Differential Targeting of Shaker-like Potassium Channels to Lipid Rafts

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Ion channel targeting within neuronal and muscle membranes is an important determinant of electrical excitability. Recent evidence suggests that there exists within the membrane specialized microdomains commonly referred to as lipid rafts. These domains are enriched in cholesterol and sphingolipids and concentrate a number of signal transduction proteins such as nicotinic acetylcholine, ligand-gated receptors, and multiple protein kinases. Here, we demonstrate that the voltage-gated K⁺ channel Kv2.1, but not Kv4.2, targets to lipid rafts in both heterologous expression systems and rat brain. The Kv2.1 association with lipid rafts does not appear to involve caveolin. Depletion of cellular cholesterol alters the buoyancy of the Kv2.1 associated rafts and shifts the midpoint of Kv2.1 inactivation by nearly 40 mV without affecting peak current density or channel activation. The differential targeting of Kv channels to lipid rafts represents a novel mechanism both for the subcellular sorting of K⁺ channels to regions of the membrane rich in signaling complexes and for modulating channel properties via alterations in lipid content.

The subcellular localization of ion channels is necessary for proper electrical signaling. In cardiac and skeletal myocytes, ion channels show a differential surface distribution (1, 2). Within the brain, voltage-gated K⁺ (Kv) channels often show not only polarized sorting to either axons or dendrites, but also isoform-specific localization within dendrites alone. Thus, there exists specific sorting mechanisms for restricting lateral distribution within a given membrane domain (3). One physiological consequence for such specific localization is that it places various signal transduction molecules near their ion channel substrates (4). Several families of intracellular proteins, PDZs and AKAPs, have been shown to cluster both ion channels and modulatory signaling enzymes. Indeed, great emphasis has been placed on the role of PDZ proteins such as PSD-95 in the targeting and localization of ion channels and neurotransmitter receptors (5). In contrast, the role of membrane lipids in differential targeting and integration of ion channels within the plane of the plasma membrane has not been addressed.

Recent advances in the study of cell membrane structure have led to the emerging idea that microdomains exist within the fluid bilayer of the plasma membrane. These dynamic structures, termed lipid rafts, are rich in tightly packed sphingolipids and cholesterol (6). The rafts, which are present in both excitable and non-excitable cells, localize a number of membrane proteins, including multiple signal transduction molecules, while excluding others (7). Different types of rafts are likely to exist based on the presence of specific marker proteins and ultrastructure data (8). Caveolae represent one well studied subpopulation of lipid raft having an invaginated morphology and containing the scaffolding protein, caveolin, which interacts directly with several intracellular proteins (7, 9–12). Here, we demonstrate that Kv2.1 K⁺ channels target to a non-caveolar lipid raft in both transfected cells and brain, whereas the Kv4.2 channel does not. In addition, cholesterol depletion dramatically alters Kv2.1 inactivation, while having no effect on Kv4.2. Thus, lipid raft association represents a new targeting mechanism for Kv channel localization that is based on protein-lipid interactions.

EXPERIMENTAL PROCEDURES

Materials—Anti-actin and anti-tubulin antibodies were purchased from Sigma. The anti-rat Na,K-ATPase β1 subunit antibody and the polyclonal anti-Kv2.1 antibody were purchased from Upstate Biotechnology (Lake Placid, NY). Anti-caveolin polyclonal antibody, which recognizes caveolin isoforms 1, 2, and 3, was obtained from Transduction Laboratories (Lexington, KY). Saponin and 2-hydroxypropyl-β-cyclodextrin were purchased from Sigma.

Raft Isolation—Low density, Triton-insoluble complexes were isolated as described by Lisanti and co-workers (13) from mouse 1-cells stably expressing either rat Kv2.1 or Kv4.2 channels (14, 15). Briefly, cells from 10 100-mm near confluent culture dishes were homogenized in 1 ml of 1% Triton X-100 and sucrose added to a final concentration of 40%. A 5–30% linear sucrose gradient was layered on top of this detergent extract followed by ultracentrifugation (39,000 rpm) for 18–20 h at 4 °C in a Beckman SW41 rotor. Gradient fractions (600 μl) were collected from the top and analyzed by Western blot. Rat brain raft isolation used one brain (approximately 0.6 g) homogenized in 10 ml Mes-buffed saline (25 mmol/liter Mes, pH 6.5, 0.15 mol/liter NaCl) containing 1% Triton X-100, 1 mmol/liter phenylmethylsulfonyl fluoride, 2 μg/ml pepstatin, and leupeptin using 18 strokes of a loose fitting dounce homogenizer. This homogenate was then centrifuged at 3000 rpm, 4 °C for 15 min to sediment debris. The supernatant was processed as described above (13). The detergent-free raft isolations were performed as described previously (16). Basically, this protocol introduces a sonication step to disrupt the cellular membranes followed by subcellular fractionation. A discontinuous 5–35% sucrose gradient in a buffer containing sodium carbonate, pH 11.0. Triton solubility experiments were performed on cells homogenized in Mes-buffered saline containing 1% Triton X-100 with or without a 0.5% saponin pretreatment for 30 min at 4 °C. The detergent-soluble (lysat) and insoluble fractions (pellet) were separated by centrifugation (14,000 rpm, for 30 min at 4 °C) and analyzed by immunoblotting.

