Ca\textsuperscript{2+}-activated IK1 Channels Associate with Lipid Rafts upon Cell Swelling and Mediate Volume Recovery\textsuperscript{a,b}

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Restoration of cell volume in the continued presence of osmotic stimuli is essential, particularly in hepatocytes, which swell upon nutrient uptake. Responses to swelling involve the Ca\textsuperscript{2+}-dependent activation of K\textsuperscript{+} channels, which promote fluid efflux to drive volume recovery; however, the channels involved in hepatocellular volume regulation have not been identified. We found that hypotonic exposure of HTC hepatoma cells evoked the opening of 50 pS \textit{K}-permeable channels, consistent with intermediate conductance (IK) channels. We isolated from rat liver and HTC cells a cDNA with sequence identity to the coding region of IK1. Swelling-activated currents were inhibited by transfection with a dominant interfering IK1 mutant. The IK channel blockers clotrimazole and TRAM-34 inhibited whole cell swelling-activated K\textsuperscript{+} currents and volume recovery. To determine whether IK1 underwent volume-sensitive localization, we expressed a green fluorescent protein fusion of IK1 in HTC cells. The localization of IK1 was suggestive of distribution in lipid rafts. Consistent with this, there was a time-dependent increase in colocalization between IK1 and the lipid raft ganglioside GM1 on the plasma membrane, which subsequently decreased with volume recovery. Pharmacological disruption of lipid rafts altered the plasma membrane distribution of IK1 and inhibited volume recovery after hypotonic exposure. Collectively, these findings support the hypothesis that IK1 regulates compensatory responses to hepatocellular swelling and suggest that regulation of cell volume involves coordination of signaling from lipid rafts with IK1 function.

Na\textsuperscript{+}-coupled amino acid uptake and insulin-stimulated changes in cation permeability (1, 2). In response to swelling, plasma membrane K\textsuperscript{+} and Cl\textsuperscript{−} channels open to promote electrolyte and fluid efflux, which restores cell volume toward its resting state. This serves to prevent a degree of swelling that would jeopardize cellular integrity and organ level function. Although volume-sensitive channels have been deemed to be of critical importance to hepatocyte physiology, their fundamental characteristics have remained elusive. We and others have shown a role for Ca\textsuperscript{2+} in channel activation. Hepatocellular swelling stimulates intracellular Ca\textsuperscript{2+} mobilization and Ca\textsuperscript{2+} influx (3–5), and swelling-mediated increases in K\textsuperscript{+} conductance and volume recovery are Ca\textsuperscript{2+}-dependent (3, 4, 6–8). These observations support the hypothesis that swelling-induced increases in membrane K\textsuperscript{+} permeability that are necessary for hepatocellular volume recovery are mediated by Ca\textsuperscript{2+}-activated K\textsuperscript{+} channels.

Two families of Ca\textsuperscript{2+}-activated K\textsuperscript{+} channels have been linked to cell volume regulation in epithelia: intermediate/small conductance (IK/SK),\textsuperscript{3} and large conductance (BK) K\textsuperscript{+} channels, and consistent with this, IK, SK, and BK channel genes have been found to be expressed in these tissues (9–12). We and others have shown functional evidence of 10 pS Ca\textsuperscript{2+}-activated K\textsuperscript{+} channels and expression of the SK isoforms SK2 and SK3 in hepatocytes (9, 13, 14). This would support a potential role for SK channels in hepatocellular volume regulation. On the other hand, alanine uptake, which elicits hepatocellular swelling, has been shown to evoke the opening of 30–90 pS K\textsuperscript{+} channels (15), and the IK channel blocker clotrimazole, but not the SK channel blocker apamin, attenuates hepatocellular swelling-induced K\textsuperscript{+} efflux (16). These findings support a potential role for IK channels in hepatocellular volume regulation. Others have reported that alanine evokes the opening of BK (200 pS) channels in hepatocytes (17). In liver, immunolabeling studies have suggested that IK1 protein (also known as SK4, KCa3.1, and KCNN4) is predominantly expressed in bile duct epithelia (18); however, volume recovery has been found to be predominantly mediated by SK2 channels in those cells (9). In colonic epithelia, IK has been suggested to be important in volume recovery after swelling (10); however, in liver there is little apparent hepatocellular IK1 immunoreactivity detected (18). Given these findings, it is not clear at the molecular level

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\textsuperscript{3} The abbreviations used are: IK, intermediate conductance K\textsuperscript{+} channels; GM1, [Gal(1→3)GalNAc(1→4)NeuAc(1→4)GlcNAc(1→3)Gal(1→4)Glc(1→1) ceramide]; SK, small conductance K\textsuperscript{+} channels; BK, large conductance K\textsuperscript{+} channels; PI, phosphatidylinositol; SES, standard extra cellular solution; CTXB, cholera toxin B subunit; GFP, green fluorescent protein; dn, dominant negative; MβCD, methyl β-cyclodextrin.
which type of $K^+$ channel governs volume regulatory responses in hepatocytes.

