14-3-3-\(\tau\) Promotes Surface Expression of Cav2.2 (\(\alpha\)1B) Ca\(^{2+}\) Channels*

Received for publication, March 23, 2014, and in revised form, December 9, 2014. Published, JBC Papers in Press, December 16, 2014, DOI 10.1074/jbc.M114.567800

Feng Liu‡1, Qin Zhou‡1, Jie Zhou‡1, Hao Sun‡, Yan Wang‡, Xiuxin Zou‡, Lingling Feng‡, Zhaoyuan Hou‡, Aiwu Zhou*, Yi Zhou§, and Yong Li‡2

From the ‡Department of Biochemistry and Molecular Cell Biology, Shanghai Key Laboratory for Tumor Microenvironment and Inflammation, Institute of Medical Sciences, Shanghai Jiao Tong University School of Medicine, Shanghai 200025, China and the §Department of Biomedical Sciences, Florida State University College of Medicine, Tallahassee, Florida 32306

Background: The mechanism and dynamics of Cav channels trafficking remains mysterious.

Results: 14-3-3\(\tau\) promotes Cav2.2 trafficking independent of Cav auxiliary subunits.

Conclusion: 14-3-3\(\tau\) enhances Cav2.2 trafficking by masking the ER retention signal at its proximal C-terminal region.

Significance: Uncovering the regulation of Cav2.2 trafficking may contribute to understanding the Cav2.2 surface expression and functional control under physiological and pathophysiological conditions.

Surface expression of voltage-gated Ca\(^{2+}\) (Cav) channels is important for their function in calcium homeostasis in the physiology of excitable cells, but whether or not and how the \(\alpha\)1 pore-forming subunits of Cav channels are trafficked to plasma membrane in the absence of the known Cav auxiliary subunits, \(\beta\) and \(\alpha\)2\(\delta\), remains mysterious. Here we showed that 14-3-3 proteins promoted functional surface expression of the Cav2.2 \(\alpha\)1B channel in transfected tsA-201 cells in the absence of any known Cav auxiliary subunit. Both the surface to total ratio of the expressed \(\alpha\)1B protein and the current density of voltage step-evoked Ba\(^{2+}\) current were markedly suppressed by the coexpression of a 14-3-3 antagonist construct, pSCM138, but not its inactive control, pSCM174, as determined by immunofluorescence assay and whole cell voltage clamp recording, respectively. By contrast, coexpression with 14-3-3\(\tau\) significantly enhanced the surface expression and current density of the Cav2.2 \(\alpha\)1B channel. Importantly, we found that between the two previously identified 14-3-3 binding regions at the \(\alpha\)1B C terminus, only the proximal region (amino acids 1706–1940), closer to the end of the last transmembrane domain, was retained by the endoplasmic reticulum and facilitated by 14-3-3 to traffic to plasma membrane. Additionally, we showed that the 14-3-3/Cav \(\beta\) subunit coregulated the surface expression of Cav2.2 channels in transfected tsA-201 cells and neurons. Altogether, our findings reveal a previously unidentified regulatory function of 14-3-3 proteins in promoting the surface expression of Cav2.2 \(\alpha\)1B channels.

Voltage-gated Ca\(^{2+}\) (Cav) channels are classified into three subfamilies including Cav1 (Cav1.1–1.4), Cav2 (Cav2.1–2.3), and Cav3 (Cav3.1–3.3). The Cav2 subfamily channels are thought to be heteromultimers comprised of one pore-forming \(\alpha\)1 subunit (Cav2.1 (\(\alpha\)1A), Cav2.2 (\(\alpha\)1B), or Cav2.3 (\(\alpha\)1E)) together with auxiliary \(\beta\) and \(\alpha\)2\(\delta\) subunits. It is well established that the Cav auxiliary subunits not only alter the biophysical properties of the pore-forming \(\alpha\)1 subunit but also modulate cell surface expression of the channel complex (1–6). In particular, the Cav \(\beta\) subunit masks an unidentified endoplasmic reticulum (ER) retention signal on the \(\alpha\)1 subunit and thus increases the trafficking of Cav channels to the plasma membrane (7, 8). Moreover, the \(\beta\) subunits may promote the Cav1 and Cav2.2 channel expression by preventing their degradation via the proteasomal pathway (9, 10).

Although coassembly with accessory proteins represents an important means of controlling the surface expression of Cav2 subfamily channels, these channels are regulated by interacting with other proteins that can influence their expression, trafficking, subcellular localization, stabilization, and biophysical properties. Previously, we have identified a protein-protein interaction between the Cav2.2 channel and 14-3-3 proteins, which are a family of homologous proteins that generally binds to targets containing specific phosphoserine motifs and participates in the regulation of a wide range of biological processes, including facilitating surface expression of several classes of ion channels and receptors (11–19). We have demonstrated that 14-3-3 binding to the C-terminal region of the \(\alpha\)1B subunit leads to profound modulation of Cav2.2 channel inactivation kinetics (20, 21). However, a potential role of 14-3-3 proteins in Cav2.2 channel trafficking has yet to be investigated.

Here, we report that 14-3-3 also promotes the surface expression of Cav2.2 channels. This effect of 14-3-3 is independent of the Cav channel auxiliary subunits and may involve the regulation of forward transport between ER and Golgi. We further determined that 14-3-3 regulates Cav2.2 channel traf-
ficking by binding to a proximal region of the α1B C terminus. Together, our findings identify a novel function of the 14-3-3/Cav2.2 protein complex in channel trafficking.