Immunostaining—Immunostaining of cells was performed using anti-Kv2.1 polyclonal antibodies and/or anti-caveolin antibodies as described previously (1). Demecolcine (0.1 μg/ml; Sigma) was dissolved in the culture medium. The bound anti-caveolin antibody was detected with BODIPY-conjugated streptavidin (green), and the Kv2.1 antibody was detected with CY3-conjugated streptavidin (red). Fluorescent sig-
Kv4.2, endogenous Na\textsuperscript{+} expressing Kv channel protein were analyzed by Western blot. The detergent-based isolation of lipid rafts. Sucrose density gradient centrifugation of insoluble protein in the pellet (P) were analyzed by Western blot. Triton X-100 and subjected to centrifugation as described under “Experimental Procedures.” Detergent-soluble protein in the lystate (L) and insoluble protein in the pellet (P) were analyzed by Western blot. C, detergent-based isolation of lipid rafts. Sucrose density gradient centrifugation of 1% Triton X-100-solubilized extracts from cells stably expressing Kv channel protein were analyzed by Western blot. The immunoblots show low density, raft-associated distribution of Kv2.1 and caveolin in contrast to the high density, non-floating distribution of Kv4.2, endogenous Na\textsuperscript{+}/K\textsuperscript{+} ATPase, actin, and tubulin. D, detergent-free raft isolation with cells stably expressing Kv2.1. Both Kv2.1 and caveolin are found exclusively in the low density, raft-associated fractions.

RESULTS AND DISCUSSION

**Kv2.1, but Not Kv4.2, Is Localized to Lipid Rafts in Transfected Fibroblasts**—Kv2.1 is a delayed rectifier K\textsuperscript{+} current that inactivates very slowly over a period of seconds. In contrast, Kv4.2 encodes for a transient current that activates and inactivates very slowly over a period of seconds. In contrast, Kv4.2 exhibited complex inactivation kinetics including a U-shaped voltage dependence and excessive cumulative inactivation. Therefore, we measured only the voltage dependence of inactivation over the range of potentials (–100 to +10 mV), which shows maximal inactivation.

Additional details are presented in the figure legends.

**Kv2.1 Is Localized in Non-caveolar Lipid Rafts**—Dual immunostaining of Kv2.1 transfected l-cells for both the channel and endogenous caveolin revealed partial colocalization. A, phase micrograph of mouse l-cell. The detection of bound anti-caveolin antibody (1:500) and anti-Kv2.1 (1:500) are shown individually in B and C, respectively. An overlay of the two images is shown in D. The white box represents enlarged area shown in E. Arrows point to regions of cells that show cell surface Kv2.1 channel but not caveolin.

**FIG. 1.** Kv channels differentially target to lipid rafts in heterologous expression systems. A, representative currents recorded from cell surface during 10 mV step depolarizations (holding potential, –80 mV) to +80 mV in mouse Ltk– cells stably expressing either Kv2.1 (top) or Kv4.2 (bottom). B, the effect of saponin on the Triton solubility of Kv2.1 and caveolin. Cell lysates were incubated with (+) or without (–) 0.5% saponin on ice for 30 min. They were then extracted with 1% Triton-X-100 on ice for 30 min. They were then extracted with 1% Triton-X-100 and subjected to centrifugation as described under “Experimental Procedures.” Detergent-soluble protein in the lystate (L) and insoluble protein in the pellet (P) were analyzed by Western blot. C, detergent-based isolation of lipid rafts. Sucrose density gradient centrifugation of 1% Triton-X-100-solubilized extracts from cells stably expressing Kv channel protein were analyzed by Western blot. The immunoblots show low density, raft-associated distribution of Kv2.1 and caveolin in contrast to the high density, non-floating distribution of Kv4.2, endogenous Na\textsuperscript{+}/K\textsuperscript{+} ATPase, actin, and tubulin. D, detergent-free raft isolation with cells stably expressing Kv2.1. Both Kv2.1 and caveolin are found exclusively in the low density, raft-associated fractions.