To address this issue, we have used complementary approaches to define the characteristics of $K^+$ channels involved in hepatocellular volume regulation. Here we report that: IK1 is expressed in rat liver and HTC rat hepatoma cells, the properties of swelling-activated $K^+$ channels are consistent with those of IK1 channels, and such channels are inhibited by a dominant interfering mutant of IK1. Moreover, we have found that cell swelling induces transient association at the plasma membrane of IK1 channels with lipid rafts. Disruption of lipid raft integrity leads to altered plasma membrane localization of IK1 and inhibits volume recovery. Taken together, these findings suggest that IK1 channels regulate cell volume in hepatocytes and raise the possibility that lipid rafts may coordinate swelling-activated fluid efflux with other events that participate in cell volume control.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Reagents**—HTC rat hepatoma cells, a well-characterized model for the study of hepatocellular volume regulation (3, 7, 19–23), were maintained in culture as previously described (21). Cells were used $\sim 18$–$24$ h after plating. The acetoxymethyl ester of calcein, Alexa 555-labeled cholera toxin B, and Alexa 647-labeled phallolidin were from Molecular Probes. All other reagents were from Sigma unless specifically noted.

**Molecular Cloning and Cell Transfection**—mRNA was isolated from the livers of male Sprague-Dawley rats, and a cDNA pool was prepared essentially as described previously (14). PCR primers corresponding to the 5’- and 3’-ends of the full-length coding sequence of rat IK1 (also known as SK4) were used (ATGGCCGAGGAGGTGGACT and CTAATGGGTGCTTCCGGATGGG) to amplify the cDNA using Pfu Turbo DNA polymerase (Stratagene). A 1278-nucleotide PCR product was cloned, and both strands of the rat liver IK1 product were analyzed by automated sequencing (Vermont Cancer Center DNA Analysis Facility). Dominant negative constructs of SK3 and IK1 were made using QuikChange XL site-directed mutagenesis kit (Stratagene), designing primers so that the signature $K^+$ channel pore motif, GYG, was altered to AAA (24). Dominant negative SK3 (dnSK3) and dominant negative IK1 (dnIK1), and their parent forms were subcloned in-frame into enhanced GFP (Clontech) on both the amino and carboxyl ends of enhanced GFP and confirmed by sequencing. HTC cells were transfected with these fusions using Amaxa nucleofector technology, and expression of all fusions was visible by fluorescence microscopy, except that of IK1 with enhanced GFP fused to its carboxyl terminus, which was not visible and thus not used. Cells transfected with GFP fusions of the ion channels were analyzed by fluorescence imaging or by patch clamp recording after $24$ h.

**Immunofluorescence and Image Analysis**—Transfected cells were seeded onto glass coverslips and treated essentially previously described (19). Live labeling of cells with Alexa 555-cholera toxin subunit B ($1 \mu g/ml$) was performed for $30$ min at $4$ °C to allow the toxin to bind to the lipid raft marker, ganglioside GM1, without substantial internalization or raft clustering. In selected experiments, cells were treated as above and then incubated with fresh isotonic or hypotonic media for $5$ min prior to fixation to promote raft coalescence. Unless otherwise specified, all experiments were performed at room temperature. The isotonic medium (SES) contained: $140$ mM NaCl, $4$ mM KCl, $1$ mM CaCl$_2$, $2$ mM MgCl$_2$, $1$ mM KH$_2$PO$_4$, $10$ mM glucose, and $10$ mM HEPES (pH 7.4). The hypotonic medium was identical to SES except that the concentration of NaCl was reduced to $84$ mM. In studies involving lipid raft disruption, pretreatment with drugs was performed at $37$ °C. Because hepatocytes are particularly sensitive to methyl-$\beta$-cyclodextrin (M$\beta$CD) (25), cells were treated for $30$ min with $10$ mM M$\beta$CD. In parallel experiments, cells were treated with filipin complex (freshly made in dry Me$_2$SO at $6 \mu g/ml$) for $30$ min. Image quantification was performed with deconvolved DeltaVision files (softWoRx version 3.5.0 from Applied Precision), using Volocity 3 software (Improvision) to calculate the overlapping voxels in three-dimensional projections of cell peripheries.

**Measurement of Membrane Currents**—Single channel and whole cell currents were measured by patch clamp recording techniques as previously described, with the exception that the microscope was fitted for fluorescence imaging (3, 13). For studies that involved expression of fluorescent fusion proteins, transfected cells were identified by visualization of the green fluorescence of GFP. The isotonic bathing solution was SES, and the pipette solution contained $10$ mM NaCl, $130$ mM KCl, 0.5 mM CaCl$_2$, $2$ mM MgCl$_2$, $1$ mM EGTA, and $10$ mM HEPES (pH 7.30). Cell swelling was evoked by perfusion with a hypotonic solution identical to SES except that the concentration of NaCl was reduced to $98$ mM.

For single channel recordings, inward currents at the resting membrane potential (approximately $-55$ mV) represent the opening of $K^+$-permeable channels under the experimental conditions employed, because there is no net Cl$^-$ flux at the resting membrane potential in these cells (26). For whole cell recordings, $K^+$ currents were measured at a holding potential of $0$ mV, and Cl$^-$ currents were measured at a holding potential of $-80$ mV (3). Currents were measured under basal conditions, and hypotonic currents were defined as the maximal current within $10$ min after hypotonic exposure. To control for variations in cell size, currents were normalized to cell capacitance and expressed as current density. All experiments were performed at room temperature.

