G Protein-coupled Receptor Kinase 2-mediated Phosphorylation of Downstream Regulatory Element Antagonist Modulator Regulates Membrane Trafficking of Kv4.2 Potassium Channel*

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Downstream regulatory element antagonist modulator (DREAM)/potassium channel interacting protein (KChIP3) is a multifunctional protein of the neuronal calcium sensor subfamily of Ca2+-binding proteins with specific roles in different cell compartments. In the nucleus, DREAM acts as a Ca2+-dependent transcriptional repressor, and outside the nucleus DREAM interacts with Kv4 potassium channels, regulating their trafficking to the cell membrane and their gating properties. In this study we characterized the interaction of DREAM with GRK6 and GRK2, members of the G protein-coupled receptor kinase family of proteins, and their phosphorylation of DREAM. Ser-95 was identified as the site phosphorylated by GRK2. This phosphorylation did not modify the repressor activity of DREAM. Mutation of Ser-95 to aspartic acid, however, blocked DREAM-mediated membrane expression of the Kv4.2 potassium channel without affecting channel tetramerization. Treatment with the calcineurin inhibitors FK506 and cyclosporin A also blocked DREAM-mediated Kv4.2 channel trafficking and calcineurin de-phosphorylated GRK2-phosphorylated DREAM in vitro. Our results indicate that these two Ca2+-dependent posttranslational events regulate the activity of DREAM on Kv4.2 channel function.

G protein-coupled receptor kinases (GRKs)3 are a family of proteins that share the ability to selectively phosphorylate the ligand-activated form of G protein-coupled receptors, thus bringing about the recognition of the phosphorylated receptors by β-arrestins and its subsequent desensitization through different mechanisms (1). GRKs fall into three subgroups, one of which includes the two visual GRKs, GRK1 and GRK7 (1, 2). The non-visual GRK subgroups divide further into the GRK2 subfamily, consisting of GRK2 and 3, and the GRK4 subfamily, with GRK4, -5, and -6. Although GRK4 is expressed primarily in testis, other non-visual GRKs are ubiquitously expressed. GRKs share a characteristic tripartite modular architecture. The central ~350-amino acid kinase domain is similar to that of other serine/threonine kinases. The C-terminal domain is of variable length and serves to facilitate interactions with lipids and other membrane proteins and is sometimes subject to different post-translational modifications. The N-terminal domains of all GRK family members are homologous to the regulator of G protein signaling family of proteins, providing additional phosphorylation-independent mechanisms for the control of cell signaling. In this regard it has been reported that GRK2/GRK3 specifically bind the Goq subunit through the G protein signaling-like domain, which inhibits the interaction between Goq and phospholipase Cβ (3).

In recent years the role of GRKs has expanded with the identification of new substrates such as non-G protein-coupled receptors including platelet-derived growth factor receptors (4) and also non-receptor substrates such as synucleins (5), tubulin (6), phosducin (7), and ezrin (8) among others. Consistent with their key roles in cell function, multiple mechanisms exist to finely tune the activity, levels, and subcellular localization of GRKs. These mechanisms include intramolecular interactions, phosphorylation by different kinases, regulation of transcription and of protein stability, and interaction with different proteins (1, 9).

A common property of all three GRK subfamilies is their ability to interact with Ca2+ binding proteins of the EF-hand class, which reduces their kinase activity and provides a mechanism to terminate the receptor desensitization (for reviews, S-transferase; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid.
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see Refs. 10 and 11). This interaction is highly selective with respect to the different EF-hand proteins and GRK subtypes. Thus, calmodulin (CaM) binds with high affinity to members of the GRK4 subfamily, with low affinity to GRK2/3 members, and does not interact with GRK1 (12). Two CaM binding sites in the N- and the C-terminal domains of GRK5 and GRK2 are responsible for the interaction (13), which results in the case of GRK5 in autophosphorylation and reduced binding to phospholipids and to its receptor binding sites (14, 15). On the other hand, neuronal calcium sensors, including recoverin, neuronal calcium sensor-1, and visinin-like protein 1, have been described as specific regulators of GRK1 activity, with no effect on GRK2 or GRK5 activity (16, 17). It has been reported that GRK2 interacts with the neuronal calcium sensor-1, resulting in the modulation of GRK2-mediated desensitization of the D2 dopamine receptor (18). Thus, the study of the functional relationships between GRKs and calcium-binding proteins is an interesting field of research.

DREAM is a multifunctional protein of the neuronal calcium sensor subfamily of EF-hand Ca\(^{2+}\)-binding proteins with specific roles in different cell compartments (19, 20). In the nucleus, DREAM acts as a Ca\(^{2+}\)-dependent transcriptional repressor, binding to specific sites in the DNA in the absence of calcium stimulation. In addition, DREAM interacts with several nucleoproteins also in a Ca\(^{2+}\)-dependent manner to affect their transcriptional properties (21–23). Outside the nucleus, DREAM, also named KChIP3 (20), interacts with potassium channels of the Kv4 class directing their trafficking to and inside the plasma membrane (24) and regulating in a Ca\(^{2+}\)-dependent manner the gating properties of the channel (20). To regulate Kv4 trafficking in the plasma membrane, it has been shown that DREAM/KChIP3 needs to be palmitoylated at the cysteines in positions 45 and 46 (24). Mutation at cysteines 45 and 46 blocks the redistribution into the plasma membrane in the presence of Kv4 channels and abolishes the increase in current amplitude observed with wild type DREAM (24). Regulation of Kv4 channel gating depends on the conformation change in the DREAM/KChIP protein upon Ca\(^{2+}\) binding to the EF-hands (20). Thus, mutation in the EF-hands of DREAM/KChIP3 causes a dramatic loss of its ability to induce faster and more complete channel inactivation recovery (20). Conversely, palmitoylation at cysteines 45/46 does not influence the gating properties of the Kv4 channels (24). In addition, a recent study has shown that DREAM/KChIP3 participates in the tetramerization of Kv4 channels, restoring channel activity of Kv4.2 tetramerization mutants (25). Finally, in the cytosol DREAM binds to the C-terminal region of the presenilins (26) and blocks the release of Ca\(^{2+}\) from the endoplasmic reticulum and the apoptosis induced by presenilin mutants associated to Alzheimer disease (27).