**FIG. 2.** Kv2.1 channel localization shows incomplete overlap with caveolin. Dual immunostaining of Kv2.1 transfected l-cells for both the channel and endogenous caveolin revealed partial colocalization. A, phase micrograph of mouse l-cell. The detection of bound anti-caveolin antibody (1:500) and anti-Kv2.1 (1:500) are shown individually in B and C, respectively. An overlay of the two images is shown in D. The white box represents enlarged area shown in E. Arrows point to regions of cells that show cell surface Kv2.1 channel but not caveolin.

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- Kv2.1, but Not Kv4.2, is Localized to Lipid Rafts in Transfected Fibroblasts
- Kv2.1 is a delayed rectifier K\textsuperscript{+} current that inactivates very slowly over a period of seconds.
- In contrast, Kv4.2 is a transient current that activates and inactivates very slowly over a period of seconds.
- Sucrose density gradient centrifugation showed low density, raft-associated distribution of Kv2.1 and caveolin.
- Differentially targeted to lipid rafts in heterologous expression systems.
- Dual immunostaining revealed partial colocalization between Kv2.1 and caveolin.
- Kv2.1 localization in non-caveolar lipid rafts.

Additional details are presented in the figure legends.
distribution after microtubule disruption. Consistent with these results, immunoprecipitation of caveolin failed to demonstrate an association between Kv2.1 and caveolin in either the L-cell system or rat heart, which expresses both Kv2.1 and caveolin (data not shown). Taken together, these data strongly suggest that Kv2.1 is associated with a non-caveolar lipid raft.

Kv2.1, but Not Kv4.2, Is Localized to Lipid Rafts in Rat Brain—The issue of Kv channel targeting to lipid rafts in vivo was addressed in the brain where the subcellular distribution and polarized sorting of voltage-gated K+ channels is critical for neural excitability. In addition, both Kv2.1 and Kv4.2 have previously been localized to neurons where they display a very high density of Kv channels at the neuronal surface (20–22). Sucrose density gradient fractions of 1% Triton X-100 extracted rat brain lysates were analyzed for Kv2.1 and Kv4.2 immunoreactivity. Kv2.1 floats in a low density lipid fraction whereas Kv4.2 is found at the bottom of the gradient in the non-raft fractions. 

Fig. 4. Kv channels differentially target to lipid rafts in rat brain. Sucrose density gradient fractions of 1% Triton X-100 extracted rat brain lysates were analyzed for Kv2.1 and Kv4.2 immunoreactivity. Kv2.1 floats in a low density lipid fraction whereas Kv4.2 is found at the bottom of the gradient in the non-raft fractions.

Depletion of membrane cholesterol alters Kv2.1 raft buoyant density and channel function. A, immunoblots of sucrose density gradient fractions of 1% Triton X-100 solubilized extracts from Kv2.1 expressing cells, with or without exposure to 2% 2-hydroxypropyl-β-cyclodextrin dissolved in the culture medium for 1 h at 37 °C. B, current record from control cells (—80 mV holding potential; step +60 mV) stably expressing Kv2.1. C, current from cell treated with 2% 2-hydroxypropyl-β-cyclodextrin for 1 h at 37 °C. D, plot showing the voltage dependence of Kv2.1 current activation, as determined from the magnitude of the tail currents, for control (○, n = 13) and cyclodextrin treated (●, n = 9) cells. E, plot showing the voltage dependence of Kv2.1 current inactivation determined using the double pulse protocol described under “Experimental Procedures” (control (□), n = 12; cyclodextrin-treated (●, n = 5). Increasing the concentration of cyclodextrin or extending the incubation time made the cells very difficult to patch clamp.

Fig. 5. Depletion of membrane cholesterol alters Kv2.1 raft buoyant density and channel function. A, immunoblots of sucrose density gradient fractions of 1% Triton X-100 solubilized extracts from Kv2.1 expressing cells, with or without exposure to 2% 2-hydroxypropyl-β-cyclodextrin dissolved in the culture medium for 1 h at 37 °C. B, current record from control cells (—80 mV holding potential; step +60 mV) stably expressing Kv2.1. C, current from cell treated with 2% 2-hydroxypropyl-β-cyclodextrin for 1 h at 37 °C. D, plot showing the voltage dependence of Kv2.1 current activation, as determined from the magnitude of the tail currents, for control (○, n = 13) and cyclodextrin treated (●, n = 9) cells. E, plot showing the voltage dependence of Kv2.1 current inactivation determined using the double pulse protocol described under “Experimental Procedures” (control (□), n = 12; cyclodextrin-treated (●, n = 5). Increasing the concentration of cyclodextrin or extending the incubation time made the cells very difficult to patch clamp.

