activity. The role of I₁ in pacemaking is well established. 22–24 f-Channels play an essential role not only in the generation of pacemaker activity, but also, importantly, in the control of pacemaker rate by autonomic neurotransmitters. This mechanism is based on the modulation of intracellular cAMP by the opposite action of β-adrenergic and muscarinic cholinergic stimuli. 1, 9, 20, 25

f-Channels are activated by direct binding of cAMP molecules to channels. 1 This property likely reflects a need for fast channel modulation. This is not surprising, because the ability to respond quickly to autonomic stimulation represents an aspect of cardiac pacemaking that is of basic physiological relevance.

Fast, efficient responses to neurotransmitter-induced modulation mediated by cAMP require not only that the second
There is evidence for restricted pools of cAMP functional to the elements contributing to the modulatory mechanism are confined to a restricted space and are sufficiently close to each other.

Indeed, the notion of restricted localization of components of the cAMP-dependent biochemical pathway has been known for some time, and in working cardiac myocytes there is evidence for restricted pools of cAMP functional to β-AR modulation of Ca2+ channels and involved in contraction.

It has been recently shown that molecules involved in β-AR signaling cascade in cardiac myocytes localize to caveolar lipid rafts. SAN cells have a large density of caveolae on their plasma membranes, which increase the surface area by about 100%, whereas in ventricular myocytes, the increase in surface due to caveolae is estimated to be about 30%. As in ventricular myocytes, caveolar rafts in SAN myocytes may therefore have a role in recruiting sets of proteins involved in specific membrane processes.

In this study, we have shown that HCN4 channels expressed in HEK cells or in SAN cells are localized to lipid rafts and that disruption of these membrane subdomains by cholesterol depletion affects cellular compartmentation of channels and their kinetic properties. In SAN cells, whose membranes are characterized by a high density of caveolar rafts according to morphological data, our data point to a caveolar localization of native f-channels.

Western blot analysis showed that HCN4 proteins are normally present exclusively in the tighter, raft-enriched fraction (first two lanes in Figure 1A). Treatment of HEK cells with the cholesterol-binding agent MβCD caused flotillin to lose its specific localization to lipid raft–enriched membranes. Under these conditions, HCN4 proteins were also distributed nonspecifically throughout all density fractions (Figure 1B), indicating that HCN4 channels are associated to lipid rafts.

Disruption of lipid rafts also affected HCN4- and f-channels kinetics. After MβCD, Ii and HCN4-induced currents activated at more depolarized voltages than in untreated cells (Figures 2 and 4). We found that both forskolin and SNP are as effective on Ii in cells treated with MβCD as they are in control cells (Figure 5), indicating that the MβCD-induced shift of the current activation curve was not due to increased basal levels of either cAMP or cGMP.

Because a rightward shift of Ii activation is responsible for the positive chronotropic action of sympathetic innervation, cholesterol depletion should affect spontaneous beating rate of isolated SAN myocytes. Indeed, MβCD-treated cells showed a much faster diastolic depolarization and beating rates than control cells (Figure 6).

As well as shifting the current activation curve, the cholesterol-depleting procedure slowed deactivation kinetics of both HCN4 current and Ii, without modifying appreciably activation (Figures 2 and 7). We do not have a ready explanation for this effect. It should be noted however that unequal modifications of activation and deactivation kinetics are known to occur for Ii. For example, the cAMP-induced shift of the Ii activation curve occurs in association with a shifting action that is stronger for deactivation than for activation time constants, suggesting that a decreased rate of channel closing, more than an increased rate of channel opening, contributes to the cAMP action. Furthermore, deactivation, but not activation of HCN2 channels, is slowed when external K+ is replaced by Na+, Li+, or NMG+.

Because changes in the concentration of cyclic-nucleotides are not responsible for the modification of HCN4/Ii kinetics, our results raise the question whether other channel modulating mechanisms may exist whose action depends on the integrity of lipid rafts.

A most natural candidate could be the MiRP1 protein (KCNE2). If MiRP1, acting as a β-subunit, modulates channel activity, then disruption of the lipid raft environment might impair its action. However, MiRP1 has a strong, nearly 2-fold slowing effect on HCN4 activation, and impairment of MiRP1 action should therefore lead to a nearly 2-fold acceleration of channel kinetics, which is not compatible with the results we have obtained with MβCD treatment (Figure 2); secondly, we did not observe significant changes in HCN4 current density after MβCD, whereas roughly a 50% reduction should be expected if MiRP1 is involved. These results indicate that MiRP1 is unlikely to be responsible for the slowing of deactivation seen on disruption of lipid rafts.

Regardless of the detailed mode of action, our data indicate the presence of an interaction between channels and lipid raft integrity that affects channel kinetics; this interaction appears to function essentially by accelerating channel deactivation.

Although our data indicate an important contribution of Ii to MβCD-dependent changes of the rate of spontaneous activity, modifications of other currents or processes (such as Ca2+ homeostasis) cannot be ruled out. For example, it is known that Kv1.5 and Kv2.1 channels normally reside in lipid rafts both in native tissues and in heterologous expression systems, and that disruption of such structures alter their properties. Furthermore, it has been reported that membrane cholesterol depletion alters the properties of the Na+-K+ pump in erythrocytes.

Physiological β-AR modulation of rate in the SAN is thought to be mediated mostly by β1-adrenergic receptors, particularly in view of the much larger density of β1 than of β2 receptors, although β2 stimulation also contributes to rate modulation in several species. In ventricular cells, the largest fraction of caveolar β-ARs belongs to the β1 subtype, and if a similar situation applied to SAN cells, our data would appear to suggest a preferential coupling of pacemaker channels to β1 receptors. However, the density of β2, as well as of β1 receptors, is reported to be several-fold higher in nodal than in surrounding atrial tissue, implying that the relative distribution of the two subtypes may be tissue-specific. The exact distribution of β-AR subtypes in SAN cell membranes requires further detailed investigation.

In conclusion, our data in HEK and SAN cells indicate that pacemaker channels are located in lipid rafts, and that disruption of the interaction between channels and lipid rafts alters the kinetic properties of channels. Finally, concentration within lipid rafts agrees with evidence collected by single-channel experiments, indicating that f-channels are densely packed in restricted areas (hot-spots), rather than
being uniformly spread across the whole surface of SAN membranes.41

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