**Measurement of Cell Volume**—Cell volume was measured two ways: in calcein-loaded cells via laser scanning confocal microscopy as previously described (3) and in suspended cells via Multisizer 3 Coulter Counter measurement (20, 21) with minor modifications. Values were normalized to basal volume. For the first method, cell swelling was elicited by exposure to a hypotonic solution, identical in composition to SES, except that the NaCl concentration was $84$ mM. In the second method, cells were suspended as follows. For studies involving the effects of channel blockers, cells were lifted off the plate by incubation in phosphate-buffered saline at $37$ °C for $20$ min with or without the appropriate drugs, resuspended in SES with or without the appropriate drugs for $10$ min more at $37$ °C with rocking, and then added to a stirred sample holder on a Multisizer 3 Coulter Counter (Beckman Coulter). Samples (0.5 ml) with a minimum
cell number of 1500 per measurement in the size range of interest (10–30 μm diameter) were aspirated and measured at 0.5- to 1-min intervals. After 1 or 2 min, SES without NaCl, with or without drug, was added to bring the concentration of NaCl to 84 mM (hypotonic), and samples continued to be aspirated and measured for at least 20 min. For studies involving lipid raft disruption, the method for preparation of cells in suspension was modified, because cells lifted using phosphate-buffered saline in the presence of lipid raft-disrupting drugs exhibited basal cell volumes that were markedly lower than those of controls (see “Results”). Thus, for these studies, cells were briefly trypsinized (1 min), rocked in serum-containing media for 20 min at 37 °C, centrifuged, and brought up in SES with or without MβCD (10 mM) and rocked for an additional 15 min at 37 °C prior to analysis.

The rate of cell volume recovery after swelling (%/min) was calculated from the equation below, where $V_{\text{max}}$ is the relative maximum cell volume after swelling, $V_0$ is the relative cell volume 20 min after hypotonic exposure, and $m$ is the linear regression coefficient of the recovery curve between $V_{\text{max}}$ and $V_0$ as follows: rate = ($m/(V_{\text{max}} - V_0)$) × 100.

Statistics—All results are presented as means ± S.E., where $n$ represents the number of cells or groups of cells. Comparisons were made with same-day controls with the use of unpaired Student’s $t$ test or analysis of variance with Bonferroni’s multiple comparison test, as appropriate, and $p < 0.05$ was considered to be significant.

RESULTS

Hepatocellular Swelling Activates IK Channels—We and others have shown that K$^+$ channels activated by hepatocellular swelling are Ca$^{2+}$-dependent (3, 4, 6, 7). Based upon the properties of known Ca$^{2+}$-activated K$^+$ channels identified in hepatocytes, it could be hypothesized that volume-sensitive K$^+$ channels are SK, IK, or BK channels. To distinguish between these possibilities, we performed single channel recordings in cell-attached membrane patches. Using a KCl-rich pipette solution (see “Experimental Procedures”), single channel activity was not detectable under isotonic conditions (data not shown). However, within 3–5 min after hypotonic exposure, inward single channel currents were observed at the resting membrane potential in 5 of 10 cell-attached patches (Fig. 1). The channels exhibited a unitary conductance of ≈50 pS with very weak inward rectification. Single channels reversed polarity at ≈45 mV positive to the resting membrane potential, consistent with K$^+$ permeability under the experimental conditions employed (26). These findings support the concept that hepatocellular swelling evokes the opening of IK channels.

Effect of IK Channel Blockade on Swelling-activated Membrane Currents—To further delineate the nature of swelling-activated K$^+$ channels, we examined the effect of the IK channel blockers clotrimazole and TRAM-34 (27) on membrane currents evoked by hypotonic challenge. We found that clotrimazole (10 μM) and TRAM-34 (1 μM) markedly attenuated swelling-activated K$^+$ currents but did not significantly affect swelling-activated Cl$^-$ currents (Fig. 2). By contrast, apamin (300 nM), which inhibits SK channels (14), and tetraethylammonium (5 mM), which inhibits BK but not IK channels at the concentration employed (28, 29), did not affect swelling-activated currents (Fig. 2). These findings add further support to the concept that IK channels are activated by hepatocellular swelling.

Molecular Identification of IK1 in Liver and HTC Cells—The functional properties of swelling-activated IK channels appear to be identical to those of the K$^+$ channel protein IK1 (30, 31). To determine whether IK1 was expressed in both HTC cells and native liver, reverse transcribed mRNA was amplified with primers derived from the rat brain IK1 sequence. The
sequenced products identified multiple forms in both rat liver and HTC cells. Primers from the 5΄- and 3΄-ends were subsequently used to amplify from rat liver mRNA, and a 1278-bp product, the sequence of which encoded a protein with high sequence similarity to other entries in GenBank™ was cloned. This nucleotide sequence is over 99% identical to rat SK4 cloned from colon (core nucleotide data base NM_023021) and varies by only three nucleotides. These nucleotide differences result in a change of only one amino acid in the S5 region; phenylalanine 213, is changed by the sequence differences to leucine. This is unlikely to have functional consequences, as a smooth muscle rat IK1 channel, and human and mouse forms of the channel also have leucine at this position (31). These findings are consistent with hepatocellular expression of IK1.

**IK1 Contributes to Swelling-activated K currents**—If IK1 channels participate in hepatocellular volume regulation, it would be predicted that reduction of functional IK1 channels would lead to attenuation of swelling-activated K currents. To test this, we expressed in HTC cells a dominant negative mutant of IK1 (dnIK1), in which the canonical pore sequence GYG was changed to AAA. We then examined the influence of dnIK1 expression on membrane currents following hypotonic challenge. In comparison with controls (vector alone), cells expressing dnIK1 exhibited a significant reduction in swelling-activated K currents but not Cl currents (Fig. 3). To exclude the possibility that the effects of dnIK1 expression resulted from interference with other Ca2+-activated K channels, we tested the effects of a dominant negative mutant of SK3 (dnSK3), an SK channel isoform that we have previously identified in hepatocytes and HTC cells (14). There was no significant difference in swelling-activated K currents in cells that expressed dnSK3, compared with vector-transfected controls (Fig. 3). These observations support the concept that IK1 channels respond to hepatocellular swelling and contribute to volume recovery.