EXPERIMENTAL PROCEDURES

cDNA Constructs and Mutagenesis—The construct encoding the full-length HA-tagged Cav2.2 α1B subunit was a generous gift from Dr. Emanuel Bourinet (Institut de Genomique Fonctionnelle, Montpellier, France). The HA epitope incorporated into this construct is located extracellularly within the S5–S6 linker of domain II. β3A-EGFP-α1B was provided by Dr. Gerald J. Obermair (22, 23). 14-3-3-3pEBFP-N1 and pcDNA3–14-3-3-7 were generous gifts from Dr. Yi Zhou (Florida State University College of Medicine, Tallahassee, FL). The plasmids for EYFP-fused doublet of R18 peptide (pSCM138) and the mutant peptide (pSCM174) were kindly provided by Dr. Haian Fu (Emory University). The truncation plasmids of the full-length HA-tagged Cav2.2 α1B subunit was a generous gift from Dr. Lan Bao (24).

Flag-Myc-tagged CT fragments were transiently transfected into tsA-201 cells. At 48 h post-transfection, cells were lysed in a buffer containing 20 mM Tris-HCl (pH 8.0), 150 mM NaCl, 2.5 mM EDTA, 0.5% Nonidet P-40, and 0.1 mM PMSF, and protease inhibitor mixture. The whole cell extracts were immunoprecipitated with anti-Flag beads (Sigma), and the coeluted pellets were resolved on SDS-PAGE and analyzed by Western blotting after transferring the resolved proteins to nitrocellulose membrane (Trans-Blot; Bio-Rad). Blots were probed with either anti-14-3-3 or anti-Flag antibodies, and the bands were detected by chemiluminescence using Lumigen PS-atto.

Immunofluorescence Assay—Cultured tsA-201 cells were transiently transfected using calcium phosphate transfection protocol according to the manufacturer’s instructions. Surface and total immunofluorescence staining of HA-α1B was performed as described (26) with minor modifications. Briefly, at least 2 days after transfection, cells were fixed at 37 °C for 8–10 min with 4% paraformaldehyde and 4% sucrose in PBS. The fixed cells were incubated in PBS containing either anti-HA or anti-Myc antibodies overnight at 4 °C to label surface proteins and then probed using Alexa Fluor 546-conjugated secondary antibodies for 1 h at room temperature. After surface staining, cells were permeabilized with 0.5% Triton X-100 in blocking solution containing 5% bovine serum in PBS for 30 min. Subsequently, intracellular HA-tagged α1B or Myc-tagged CD8α fusion protein was stained with the primary antibodies (either anti-HA or anti-Myc antibodies), followed by Alexa Fluor 633-conjugated secondary antibody for 1 h at room temperature to visualize intracellular HA-tagged α1B or Myc-tagged CD8α fusion protein. Fluorescence images were acquired using a Zeiss LSM-510 Meta confocal microscope (Zeiss, Germany) with a 40× 1.3NA oil immersion lens in the inverted position, performed with identical gain, contrast, laser excitation, pinhole aperture, and laser scanning speed for each round of cultures. Image processing was performed using Imagel software. Because the HA- or Myc-tagged protein is extracellular, signal intensity from nonpermeabilized cells was used as a measure of surface channel expression, and that from permeabilized cells was used as a measure of intracellular channel expression. The ratio of cell surface to total channel expression (surface expression to surface plus intracellular expression), representing either membrane:total HA-α1B or membrane:total Myc-CD8α, allowed comparisons between various conditions assayed from different batches of cells.

Antibodies, Coinmunoprecipitation, and Western Blot—The Western blotting and coimmunoprecipitation assays were performed as previously described with minor modifications (27). Plasmids encoding 14-3-3-7 and Flag-Myc-tagged CT fragments were transiently transfected into tsA-201 cells. At 48 h post-transfection, cells were lysed in a buffer containing 20 mM Tris-HCl (pH 8.0), 150 mM NaCl, 2.5 mM EDTA, 0.5% Nonidet P-40, 0.1 mM PMSF, and protease inhibitor mixture. The whole cell extracts were immunoprecipitated with anti-Flag beads (Sigma), and the coeluted pellets were resolved on SDS-PAGE and analyzed by Western blotting after transferring the resolved proteins to nitrocellulose membrane (Trans-Blot; Bio-Rad). Blots were probed with either anti-14-3-3 or anti-Flag antibodies, and the bands were detected by chemiluminescence using Lumigen PS-atto.

GTC, and R, TGTCTACCTCGCTGACTCTGTAAT (product length, 288 bp); α281 F, AACAGATCTAAACCGCTTGTGGGCCC, and R, ACCATGGAAGCTTGGAATAATGCG (product length, 398 bp); α282 F, ATTTGAGTGGTTAGTGGCAGATTTTTG, and R, GACATCTCAGCTCACTTCTTGGT (product length, 588 bp); and α283 F, GTGGGGAGTAAAATCCATGCGT, and R, GCTCCTTCAACTGCGACATCTTGTC (product length, 1377 bp); and α284 F, TTGATCCAGTGCTGACCTGACC, and R, ACAGATTGATGTCGCTCCTTCTC (product length, 269 bp).
Size Fractionation—The fractionation of the cell extracts was carried out according to the manufacturer's instructions. Briefly, the tsA-201 cells expressing 14-3-3\textsuperscript{t}, Flag-Myc-CT1 were lysed by sonication in buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.5% Nonidet P-40 and 1% dodecyl maltoside) supplemented with protease inhibitors. The lysates were then centrifuged at 4 °C at 10,000 rpm for 10 min. The supernatants were transferred to a fresh tube, and protein concentrations were determined by using the BCA protein assay kit (Thermo Scientific). 4–8 mg of protein was brought up to 0.5 ml of total volume using chromatography running buffer (PBS and 0.1% dodecyl maltoside, pH 7.4). The lysates were run on a Superose 6 10/300 GL size exclusion chromatography column (GE Healthcare) using the ÄKTA Purifier system (GE Healthcare), which was pre-equilibrated with PBS. After sample injection (using a 1-ml loop), the running buffer was set at a flow rate of 0.5 ml/min, and 1 ml per fraction was collected. Each fraction was separated by SDS-PAGE on 12% gels and fractionated proteins were detected with anti-Myc or 14-3-3 antibodies.