Kv2.1 channel function. The current density and activation kinetics were not affected by cyclodextrin treatment (compare Fig. 5, B and C). Voltage sensitivity was also unaltered as shown in Fig. 5D. However, cyclodextrin significantly altered the steady-state inactivation of Kv2.1 as evidenced by a 36-mV hyperpolarizing shift in the inactivation curve (Fig. 5E). The V_{1/2} for inactivation of control and cyclodextrin-treated cells was $-15.7 \pm 0.59$ and $-51.6 \pm 0.44$, respectively. The drug effect was not due to a direct interaction with channel protein because acute application of cyclodextrin in the bath solution did
not affect channel function. Therefore, cyclodextrin treatment did not non-selectively modify channel gating or cell surface expression but rather specifically altered inactivation. Treatment of cells stably expressing Kv4.2 showed no observable effect (data not shown). These data show that altering raft structure significantly affects the function of raft-associated channels. Such a large shift in both resting potential and/or action potential duration.

Possible Functions of the Lipid Raft-Channel Complex—Since rafts often localize signaling proteins such as protein kinase C, nitric-oxide synthase, tyrosine kinases, Ha-Ras, mitogen-activated protein kinase, glycosylphosphatidylinositol-anchored proteins and G-proteins, channel/raft association could serve primarily to cluster signaling molecules with ion channels (9–12). Kv channels are known to be modulated by activation of various signal transduction pathways and often contain multiple consensus phosphorylation sites (4). In fact, Kv2.1 is constitutively tyrosine phosphorylated and physically associates with tyrosine kinases in Schwann cells (25). Multiple reports have localized tyrosine kinases to lipid raft microdomains including those from neuronal plasma membranes (26). Differential targeting to various lipid raft subpopulations may serve to organize signaling molecules and their K⁺ channel substrates. It is possible that the functional effects of cyclodextrin treatment are a consequence of kinase disruption as opposed to direct effects of altered lipid on channel activity.

It is also tempting to hypothesize a possible role for lipid-protein rafts in the polarized sorting of K⁺ channels in the brain. Certainly, this appears to be a mechanism for the polarized sorting of other neuronal proteins (27–29). Although both channels target to dendrites, it is clear that even within the dendritic region multiple plasma membrane subdomains exist (29), for the Kv2.1 and Kv4.2 channels segregate to somatodendritic and distal regions, respectively (22).

Potential Mechanisms of Channel-Raft Association—One obvious question deals with the mechanism of channel targeting to rafts. The channel could bind raft-associated proteins or it could directly target to, or interact with, the raft lipid. The cytoplasmic COOH-terminal domain of Kv2.1 has been implicated in the polarized sorting and clustering of Kv2.1 in Madin-Darby canine kidney cells (3). However, preliminary results based on truncation mutants of Kv2.1 suggest that neither the amino or carboxyl terminus is necessary for targeting to lipid rafts (data not shown). This finding is consistent with reports that transmembrane regions of integral membrane proteins may contain the information that determine raft association (30).

An alternative mechanism for channel-raft association may involve the channel binding to other raft-associated proteins. One candidate is PSD-95, which has been reported to associate with low density lipid rafts in both COS cells and rat brain (31). In addition, protein-lipid interactions are necessary for clustering of this PDZ protein at the synapse (32). Therefore, raft-bound PDZ proteins could localize ion channels to raft domains. However, Kv2.1 does not contain standard PDZ binding sequences nor does it interact with PSD-95 (3). Even if it did contain a PDZ binding site, the Kv2.1 truncation mentioned above still targets to rafts. In addition, glutamate receptors bind PDZ proteins but are not raft associated (26). Thus, PDZ proteins are not likely to be responsible for raft localization. Recent work from the Clapham laboratory (33) indicates that PSD-95, when expressed in slices of rat cortex, only targets to axons in the presence of Kv1.4 channel coexpression. Thus, the ion channel, or another protein which recognizes the PSD-95-channel complex, is responsible for localizing PSD-95 to the axon. It is tempting to speculate that localization of channels to rafts is part of a mechanism by which PDZ proteins are themselves localized. Another candidate protein for targeting K⁺ channels to rafts is the K⁺ channel beta subunit. This protein has been implicated in channel association with the cytoskeleton. However, Kv2.1 does not associate with the Kvbeta 2.1 subunit present in the l-cell expression system (data not shown), making the β subunit a poor candidate (34). Additional mutagenesis experiments are necessary to determine a potential raft association signal within the channel.

Conclusion—This report is the first description of ion channels localizing to lipid microdomains and provides a dramatic example of the differential targeting of protein isoforms to lipid rafts. Although progress has been made in identifying elements involved in channel targeting, clustering, and anchoring, it is not yet clear how the number and location of channel complexes within the plane of the membrane are determined (32). Our data indicate that protein-lipid interactions should be considered as a mechanism of Kv channel localization. The finding that cyclodextrin treatment shifts steady-state inactivation in the hyperpolarizing direction by more than 30 mV suggests that alteration of membrane lipid, either by disease (35) or the clinical use of lipid-lowering drugs, can affect membrane excitability by altering the function of raft-localized channels.

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