**Volume-sensitive Localization of IK1**—We have recently shown that critical proteins required for early signaling events in volume recovery appear to be translocated to the cell membrane upon hepatocellular swelling (19). We therefore sought to determine whether IK1 localization was altered in response to cell swelling. Because antibodies that recognize IK1 in immunofluorescence applications are not yet avail-
able, we constructed amino-terminal GFP fusions of the IK1 gene (GFP-IK1) and, as a control, a GFP fusion of the closely related SK3 gene (GFP-SK3), in mammalian expression plasmids. We first confirmed that these constructs exhibited functional properties expected for IK1 and SK3. Human embryonic kidney HEK293 cells transiently transfected with GFP-IK1 were treated with fluorescently tagged cholera toxin B (CTXB, 1 µg/ml) at 4 °C for 30 min in isotonic (ISO) or hypotonic (HYPO) buffer prior to fixation. Arrows indicate areas on the plasma membrane where IK1 and lipid raft signals coincide. Green, GFP-IK1; red, CTXB. All bars are 15 µm.

FIGURE 5. IK1 associates with lipid rafts at the periphery of the cell. A, examples of cells treated a lipid raft marker under conditions that suppress endocytosis and lipid raft coalescence. HTC cells transiently transfected with GFP-IK1 were treated with fluorescently tagged cholera toxin B (CTXB, 1 µg/ml) at 4 °C for 30 min in isotonic (ISO) or hypotonic (HYPO) buffer prior to fixation. B, examples of cells treated as in A, but then warmed to promote lipid raft coalescence. Live CTXB labeling was performed as in A, but cells were treated with fresh buffer and incubated at room temperature for 5 min more. Arrows indicate areas on the plasma membrane where IK1 and lipid raft signals coincide. Green, GFP-IK1; red, CTXB. All bars are 15 µm.

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and GFP-SK3 signals were largely diminished in cells, and there appeared to be substantially less K+ channel protein on the plasma membrane. Intriguingly, although there was substantial overlap between peripheral GFP-SK3 and actin signals, there was little between GFP-IK1 and actin. Thus, in response to cell swelling, GFP-IK1 appeared to be clustered in the plasma membrane in a time-dependent manner, and it exhibited different plasma membrane and cytoskeletal association from that of the closely related GFP-SK3.

The volume-sensitive localization of GFP-IK1 was suggestive of spatially controlled ion channel occupancy in the plasma membrane. Among the mechanisms that could be responsible for this are association with lipid rafts, plasma membrane microdomains that are enriched in cholesterol and sphingolipids, and signaling molecules such as Src (32), which are key cell volume regulators (19). Lipid rafts are thought to form primarily as structures too small to be detected by fluorescence microscopy (33, 34). However, in cells polarized by stimulation, lipid rafts can coalesce into structures that can be detected by fluorescence methods (35, 36). Lipid rafts isolated from the plasma membrane in polarized hepatocytes have been detected with fluorescently labeled cholera toxin subunit B (CTXB), which binds to the lipid raft component, ganglioside GM1 (37). Moreover, because lipid rafts have been implicated in volume control in selected cell types (38, 39), we tested whether IK1 associated with lipid rafts in response to cell swelling.

Live GFP-IK1-transfected HTC cells were exposed to fluorescently labeled CTXB in isotonic or hypotonic solutions at 4 °C for 30 min prior to fixation, conditions that allow binding of the fluorescent marker on the outside of the cell, but suppress endocytosis and reduce raft coalescence (40). These conditions led to asymmetric accumulation of the CTXB raft marker with peripheral GFP-IK1, which appeared to be enhanced in swollen cells (Fig. 5). To determine whether GFP-IK1 signal would exhibit continued association when the apparent rafts coalesced into larger rafts, cells treated as above were warmed to room temperature for 5 min in the continued presence of isotonic and hypotonic conditions (41). Larger clusters of lipid rafts and GFP-IK1 resulted, and IK1 signal continued to colocalize with CTXB signal particularly under hypotonic conditions (Fig.
5). To confirm that the labeling was specific for lipid rafts, cells were pretreated with the cholesterol-sequestering drug, filipin (6 μg/ml), or the cholesterol-depleting drug MβCD (10 mM) for 30 min at 37 °C prior to hypotonic exposure. Under these conditions, there was considerably reduced CTXB staining at the periphery, and the patterns of GFP-IK1 signal were dramatically altered: either no signal on the plasma membrane or continuous signal along the plasma membrane (Fig. 6). Thus, GFP-IK1 localization is altered in cells that have been treated with agents that disrupt lipid raft integrity. Taken together, these data are consistent with IK1 channel association with lipid rafts at the periphery in response to cell swelling.