Primary Hippocampal Neuron Cultures—All animal procedures were carried out in accordance with the guidelines for the Care and Use of Laboratory Animals of Shanghai Jiao Tong University School of Medicine and approved by the Institutional Animal Care and Use Committee. Cultures of embryonic hippocampal neurons were prepared using a standard procedure. Briefly, hippocampi were dissected from embryonic day 18 rats (Sprague-Dawley) with the aid of a stereo microscope. Isolated hippocampi were digested with papain for 15 min at 37 °C. Isolated neurons were seeded on poly-d-lysine-precoated coverslips in appropriate density and cultured in neurobasal medium supplemented with B-27 and 0.5 mM glutamine at 37 °C and 5% CO\textsubscript{2}. Cultures were used after 7–10 days in vitro.

Electrophysiology—Calcium channel currents were recorded in tsA-201 cells as described previously (20) using the whole cell configuration of the patch-clamp technique. Briefly, a standard calcium phosphate protocol was used to transfect the cells with cDNAs encoding Cav2.2 α1B-EGFP, either alone or in combination with 14-3-3-pEBFP-N1, pSCM138, or pSCM174 as indicated. At least 2 days after transfection, Ca\textsuperscript{2+} channel currents were recorded from transfected cells with an EPC10 amplifier (HEKA). α1B- and 14-3-3-cotransfected cells bearing both green and blue fluorescence were identified with the FITC and blue fluorescent protein (BFP) filter sets on the Olympus IX71 inverted fluorescence microscope. To ensure that the BFP tag did not affect 14-3-3 function, similar experiments were performed by transfecting cells with α1B-pRc/CMV and 14-3-3-pIRES2-EGFP. Under this condition, recordings were done from green cells. Recording microelectrodes with resistances of 3–5 megohms were pulled from thin walled borosilicate glass with inner filament (Sutter Instrument) and filled with the intracellular solution containing 135 mM CsCl, 10 mM EGTA, 4 mM Mg\textsubscript{2+}-ATP, and 10 mM HEPES (pH 7.5 with CsOH). The extracellular solution contained 125 mM NaCl, 20 mM BaCl\textsubscript{2}, 1 mM MgCl\textsubscript{2}, and 10 mM HEPES (pH 7.3 with NaOH). Whole cell currents, which were filtered at 4 kHz and sampled at 50 kHz, were elicited by 400-ms depolarizing steps from −80 to +60 mV in 5-mV increments every 5 s from a holding potential of −80 mV (to allow full recovery from inactivation). Data analysis was performed using Clampfit 10.0 (Axon Instruments), Prism 5.0 (GraphPad), and Origin 8.0 (OriginLab) software. Current densities (pA/pF) were obtained for each cell by normalization of the whole cell current to cell capacitance to account for differences in cell membrane surface area. Activation curve were fitted using the Boltzmann equation: 

\[ G/G_{\text{max}} = \frac{1 + \exp[(V_m - V_{1/2})/k]}{1 + \exp[(V_m - V_{1/2})/k]}, \]

where \( G \) is conductance, \( G_{\text{max}} \) is the maximum conductance, \( V_m \) is membrane voltage, \( V_{1/2} \) is the half-activation potential, and \( k \) is the slope factor.

Statistical Analysis—Statistical analyses of surface and intracellular staining of HA-α1B and Myc-CD8α were performed using ImageJ software. The data are expressed as means ± S.E. with statistical significance assessed by Student's \( t \) test for two group comparison or one-way analysis of variance (ANOVA) tests for multiple comparisons. The value of \( p < 0.05 \) was considered to have statistically significant difference.

RESULTS

14-3-3\textsuperscript{t} Enhances Membrane Expression of Cav2.2 Independently of Cav Auxiliary Subunits—Functional Cav channel expression is known to be promoted by the auxiliary subunits (1, 2, 4–7, 9, 28). To specifically assess the effect of 14-3-3 proteins on surface expression of the Cav2.2 channel, we expressed Cav2.2 α1B subunits in tsA-201 cells in which neither endogenous β or α28 subunit was expressed (Fig. 1A and Ref. 25). In addition, we did not detect any voltage-gated Cav channel current in the absence of exogenously expressed α1B subunit (Fig. 1B), suggesting a lack of endogenous Cav channel activity in the tsA-201 cells.

14-3-3 proteins bind to the C terminus of Cav2.2 α1B subunit at two putative regions (20). To test whether the interaction with 14-3-3 affects Cav2.2 channel trafficking, we used a previously characterized high affinity 14-3-3 antagonist expression construct, pSCM138, which encodes EYFP-tagged R18 peptide dimer (29). The construct coding for the inactive mutant of the R18 peptide pSCM174 was used as a negative control (20, 29). The surface expression of Cav2.2 was examined by transfection into tsA-201 cells of a HA-tagged α1B construct (HA-α1B), in which the HA epitope was inserted into the second extracellular loop (between transmembrane segments 5 and 6) of the domain II transmembrane repeat of the α1B coding sequence. This allowed detection of surface expressed and total Cav2.2 α1B protein levels by immunofluorescence labeling under nonpermeabilized and permeabilized conditions, respectively. As shown in Fig. 1C, the surface expressed HA-α1B, as labeled by the anti-HA antibody and the Alexa Fluor 546-conjugated secondary antibody under nonpermeabilized conditions, was decreased by the cotransfection of pSCM138, but not pSCM174. Quantification of fluorescence intensity ratio (nonpermeabilized intensity to nonpermeabilized plus permeabilized intensity) revealed that inhibiting 14-3-3 by pSCM138 reduced the plasma membrane expression of Cav2.2 α1 subunit by ~40% (Fig. 1D). By contrast, coexpression of exogenous 14-3-3\textsuperscript{t} significantly enhanced the surface expression of HA-α1B, which was completely abolished by cotransfected pSCM138, but not pSCM174 (Fig. 1, C and D). The 14-3-3-dependent changes in the surface expression were also reflected
Regulation of Cav2.2 Surface Expression by 14-3-3τ

by alterations in the densities of Cav2.2 whole cell currents over a wide range of test potentials in the transfected tsA-201 cells. The current density of Cav2.2 was decreased by ~70% with cotransfected pSCM138, whereas it more than doubled with 14-3-3τ coexpression (Fig. 1E). Again, the enhancing effect of 14-3-3τ on Cav2.2 current density and the ratio of surface expressed Cav2.2 α1 subunit was completely prevented by the cotransfection with pSCM138, suggesting a requirement for 14-3-3 binding. All the 14-3-3 isoforms were tested in this study, 14-3-3τ exhibited quantitatively strongest ratio of sur-
Regulation of Cav2.2 Surface Expression by 14-3-3

14-3-3 regulates Endoplasmic Reticulum Retention of Cav2.2 α1B—14-3-3 proteins have been shown to modulate surface expression of several membrane proteins by interfering with the interaction between the coat protein I (COP1) complex and cargo. To determine whether such mechanism applies to 14-3-3-mediated surface expression of Cav2.2 α1B channel, we examined the influence of α1B C-terminal fragments on retention of the rat CD8α subunit, which is a transmembrane glycoprotein predominantly expressed on the cell surface. Two regions (CT1 and CT2, encompassing amino acids 1706–1940 and 2102–2220, respectively; Fig. 2A) of the α1B C terminus previously shown to bind to 14-3-3 were fused individually to the C terminus of rat CD8α that contained a Myc tag at its N terminus. Another α1B C-terminal fragment (Myc-CD8α-CT3; Fig. 2A) was tested in parallel as a negative control. Consistent with our previous finding (20), CT1 and CT2, but not CT3, exhibited robust binding to 14-3-3 as shown by coimmunoprecipitation between 14-3-3 and these Flag-Myc-tagged CT fragments in cotransfected tsA-201 cells (Fig. 2B). To further strengthen this observation, we analyzed 14-3-3 binding to Flag-Myc-tagged CT1 using size exclusion chromatography, which is another widely established method for assessing protein complexes. As shown in Fig. 2C, either the 14-3-3α or Flag-Myc-CT1 protein eluted in fractions that correspond to monomeric and/or dimeric forms of the respective proteins (Fig. 2C, fractions 17 and 18), whereas the mixture of Flag-Myc-CT1 and 14-3-3 proteins coeluted in fractions with higher molecular weights (Fig. 2C, fractions 16–18). These observations demonstrate that 14-3-3 and CT1 coexist in the same protein complex under this condition. Subsequently, we assessed the cell surface expression of the three Myc-CD8α fusion proteins by immunofluorescence labeling of the transfected tsA-201 cells under nonpermeabilized and permeabilized conditions. The ratio of membrane to total Myc-CD8α-CT1 was significantly lower than that of Myc-CD8α-CT2 and Myc-CD8α-CT3 (Fig. 2, D and E), suggesting that CT1 was able to obstruct CD8α from trafficking to the plasma membrane. The effect of CT1 on CD8α surface expression is in agreement with previous studies showing that the Cav2.2 C terminus contains an ER retention motif at its proximal region (7, 9). It thus raises a possibility that 14-3-3 may facilitate Cav2.2 channel trafficking by promoting its ER export.

To test this hypothesis, we first utilized the 14-3-3 antagonist to assess the participation of endogenous 14-3-3 proteins in forward transport of the Myc-CD8α and Cav2.2-CT fusion. Cotransfection of pSCM138, but not pSCM174, significantly reduced the surface expression of Myc-CD8α-CT1 (Fig. 3, A and B). Moreover, the level of surface Myc-CD8α-CT1 was increased by coexpression of 14-3-3α, an effect that was also abolished by cotransfection of pSCM138 (Fig. 3, A and B). By contrast, neither exogenous 14-3-3α, pSCM138 nor pSCM174 affected the surface expression of Myc-CD8α-CT2 (Fig. 3, C and D), which also binds to 14-3-3 but does not contain an ER retention motif (Fig. 2B). Together, these data suggest that 14-3-3α may promote forward transport of the Cav2.2 channel by masking the ER retention signal at its proximal C-terminal region (CT1).

In addition, we have previously showed that phosphorylation of a serine residue (Ser-2126) situated within CT2 of α1B was involved in the interaction with 14-3-3 proteins (20). Consistent with the idea that only CT1, but not CT2, of Cav2.2 α1B is involved in the ER retention regulated by 14-3-3, the current density of the α1B S2126A mutant was enhanced by 14-3-3α to a similar degree as that of the wild type channel (Fig. 4A), despite the obvious increase in the rate of current inactivation as previously described (20). Furthermore, coexpression of S2126α1B with the 14-3-3α antagonist construct pSCM138, but not its nonfunctional control pSCM174, dramatically reduced the current density regardless of whether 14-3-3α was coexpressed or not (Fig. 4B). These observations provide additional evidence to support that 14-3-3 binding to the CT1 region, but not the CT2 region, of the Cav2.2 α1B subunit is responsible for 14-3-3-mediated regulation of Cav2.2 surface expression.