The skate anion-exchange protein AE1 has been shown to translocate with lipid rafts from an intracellular compartment to the plasma membrane on hypotonic exposure (39). Because IK1 appeared to be localized to lipid rafts, we sought to determine whether GFP-IK1 was constitutively associated with lipid rafts, or if the association occurred transiently in response to swelling. To test this, we performed imaging studies in which transfected cells were fixed under isotonic or hypotonic conditions before staining with CTXB to label both intracellular and peripheral pools of lipid rafts. Under isotonic conditions, discrete areas of GFP-IK1 signal were found to overlap with raft-specific signal at the periphery in extensions from the body of cells apparently undergoing spreading (Fig. 7). However, there did not seem to be extensive overlap of the intracellular pools of GFP-IK1 and raft-specific vesicles. Upon swelling, there was extensive overlap of GFP-IK1 signal and raft-specific signal in peripheral extensions (Fig. 7A); colocalization increased significantly at 1 and 5 min following hypotonic exposure, and this returned to basal levels by 10 min (Fig. 7B). Near the periphery of the cell where they could be more easily distinguished, the GFP-IK1 and lipid raft signals did not appear to overlap in intracellular vesicles as the plasma membrane signal diminished, suggesting that they were not internalized together. Thus, IK1 does not appear to be constitutively associated with lipid rafts but appears to accumulate with lipid rafts at the periphery of the cell on cell swelling, before being internalized independently of lipid rafts.

**Effect of IK1 Channel Blockade and Lipid Raft Disruption on Cell Volume Recovery**—The findings above are consistent with the concept that IK1 channels are activated upon hepatocellular swelling and undergo time-dependent association with lipid rafts. To test whether IK1 channels regulate volume recovery after hepatocellular swelling, we examined the effect of clotrimazole (10 μM) on changes in cell volume induced by hypotonic challenge using cell imaging (Fig. 8A). The rate of volume recovery was significantly attenuated in the presence of clotrimazole (1.38 ± 0.08%/min, n = 42 cells) compared with that of controls (rate recovery = 5.81 ± 0.07%/min, n = 14 cells).

To extend these observations to a system that would efficiently assay more cells per time point, we pursued Coulter Multisizer measurements of cells in suspension, the volume regulatory behavior of which is similar to that of adherent cells.
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As in adherent cells, clotrimazole significantly inhibited volume recovery after hypotonic exposure (Fig. 8C). Furthermore, TRAM-34 (5 μM) also significantly inhibited volume recovery. The rates of volume recovery for cells treated with clotrimazole and TRAM-34 were 3.78 ± 0.40%/min (n = 3 groups of cells) and 4.04 ± 1.27%/min (n = 3 groups of cells), respectively, compared with that of controls (14.01 ± 1.98%/min, n = 3 groups of cells). Collectively, these observations support the hypothesis that IK1 channels function as principal regulators of hepatocellular volume control.

Because GFP-IK1 could still be found on the plasma membrane under conditions in which the integrity of lipid rafts was disrupted (see Fig. 6), we sought to evaluate whether abrogation of the association of the channel with lipid rafts affected cell volume recovery. To assess this, we first examined the effect of filipin on cell volume responses to hypotonic challenge. Intriguingly, although hypotonic challenge elicited the same rate of cell swelling in the presence of filipin, the extent of cell swelling was less, and the shape of the mean cell volume recovery curve was altered in comparison with its absence (data not shown). However, the initial volume filipin-treated cells suspended under these conditions (1243.4 ± 27.8 fl, n = 6 groups of cells) was almost half that of the controls (2361.3 ± 19.2 fl, n = 8 groups of cells). To ensure that there was no potential contribution from processes other than those involved in cell volume control, we modified the method of cell detachment (incorporating a brief trypsinization, see “Experimental Procedures”) to yield cells with comparable initial cell volumes despite lipid raft depletion. With this method, the basal volume of cells pretreated with MβCD (10 mM) was 2405.2 ± 11.1 fl (n = 14 groups of cells), similar to that of controls (2491.6 ± 20.7 fl, n = 14 groups of cells). Moreover, in cells prepared in this fashion, clotrimazole still significantly inhibited volume recovery (see supplementary Fig. S2). As shown in Fig. 8C, MβCD also significantly attenuated volume recovery under these conditions (recovery rate = 2.95 ± 0.41%/min, n = 7 groups of cells), compared with controls (recovery rate = 5.62 ± 0.83%/min, n = 7 groups of cells). Although lipid raft disruption may make IK-independent contributions to cell volume recovery, these observations support the hypothesis that the association of IK1 channels with lipid rafts coordinates ion channel-mediated fluid efflux with other processes required to facilitate cell volume recovery.

DISCUSSION

We have provided evidence that supports a role for IK1 channels and lipid rafts in regulating volume recovery after hepatocellular swelling. This is based upon our previous work that swelling-activated increases in K⁺ conductance are Ca²⁺-dependent (3) and the present work that indicates that: (a) the single channel and pharmacological properties of swelling-activated K⁺ channels are consistent with those of IK1 channels, (b) IK1 is expressed in HTC cells and rat liver, (c) IK1 and lipid rafts undergo volume-sensitive association, and (d) volume recovery after swelling is inhibited by IK1 channel blockade and also by disruption of lipid rafts with which IK1 channels localize on cell swelling.

There is substantial physiological and pharmacological evidence to support a role for IK1 channels in volume regulation in cells of hematopoietic origin, and IK1 is the likely candidate protein. In IK1 knock-out mice, Ca²⁺ ionophore-mediated lymphocyte shrinkage was ablated in lymphocytes, as were Ca²⁺ ionophore-stimulated increases in erythrocyte K⁺ permeability and osmotic fragility, respectively (42). IK1 is expressed in selected epithelia such as airway and intestine,
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FIGURE 8. IK1 blockade and lipid raft disruption inhibit volume recovery after swelling. A, effect of clotrimazole (10 μM) on cell volume recovery following hypotonic exposure. Volume (normalized to basal values) was measured by imaging individual cells in the absence (control, n = 14 cells) versus presence of clotrimazole (CLTZ, n = 42 cells). Data represent means ± S.E. for each condition. B, effect of clotrimazole (10 μM) and TRAM-34 (5 μM) on volume recovery in cell suspensions. Cells underwent hypotonic exposure at 0 min. Mean cell volume (normalized to basal values) was determined by Coulter Multisizer measurements in the absence (Control) versus presence of clotrimazole (CLTZ) or TRAM-34 (TRAM). Data represent means ± S.E. for three groups of cells for each condition. C, effect of MβCD (10 mM) on cell volume recovery following hypotonic exposure (time = 0 min). Cell volume was determined as in B in the absence (control) versus presence of MβCD. Data represent means ± S.E. for seven groups of cells for each condition.

where swelling-activated IK channels have been described (10, 12, 43). A recent study suggests that the expression of IK1 among epithelia is widespread (18). However, a role for IK1 per

se in epithelial responses to osmotic stress has not yet been established at the molecular level. The results of the present studies, in which dnIK1 attenuated swelling-activated K+ currents, suggest that IK1 is the major volume-sensitive hepatocellular K+ channel isoform, and lend support to a volume regulatory role for IK1 in tissues in which swelling-activated IK channels are found.

Two points merit comment. First, it should be emphasized that the present studies were performed in a model liver cell line. Although HTC cells bear substantial resemblance to hepatocytes in situ with respect to volume regulatory responses, such as dependence of activation of Src, phospholipase C, and intracellular Ca2+ mobilization (5, 19, 22, 44, 45), the role of IK1 channels, and in particular IK1, in cell volume regulation in native liver awaits confirmation. Studies in knock-out mice are likely to be fruitful in this regard. Second, although our studies suggest a predominant role for IK1 in hepatocellular volume control, they do not exclude a role for other K+ channel isoforms. For example, it has been recently shown that the voltage-activated K+ channel isoform KCNQ1 is expressed in hepatocytes and that the KCNQ1 inhibitor chromanol 293B partially inhibits swelling-induced K+ efflux and K+ conductance in perfused rat liver and hepatocytes, respectively (45). It is thus possible that KCNQ1-encoded channels participate in hepatocellular volume regulation, but their relative contribution to swelling-induced changes in K+ permeability remains to be defined.

How IK1 channels might be activated by hepatocellular swelling awaits further exploration. The simplest model for channel activation would be by intracellular Ca2+ mobilization, which we have shown to be triggered by Src-mediated activation of phospholipase Cγ (19, 22). Increases in cytosolic Ca2+ concentration have been shown to activate IK1 via conformational changes in calmodulin, which is tightly associated with the channel protein (46). Our observations concerning the volume-sensitive association of plasma membrane IK1 with lipid rafts raise the possibility that localization could represent an additional mechanism for channel regulation or functional integration with other processes involved in volume recovery. Indeed, in T lymphocytes, lipid rafts play essential roles in immune function (47), and IK1 has been shown recently to undergo stimulus-dependent localization to the immunological synapse (48), a specialized plasma membrane domain that contains critical signaling molecules that are clustered by lipid rafts (49). By analogy, the observations from the present studies support the concept that recruitment of IK1 to the immune synapse is facilitated by lipid rafts.

There is precedent for volume-sensitive localization of selected membrane transport proteins in hepatocytes. For example, the bile acid transport protein sodium-taurocholate cotransporting polypeptide undergoes osmosensitive translocation to the plasma membrane via a mechanism that requires phosphatidylinositol (PI) 3-kinase activation (50). This is of interest, because PI 3-kinase is required for hepatocellular volume recovery (50, 51), it can associate with lipid rafts (52), and lipid products of PI kinases have been suggested to regulate IK1 activation (53). In particular, in hepatocytes, the lipid kinase product PI 4,5-bisphosphate, which causes lipid raft coales-
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cence during cell migration (54), augments swelling-mediated increases in hepatocellular K+ conductance (45).

The lipid raft association of IK1 channels has additional implications for the coordination of swelling-induced cell signaling. Many of the proteins (and/or their substrates) that have been implicated in cell volume recovery in liver have been shown in other systems not only to be resident in lipid rafts but also to be activated by delivery to lipid rafts. This includes integrins (55), which are thought to sense cell swelling and activate cell volume recovery (56, 57). Simple changes in the lipid composition of rafts are sufficient to cause clustering and activation of integrins, and this clustering is sufficient to activate Src (41), which we and others have shown is critical for hepatocellular cell volume recovery (19, 56). Similarly, lipid raft localization of phospholipase Cγ1, another volume-sensitive signaling protein (22), leads to its constitutive activation (58). Other actin cytoskeleton-regulating proteins that are activated by cell swelling, such as Rac1 (57) and Vav (19), regulate the integrin-mediated translocation of GM1 ganglioside lipid rafts to the cell surface during cell migration (59) and are required for stimulus-induced lipid raft clustering in some cells (60). Thus, cell swelling may lead to lipid raft recruitment of a group of proteins, including IK1, which would contribute to the formation of a volume recovery complex that coordinates fluid efflux with the restoration of an organized actin cytoskeleton. In support of this, our findings demonstrate that lipid raft disruption attenuates volume recovery but does not prevent association of IK1 with the plasma membrane (although the pattern of IK1 localization is altered). Thus, under these conditions, it is possible that volume recovery would be impeded, because the interaction between IK1 and other raft-associated proteins that reorganize the cytoskeleton (e.g. Src, phospholipase Cγ, and Vav) would be disabled even though the channel is still present on the plasma membrane. This concept warrants future exploration.