14-3-3 and Cav β Subunit Coregulate Surface Expression of Cav2.2 Channels—As reported previously (20), 14-3-3 and the Cav β subunit can simultaneously bind to the α1B subunit. Considering that 14-3-3 and the β subunit may mask ER retention signals localized to different regions of the α1B subunit (7, 9), we anticipated the possibility that they could act in concert...
to regulate the surface expression of Cav α1 subunits. Thus, we compared the Cav current density in tsA-201 cells that were cotransfected Cav α1B and β1 with either pSCM138 or pSCM174. Coexpression of pSCM138, but not pSCM174, significantly decreased the current density of Cav2.2 channel (Fig. 5A), suggesting that endogenous 14-3-3 and Cav β1 subunit may coregulate the surface expression of Cav channels in tsA-201 cells. Next, we tested whether endogenous 14-3-3 and Cav β subunits comodulate Cav2.2 channels in neurons, where both Cav2.2 channels and 14-3-3 proteins are abundantly expressed (20). Cultured neurons were transfected with either pSCM138 or pSCM174. Two days following transfection, endogenous Ca2+ channel currents were recorded from these neurons with bath solution contained tetrodotoxin (1 μM) to block Na+.

FIGURE 2. An ER retention signal is present in the 14-3-3 binding region at the proximal C terminus of Cav2.2 α1B. A, schematic diagram showing the relative positions of the three C-terminal fragments [CT1 (red), CT2 (yellow), and CT3 (blue)]. Note: the green color represents the overlapping area between CT2 and CT3) of Cav2.2 α1B fused to the C terminus of Myc-CD8α or dual Flag-Myc epitope tags. B, association of α1B C-terminal fragments with 14-3-3. Lysates of tsA-201 cells cotransfected with 14-3-3 and Flag-Myc-tagged CT1, CT2, and CT3 were subjected to immunoprecipitation (IP) with the anti-Flag beads at 48 h post-transfection. The precipitants were analyzed by Western blotting using anti-14-3-3 or anti-Flag antibody as indicated. C, Western blots show the patterns of the 14-3-3, Flag-Myc-tagged-CT1, and the mixture of 14-3-3 and CT1 proteins eluted from Superose 6 10/300 GL size column. Whole cell extract (4 mg) was prepared from tsA-201 cells transiently expressing 14-3-3, and Flag-Myc-CT1 or their mixture and was loaded onto a Superose 6 10/300 GL gel filtration column. D, representative images of tsA-201 cells transfected with Myc-CD8α-tagged CT1, CT2, and CT3 by immunofluorescence assay under nonpermeabilized conditions as indicated. Scale bar, 10 μm. E, quantification of membrane total ratios of Myc-CD8α. ***, p < 0.001 compared with Myc-CD8α-CT3, one-way ANOVA followed by Tukey’s post hoc test. The data are means ± S.E. from four to six experiments; the total numbers of cells analyzed (n) ranged from 24 to 61 cells per condition.
channels and nifedipine (10 μM) and ω-agatoxin IVA (200 nM) to block Cav1 and Cav2.1 channels, respectively. Under these conditions, the inward currents are mediated primarily by Cav2.2 channels (20). Transfection of pSCM138, but not pSCM174, significantly reduced the density of endogenous Cav2.2 channel currents (Fig. 5B). This is in line with our obser-

FIGURE 3. 14-3-3 masks the ER retention effect of α1B CT1 fragment. A, representative images of surface stained Myc-CD8α-CT1 in tsA-201 cells transfected with Myc-CD8α-CT1 without (upper row) or with (lower row) 14-3-3 and together with vector, pSCM138, or pSCM174 as indicated. Scale bar, 10 μm. B, quantification of membranetotal ratios of Myc-CD8α. **, p < 0.01; ***, p < 0.001 compared with Myc-CD8α-CT1 alone or Myc-CD8α-CT1 plus 14-3-3; one-way ANOVA followed by Tukey’s post hoc test. The data are means ± S.E. from four to six experiments; the total numbers of cells analyzed (n) ranged from 20 to 40 cells per condition. C and D, similar to A and B, but Myc-CD8α-CT2 was used in place of Myc-CD8α-CT1. D represents data (means ± S.E.) from four to six experiments; the total numbers of cells analyzed (n) ranged from 33 to 61 cells per condition. Note: 14-3-3 only affected surface expression of Myc-CD8α-CT1, but not Myc-CD8α-CT2.

FIGURE 4. The S2126A α1B mutation did not affect the modulation of 14-3-3 on current density of Cav2.2 α1B. A, representative whole cell Ba2+ currents were elicited by voltage steps to 20 mV (left panel), current-voltage relationships (middle panel), and peak current densities at 20 mV (right panel) from tsA-201 cells expressing wild type α1B or its S2126A mutant without or with 14-3-3 (α1B, n = 17; S2126Aα1B, n = 22; α1B+14-3-3, n = 94; S2126Aα1B+14-3-3, n = 20). B, current-voltage relationships (left panel) and peak current densities at 25 mV (right panel) recorded from tsA-201 cells transfected with S2126Aα1B alone (n = 22), S2126Aα1B+pSCM138 (n = 14), S2126Aα1B+pSCM174 (n = 13), S2126Aα1B+14-3-3 (n = 20), S2126Aα1B+14-3-3+pSCM138 (n = 22), and S2126Aα1B+14-3-3+pSCM174 (n = 20). **, p < 0.01; ***, p < 0.001 compared with S2126Aα1B alone or S2126Aα1B plus 14-3-3; one-way ANOVA followed by the Tukey’s post hoc test.
vation in tsA-201 cells and indicates the involvement of endogenous 14-3-3 in promoting surface expression of Cav2.2 channels. Thus, these data support that the surface expression of Cav2.2 channels could be coordinately regulated by both 14-3-3 and Cav subunits.