The identification of IK1 as a potential contributor to hepatocellular volume regulation has implications in terms of liver pathobiology. Specifically, disorders characterized by hepatocyte ballooning, such as alcoholic liver disease, and ischemia-reperfusion injury, may be attributable to alterations in the activity of volume-sensitive membrane transporters and resultant irreversible cell swelling (61, 62). It is tempting to speculate that, in the setting of such disorders, there would be increased susceptibility to liver failure among individuals with either reduced hepatocellular IK1 expression or with genetic polymorphisms in IK1 that reduce volume-sensitive K+ channel function. This area is worthy of exploration.

In summary, the results of the present studies support the concept that IK1 encodes a K+ channel that couples swelling-mediated increases in intracellular Ca2+ mobilization to stimulation of K+ efflux necessary for volume recovery in hepatocytes. In view of the critical roles of K+ channels in volume control and organ level function, it is reasonable to speculate that IK1 channels would represent attractive pharmacological targets for prevention of hepatic injury during pathological states.

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REFERENCES

1. Haussinger, D. (1996) Prog. Liver Dis. 14, 29–53
2. Dunkelberg, J. C., Feranchak, A. P., and Fitz, J. G. (2001) Hepatology 33, 1349–1352
3. Roe, M. W., Moore, A. L., and Lidofsky, S. D. (2001) J. Biol. Chem. 276, 30871–30877
4. Junanakar, P. R., Karjalainen, A., and Kirk, K. (2002) J. Biol. Chem. 277, 40324–40334
5. Wang, Y. J., Gregory, R. B., and Barritt, G. J. (2002) Biochem. J. 363, 117–126
6. Khalilutt, W. E., and Wondergem, R. (1991) Hepatology 13, 962–969
7. Roman, R. M., Bodily, K. O., Wang, Y., Raymond, J. R., and Fitz, J. G. (1998) Hepatology 28, 1073–1080
8. Feranchak, A. P., Fitz, J. G., and Roman, R. M. (2000) J. Hepatol. 33, 174–182
9. Roman, R., Feranchak, A. P., Troetsch, M., Dunkelberg, I. C., Kilic, G., Schlenker, T., Schauack, J., and Fitz, J. G. (2002) Am. J. Physiol. 282, G116–G122
10. Wang, J., Morishima, S., and Okada, Y. (2003) Am. J. Physiol. 284, C77–C84
11. Morita, T., Hanaoka, K., Morales, M. M., Montrose-Rafizadeh, C., and Guggino, W. B. (1997) Am. J. Physiol. 273, F615–F624
12. Vazquez, E., Nobles, M., and Valverde, M. A. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 5329–5334
13. Lidofoxy, S. D. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 7115–7119
14. Barford, E. T., Moore, A. L., and Lidofsky, S. D. (2001) Am. J. Physiol. 280, C836–C842
15. Bear, C. E., and Petersen, O. H. (1987) Pflugers Arch. 410, 342–344
16. Junanakar, P. R., Karjalainen, A., and Kirk, K. (2004) Cell Physiol. Biochem. 14, 143–154
17. Pon, D. C., and Hill, C. E. (1997) J. Cell Physiol. 171, 87–94
18. Thompson-Vest, N., Shimizu, Y., Hunne, B., and Farness, J. B. (2006) J. Anat. 208, 219–229
19. Barford, E. T., Moore, A. L., Melnick, R. F., and Lidofsky, S. D. (2005) J. Biol. Chem. 280, 25548–25557
20. Bodily, K., Wang, Y., Roman, R., Sostman, A., and Fitz, J. G. (1997) Hepatology 25, 403–410
21. Lidofoxy, S. D., and Roman, R. M. (1997) Am. J. Physiol. 273, G849–G853
22. Moore, A. L., Roe, M. W., Melnick, R. F., and Lidofsky, S. D. (2002) J. Biol. Chem. 277, 34030–34035
23. Wang, Y., Roman, R., Lidofsky, S. D., and Fitz, J. G. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 12020–12025
24. Kuzhikandathil, E. T., and Oxford, G. S. (2000) J. Gen. Physiol. 115, 697–706
25. Nyasae, L. K., Hubbard, A. L., and Tuma, P. L. (2003) Mol. Biol. Cell 14, 2689–2705
26. Fitz, J. G., Sostman, A. H., and Middleton, J. P. (1994) Am. J. Physiol. 266, G677–G684
27. Wulff, H., Miller, M. I., Hansel, W., Grissmer, S., Cahalan, M. D., and Chandy, K. G. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 8151–8156
28. Herrera, G. M., and Nelson, M. T. (2002) J. Physiol. 541, 483–492