DISCUSSION
Cav2.2-mediated signaling is determined by the channel abundance at the cell surface. Thus, appropriate cellular trafficking and localization are crucial for the physiological function of Cav2.2 channels. The foregoing results provide evidence and elucidate the possible mechanisms underlying the effect of 14-3-3 on Cav2.2 surface expression in transfected tsA-201 cells in the absence of known Cav auxiliary subunits. Coexpression of 14-3-3 led to enhanced surface expression of Cav2.2 α1B channels via binding of its proximal C-terminal region (CT1, amino acids 1706–1940) to the 14-3-3 protein, which may modulate the export of α1B from the ER. Consistent with the immunofluorescence assay, biochemical analysis and electrophysiological recordings in transfected tsA-201 cells demonstrated that the CT1 region of the Cav2.2 α1B C terminus accounted for the observed effect of 14-3-3 on Cav2.2 α1B surface expression. In addition, the 14-3-3/Cav β subunit coregulates the surface expression of Cav2.2 channels in transfected tsA-201 cells and neurons. Our data thus show a critical role for the CT1 region of Cav2.2 α1B C terminus in regulating channel trafficking to the plasma membrane, and this regulation appears to be mediated by 14-3-3 proteins via direct protein-protein interaction.

The Cav channels are thought to be heteromultimers composed of the pore-forming α1 subunit and auxiliary β and α2δ subunits. The β subunit has been proposed to both enhance the functional expression and influence the biophysical properties of the Cav1 and Cav2 channels. Whereas some studies have described that β subunit hyperpolarizes the voltage dependence of activation, increases the maximal open probability, and consequently results in increased current through individual channel and macroscopic current density (3, 10, 30, 31), others observed that the β subunit either promotes the insertion of Cav channels into the plasma membrane, as determined by gating charge measurements, imaging, and biochemical methods (7, 32–37) or has no effect on membrane insertion at all (38). The mechanism of the Cav subunit effect on surface expression has generally been attributed to masking an ER retention signal in the α1 subunits (7, 8). However, it was recently reported that the Cav β subunit enhances the channel translocation from ER to plasma membrane via preventing its degradation by the proteasomal pathway and thereby leading to increased cell surface expression of α1 subunits rather than masking an ER retention signal (9, 10). Our current results suggest that 14-3-3 enhances surface expression of Cav2.2 α1B by binding to the CT1 region at the α1B C terminus via possible masking its ER retention effect. This would fit well with the

FIGURE 5. **Endogenous 14-3-3 and Cav β subunit may coordinately regulate the surface expression of Cav2.2 channels.** A, representative whole cell Ba2+ currents were elicited by voltage steps to 20 mV (left panel), current-voltage relationships (middle panel), and peak current densities at 20 mV (right panel) from tsA-201 cells expressing Cav2.2 α1B and β1 with pSCM138 (n = 8) or pSCM174 (n = 9). *, p < 0.05, by Student’s t test. B, representative whole cell Ba2+ currents were elicited by voltage steps to 15 mV (left panel), current-voltage relationships (middle panel), and peak current densities at 15 mV (right panel) from cultured hippocampal neurons transfected with either pSCM138 (n = 11) or pSCM174 (n = 11). *, p < 0.05, by Student’s t test.
previous reports showing that the Cav β subunits mask an unidentified ER retention signal on the α1 subunit (7, 9). In this context, our findings support that 14-3-3 binding to CT1 of the Cav2.2 α1B C terminus is critical for mediating the surface expression of Cav2.2 channels via masking the ER retention signal at this site and consequently enabling the channel to escape the ER.

Our previous study revealed two putative 14-3-3 interaction sites in the C terminus of the α1B subunit, including a phosphoserine-containing motif that directly binds to 14-3-3 and an upstream region near the EF hand and IQ domain (20). Using immunofluorescence assay and electrophysiological recording, we confirmed that 14-3-3 binding to α1B C terminus is important for Cav2.2 channel surface expression, as reflected by decreases in the ratio of surface to total Cav2.2 α1B protein expression and the current density of the Cav2.2 channel caused by coexpression with the 14-3-3 antagonist, pSCM138 (Fig. 1, C–E). The mutant S2126A α1B subunit did not affect the effect of 14-3-3 on Cav2.2 current density (Fig. 4), suggesting that the Ser-2126 phosphoserine is not involved in the 14-3-3-mediated regulation on Cav2.2 α1B surface trafficking. This is consistent with the finding using Myc–CD8α fusion proteins that although both interact with 14-3-3 proteins, only CT1, but not CT2 (which contains Ser-2126), fragment of the α1B C terminus is involved in ER retention, the effect that can be masked by 14-3-3 proteins (Figs. 2 and 3). Taken together, our results indicate that only one of the putative 14-3-3-binding sites at α1B C terminus, the one closer to the last transmembrane domain (amino acids 1706–1940), contains the ER retention signal that is subjected to regulation by 14-3-3 proteins, which enable the channel to escape from the ER. The other more downstream 14-3-3-binding site that contains the phosphoserine may be more dedicated to regulation of biophysical properties of Cav2.2 channels (20).

Based on our previous study, 14-3-3 and Cav β subunits are known to simultaneously bind to the pore-forming α1B subunit of Cav2.2 (20). We showed that the current density of Cav2.2 channels can be regulated by both 14-3-3 and Cav β subunit in either tsA-201 cells or neurons (Fig. 5), suggesting that the surface expression of Cav2.2 could be coordinately regulated by Cav β subunit and 14-3-3. Certainly, these results may not be sufficient to establish a direct correlation between 14-3-3 and Cav β subunits. Therefore, it will be interesting to determine whether 14-3-3 and Cav β subunit might cooperatively or competitively regulate the surface expression of Cav2.2 channels and elucidate the detailed mechanism on 14-3-3-dependent modulation of the Cav2.2 channel trafficking in the future.

14-3-3 protein interaction with ion channels has been shown to not only regulate the functional properties of the ion channels (20, 39–41) but also to modulate their trafficking without affecting their biophysical properties (14–18). Interestingly, our previous and current results suggest that 14-3-3R modifies both function (20) and trafficking of Cav2.2 α1B channels. In light of the findings reported here, we propose a model for 14-3-3-mediated Cav2.2 α1B surface expression through a possible pathway that masks an ER retention signal and consequently enables the channel to escape the ER in the absence of known Cav auxiliary subunits. The proximal C-terminal region of α1B that binds to 14-3-3 proteins contains the ER retention motif important for the functional surface expression of the Cav α1B subunit. Together, 14-3-3 binding to the C-terminal ER retention signal of Cav2.2 α1B plays a critical role in the functionality of the Cav channel because of its regulation on channel protein trafficking to the cell surface. Uncovering the regulation of forward trafficking of Cav2.2 channels is pivotal for understanding the molecular mechanisms underlying their surface expression and functional control under physiological and pathophysiological conditions. This knowledge is fundamental for the development of therapeutic approaches to treat human diseases caused by dysfunctions in channel surface expression.

REFERENCES

1. Pragnell, M., De Waard, M., Mori Y, Tanabe, T., Snutch, T. P., and Campbell, K. P. (1994) Calcium channel β-subunit binds to a conserved motif in the I-II cytoplasmic linker of the α1-subunit. Nature 368, 67–70.
2. Birnbaumer, L., Qin, N., Olcese, R., Tareilus, E., Platano, D., Costantin, L. and Stefani, E. (1998) Structures and functions of calcium channel β subunits. J. Bioenerg. Biomembr. 30, 357–375.
3. Meir, A., Bell, D. C., Stephens, G. J., Page, K. M., and Dolphin, A. C. (2000) Calcium channel β subunit promotes voltage-dependent modulation of α1B by G βγ. Biochem J. 371, 731–746.
4. Dolphin, A. C. (2003) β Subunits of voltage-gated calcium channels. J. Bioenerg. Biomembr. 35, 599–620.
5. Opatowsky, Y., Chen, C. C., Campbell, K. P., and Hirsch, J. A. (2004) Structural analysis of the voltage-dependent calcium channel β subunit functional core and its complex with the α1 interaction domain. Neuron 42, 387–398.
6. Hidalgo, P., and Neely, A. (2007) Multiplicity of protein interactions and functions of the voltage-gated calcium channel β-subunit. Cell Calcium 42, 389–396.
7. Bichet, D., Cornet, V., Geib, S., Carlier, E., Volsen, S., Hoshi, T., Mori, Y., and De Waard, M. (2000) The I-II loop of the Ca۲⁺ channel α1 subunit contains an endoplasmic reticulum retention signal antagonized by the β subunit. J. Biol. Chem. 275, 177–190.
8. Cornet, V., Bichet, D., Sandoz, G., Marty, L., Brocard, J., Bourinet, E., Mori, Y., Villaz, M., and De Waard, M. (2002) Multiple determinants in voltage-dependent P/Q calcium channels control their retention in the endoplasmic reticulum. Eur. J. Neurosci. 16, 883–895.
9. Altier, C., Garcia-Caballerio, A., Simms, B., You, H., Chen, L., Walcher, J., Tedford, H. W., Hermosilla, T., and Zamponi, G. W. (2011) The Cavβ subunit prevents RFP2-mediated ubiquitination and proteasomal degradation of L-type channels. Nat. Neurosci. 14, 173–180.
10. Waite, D., Ferron, L., Page, K. M., Chaggar, K., and Dolphin, A. C. (2011) β-subunits promote the expression of Cav(V)2.2 channels by reducing their proteasomal degradation. J. Biol. Chem. 286, 9598–9611.
11. Cao, T. T., Deacon, H. W., Reczek, D., Bretscher, A., and von Zastrow, M. (1999) A kinase-regulated PDZ-domain interaction controls endocytic sorting of the β2-adrenergic receptor. Nature 401, 286–290.
12. Fu, H., Subramaniam, R. R., and Masters, S. C. (2000) 14-3-3 proteins: Structure, function, and regulation. Annu. Rev. Pharmacol. Toxicol. 40, 617–647.
13. Tzivion, G., and Avrutich, J. (2002) 14-3-3 proteins: Active cofactors in cellular regulation by serine/threonine phosphorylation. J. Biol. Chem. 277, 3061–3064.
14. O’Kelly, I., Butler, M. H., Zilberberg, N., and Goldstein, S. A. (2002) Forward transport. 14-3-3 binding overcomes retention in endoplasmic reticulum by dibasic signals. Cell 111, 577–588.
15. Rajan, S., Preisig-Müller, R., Wichaemeyer, E., Nehring, R., Hanley, P. J., Renigunta, V., Musset, B., Schlichthörl, G., Derst, C., Karschin, A., and Daut, J. (2002) Interaction with 14-3-3 proteins promotes functional expression of the potassium channels TASK-1 and TASK-3. J. Physiol. 545, 13–26.
16. Yuan, H., Michelsen, K., and Schwappach, B. (2003) 14-3-3 dimers probe
Regulation of Cav2.2 Surface Expression by 14-3-3π

the assembly status of multimeric membrane proteins. *Curr. Biol.* 13, 638–646

17. Heusser, K., Yuan, H., Neagoe, I., Tarasov, A. I., Ashcroft, F. M., and Schwappach, B. (2006) Scavenging of 14-3-3 proteins reveals their involvement in the cell-surface transport of ATP-sensitive K⁺ channels. *J. Cell Sci.* 119, 4353–4363

18. O’Kelli, L. C., and Goldstein, S. A. (2008) Forward transport of K(2P)3.1 and K(2P)9.1. *Traffic* 9, 72–78

19. Mant, A., Evert, D., Evers, P. A., and O’Kelli, I. M. (2011) Protein kinase A is central for forward transport of two-pore domain potassium channels K(2P)3.1 and K(2P)9.1. *J. Biol. Chem.* 286, 14110–14119

20. Li, Y., Wu, Y., and Zhou, Y. (2006) Modulation of inactivation properties of Cav2.2 channels by 14-3-3 proteins. *Neuron* 51, 755–771

21. Li, Y., Wu, Y., Li, R., and Zhou, Y. (2007) The role of 14-3-3 dimerization in its modulation of the Cav2.2 channel. *Channels (Austin)* 1, 1–2