29. Hayashi, K., Kuni, C., Takahata, T., and Ishikawa, T. (2004) Am. J. Physiol. 286, C635–C646
30. Ishii, T. M., Silvia, C., Hirschberg, B., Bond, C. T., Adelman, J. P., and Maylie, J. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 11651–11656
31. Neylon, C. B., Lang, R. J., Fu, Y., Bobik, A., and Reinhart, P. H. (1999) Circ. Res. 85, e33–e43
32. Simons, K., and Ehehalt, R. (2002) J. Clin. Invest. 110, 597–603
33. Meder, D., Moreno, M. J., Verkade, P., Vaz, W. L., and Simons, K. (2006) Proc. Natl. Acad. Sci. U. S. A. 103, 329–334
34. Kusumi, A., and Suzuki, K. (2005) *Biochim. Biophys. Acta* **1746**, 234–251
35. Manes, S., Mira, E., Gomez-Mouton, C., Lacalle, R. A., Keller, P., Labrador, J. P., and Martinez, A. C. (1999) *EMBO J.* **18**, 6211–6220
36. Kindzelskii, A. L., Sitrin, R. G., and Petty, H. R. (2004) *J. Immunol.* **172**, 4681–4685
37. Mazzone, A., Tietz, P., Jefferson, J., Pagano, R., and LaRusso, N. F. (2006) *Hepatology* **43**, 287–296
38. Caprini, M., Gomis, A., Cabedo, H., Planells-Cases, R., Belmonte, C., Viana, F., and Ferrer-Montiel, A. (2003) *EMBO J.* **22**, 3004–3014
39. Musch, M. W., Koomoa, D. L., and Goldstein, L. (2004) *J. Biol. Chem.* **279**, 39447–39453
40. Magee, A. I., Adler, J., and Parmryd, I. (2005) *J. Cell Sci.* **118**, 3141–3151
41. Sharma, D. K., Brown, J. C., Cheng, Z., Holicky, E. L., Marks, D. L., and Pagano, R. E. (2005) *Cancer Res.* **65**, 8233–8241
42. Begenisich, T., Nakamoto, T., Ovitt, C. E., Nehrke, K., Brugnara, C., Alper, S. L., and Melvin, J. E. (2004) *J. Biol. Chem.* **279**, 47681–47687
43. Sand, P., Anger, A., and Rydqvist, B. (2004) *Acta Physiol. Scand.* **182**, 361–368
44. Haussinger, D., Kurz, A. K., Wettstein, M., Graf, D., Vom Dahl, S., and Schliess, F. (2003) *Gastroenterology* **124**, 1476–1487
45. Lan, W. Z., Wang, P. Y., and Hill, C. E. (2006) *Am. J. Physiol.* **291**, C93–C103
46. Khanna, R., Chang, M. C., Joiner, W. J., Kaczmarek, L. K., and Schlichter, L. C. (1999) *J. Biol. Chem.* **274**, 14838–14849
47. Dykstra, M., Cherukuri, A., Sohn, H. W., Tzeng, S. J., and Pierce, S. K. (2003) *Annu. Rev. Immunol.* **21**, 457–481
48. Nicolau, S. A., Neumeier, L., Peng, Y., Devor, D., and Conforti, L. (2007) *Am. J. Physiol.,* in press
49. Bi, K., Tanaka, Y., Couadronniere, N., Sugie, K., Hong, S., van Stipdonk, M. J., and Altman, A. (2001) *Nat. Immunol.* **2**, 556–563
50. Webster, C. R., Blanch, C. J., Phillips, J., and Anwer, M. S. (2000) *J. Biol. Chem.* **275**, 29754–29760
51. Feranchak, A. P., Roman, R. M., Schwiebert, E. M., and Fitz, J. G. (1998) *J. Biol. Chem.* **273**, 14906–14911
52. Li, X., Leu, S., Cheong, A., Zhang, H., Baibakov, B., Shih, C., Birnbaum, M. J., and Donowitz, M. (2004) *Gastroenterology* **126**, 122–135
53. Srivastava, S., Li, Z., Lin, L., Liu, G., Ko, K., Coetzee, W. A., and Skolnik, E. Y. (2005) *Mol. Cell Biol.* **25**, 3630–3638
54. Golub, T., and Caroni, P. (2005) *J. Cell Biol.* **169**, 151–165
55. Leitinger, B., and Hogg, N. (2002) *J. Cell Sci.* **115**, 963–972
56. vom Dahl, S., Schlies, F., Reissmann, R., Gorg, B., Weiergraber, O., Kocalkova, M., Dombrowski, F., and Haussinger, D. (2003) *J. Biol. Chem.* **278**, 27088–27095
57. Carton, I., Hermans, D., and Eggermont, J. (2003) *Am. J. Physiol.* **285**, C935–C944
58. Veri, M. C., DeBell, K. E., Seminario, M. C., DiBallassarre, A., Reischl, L, Rawat, R., Graham, L., Noviello, C., Rellahan, B. L., Miscia, S., Wange, R. L., and Bonvini, E. (2001) *Mol. Cell Biol.* **21**, 6939–6950
59. del Pozo, M. A., Alderson, N. B., Kiosses, W. B., Chiang, H. H., Anderson, R. G., and Schwartz, M. A. (2004) *Science* **303**, 839–842
60. Villalba, M., Bi, K., Rodriguez, F., Tanaka, Y., Schoenberger, S., and Altman, A. (2001) *J. Cell Biol.* **155**, 331–338
61. Vom Dahl, S., and Haussinger, D. (1998) *Gastroenterology* **114**, 1046–1053
62. Carini, R., De Cesaris, M. G., Splendore, R., Bagnati, M., Bellomo, G., and Albano, E. (2000) *Biochim. Biophys. Acta* **1500**, 297–305