22. Grabner, M., Dirksen, R. T., and Beam, K. G. (1998) Tagging with green fluorescent protein reveals a distinct subcellular distribution of L-type and non-L-type Ca²⁺ channels expressed in dysgenic myotubes. *Proc. Natl. Acad. Sci. U.S.A.* 95, 1903–1908

23. Obermair, G. J., Szabo, Z., Bourinet, E., and Flucher, B. E. (2004) Differential targeting of the L-type Ca²⁺ channel α1C (CaV1.2) to synaptic and extrasynaptic compartments in hippocampal neurons. *Eur. J. Neurosci.* 19, 2109–2122

24. Zhang, Z. N., Li, Q., Liu, C., Wang, H. B., Wang, Q., and Bao, L. (2008) The voltage-gated Na⁺ channel Nav1.8 contains an ER-retention/retrograde signal antagonized by the β3 subunit. *J. Cell Sci.* 121, 3243–3252

25. Yasuda, T., Chen, L., Barr, W., McRory, J. E., Lewis, R. J., Adams, D. J., and Zamponi, G. W. (2004) Auxiliary subunit regulation of high-voltage-activated calcium channels expressed in mammalian cells. *Eur. J. Neurosci.* 20, 1–13

26. Lee, S. H., Simonetta, A., and Sheng, M. (2004) Subunit rules governing the sorting of internalized AMPA receptors in hippocampal neurons. *Neuron* 43, 221–236

27. Sun, C., Qiao, H., Zhou, Q., Wang, Y., Wu, Y., Zhou, Y., and Li, Y. (2013) Modulation of GluK2α subunit-containing kainate receptors by 14-3-3 proteins. *J. Biol. Chem.* 288, 24676–24690

28. Carterall, W. A., Perez-Reyes, E., Snutch, T. P., and Striessnig, J. (2005) International Union of Pharmacology: XLVIII. nomenclature and structure-function relationships of voltage-gated calcium channels. *Pharmacol. Rev.* 57, 411–425

29. Wang, B., Yang, H., Liu, Y. C., Jelinek, T., Zhang, L., Rusolathi, E., and Fu, H. (1999) Isolation of high-affinity peptide antagonists of 14-3-3 proteins by phage display. *Biochemistry* 38, 12499–12504

30. Matsuyma, Z., Wakamori, M., Moriy, Y., Kawakami, H., Nakamura, S., and Imoto, K. (1999) Direct alteration of the P/Q-type Ca²⁺ channel property by polyglutamine expansion in spino-cerebellar ataxia 6. *J. Neurosci.* 19, RC14

31. Neely, A., Garcia-Olivares, J., Yoswielnk, S., Horstkott, H., and Hidalgo, P. (2004) Folding of active calcium channel β₂α₁-subunit by size-exclusion chromatography and its role on channel function. *J. Biol. Chem.* 279, 21689–21694

32. Josephson, I. R., and Varadi, G. (1996) The β subunit increases Ca²⁺ currents and gating charge movements of human cardiac L-type Ca²⁺ channels. *Biophys. J.* 70, 1285–1293

33. Kamp, T. J., Pérez-García, M. T., and Marban, E. (1996) Enhancement of ionic current and charge movement by coexpression of calcium channel β₁ subunit with α₁C subunit in a human embryonic kidney cell line. *J. Physiol.* 492, 89–96

34. Brice, N. L., Berrow, N. S., Campbell, V., Page, K. M., Brickley, K., Tedder, I., and Dolphin, A. C. (1997) Importance of the different β subunits in the membrane expression of the α1A and α2 calcium channel subunits: studies using a depolarization-sensitive α1A antibody. *Eur. J. Neurosci.* 9, 749–759

35. Allier, C., Dubel, S. I., Barrère, C., Jarvis, S. E., Stotz, S. C., Spatens, L. G., Scott, J. D., Cornet, V., De Ward, M., Zamponi, G. W., Nargeot, J., and Bourinet, E. (2002) Trafficking of L-type calcium channels mediated by the post-synaptic scaffolding protein AKAP79. *J. Biol. Chem.* 277, 33598–33603

36. Cohen, R. M., Foell, J. D., Balieppali, R. C., Shah, V., Hell, J. W., and Kamp, T. J. (2005) Unique modulation of L-type Ca²⁺ channels by short auxiliary β₁ subunit present in cardiac muscle. *Am. J. Physiol. Heart Circ. Physiol.* 288, H2363–H2374

37. Leroy, J., Richards, M. W., Butcher, A. J., Nieto-Rostro, M., Pratt, W. S., Davies, A., and Dolphin, A. C. (2005) Interaction via a key tryptophan in the I-II linker of N-type calcium channels is required for Bl but not for palmitoylated β₂, implicating an additional binding site in the regulation of channel voltage-dependent properties. *J. Neurosci.* 25, 6984–6996

38. Neely, A., Wei, X., Olcese, R., Birnbaumer, L., and Stefani, E. (1993) Potentiation by the β subunit of the ratio of the ionic current to the charge movement in the cardiac calcium channel. *Science* 262, 575–578

39. Kagan, A., Melman, Y. F., Krumerman, A., and McDonald, T. V. (2002) 14-3-3 proteins: implications for HERG K⁺ channel activity. *EMBO J.* 21, 1889–1898

40. Allouis, M., Le Bouffant, F., Wilders, R., Péroz, D., Schott, J. I., Noireaud, J., Le Marec, H., Mérot, J., Escande, D., and Baró, I. (2006) 14-3-3 binds to palmitoylated α₁C subunit in a human embryonic kidney cell line. *J. Biol. Chem.* 281, 7034–7041

41. Czirják, G., Vuitry, D., and Enyedi, P. (2008) Phosphorylation-dependent binding of 14-3-3 proteins controls TRESK regulation. *J. Biol. Chem.* 283, 15672–15680