In this study we describe the interaction between the neuronal calcium sensor DREAM/KChIP3 and GRK2 or GRK6 and analyzed the consequences of this interaction for different cellular functions of DREAM. We show that the phosphorylation state of DREAM at Ser-95 is regulated by GRK2 and calcineurin and that this is important for the regulation by DREAM of Kv4 channel cell surface expression.

EXPERIMENTAL PROCEDURES

Cell Cultures and Transfections—HEK 293 cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM; Sigma) containing 10% fetal bovine serum (FBS), Chinese hamster ovary cells in DMEM-F-12 (1:1) with 10% FBS, and PC12 cells in DMEM with 10% horse serum and 5% FBS. All media were supplemented with Glutamax and 1% penicillin-streptomycin (Invitrogen), and incubation was at 37 °C under 5% CO\(_2\) atmosphere. For coimmunoprecipitations, HEK 293 cells were cotransfected using calcium phosphate precipitation with DREAM and GRK cDNAs at a total amount of plasmid DNA of 10 μg. In some experiments, PC12 cells were treated with KCl (60 mM) or isoproterenol (100 nM) for 30 min. For electrophysiological recordings, HEK 293 cells were plated 2 days before transfection on 20-mm sterile glass coverslips at 50% confluency. Before transfection, cells were washed once with Dulbecco’s modified Eagle’s medium without fetal bovine serum. Two μg of DNA of interest and 1 μg of EGFP-DNA were used for transfection in serum-free conditions with Lipofectamine 2000 (Invitrogen). After 5 h, fetal bovine serum was added, and cells were recorded 35 h post-transfection. For confocal microscopy, HEK 293 cells on polylysine-coated glass coverslips in 35-mm plates were transfected using calcium phosphate precipitation with Fusiform fungus (26) and blocks the redistribution into the plasma membrane in the presence of Kv4 channels and abolishes the increase in current amplitude observed with wild type DREAM (24). Regulation of Kv4 channel gating depends on the conformation change in the DREAM/KChIP protein upon Ca\(^{2+}\) binding to the EF-hands (20). Thus, mutation in the EF-hands of DREAM/KChIP3 causes a dramatic loss of its ability to induce faster and more complete channel inactivation recovery (20). Conversely, palmitoylation at cysteines 45/46 does not influence the gating properties of the Kv4 channels (24). In addition, a recent study has shown that DREAM/KChIP3 participates in the tetramerization of Kv4 channels, restoring channel activity of Kv4.2 tetramerization mutants (25). Finally, in the cytosol DREAM binds to the C-terminal region of the presenilins (26) and blocks the release of Ca\(^{2+}\) from the endoplasmic reticulum and the apoptosis induced by presenilin mutants associated to Alzheimer disease (27).

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Coimmunoprecipitation and Pulldown Assays—For coimmunoprecipitation experiments, whole cell extracts were prepared in Nonidet P-40 lysis buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 1% Nonidet P-40) containing protease inhibitor mixture (Calbiochem). Cell membrane extract preparation was done as previously described (29). Coimmunoprecipitations were performed overnight at 4 °C using a mouse monoclonal antibody 1B1 for DREAM (22) or antibodies against GRK2 (28), GRK6 (C-20, Santa Cruz), and for the Myc-tagged GRK truncated constructs, the mouse monoclonal antibody (9E10) against c-Myc (Pharmingen). Immunocomplexes were captured with protein A/G-Sepharose (Amersham Biosciences) for 1 h, and pellets were washed three times in Nonidet P-40 lysis buffer. Protein complexes were eluted in SDS sample buffer and resolved by SDS-PAGE. Detection of DREAM after immunoprecipitation was performed with an affinity-purified rabbit polyclonal antibody (Ab1014) raised against recombinant full-length DREAM (30). For pulldown studies, GRK2 or GRK6 and its truncated forms were 35S-labeled in vitro using the transcription/translation T7-TNT system (Promega). Equimolar amounts, confirmed by PAGE and silver staining (data not shown), of recombinant GST and GST-DREAM proteins (20 pmol) bound to glutathione-Sepharose (Amersham Biosciences) were incubated with the labeled proteins in interaction buffer (20 mM K-Hepes, pH 7.5, 10% glycerol, 150 mM KCl, 2 mM MgCl2, 0.5 mM EGTA, 1 mM dithiothreitol, 0.1% Nonidet P-40, 0.5% Blotto (Bio-Rad)) containing protease inhibitor mixture (Calbiochem) and with the addition of 2 mM CaCl2, or 4 mM EDTA for calcium-dependence experiments. After five washes in the same buffer, bound proteins were eluted with SDS sample buffer, resolved in SDS-PAGE, detected by fluorography, and quantified using the NIH Image software. -Fold change was calculated by the ratio of glutathione-bound protein to GST protein alone.

Phosphorylation Assays—GRK activities were assessed by a rhodopsin phosphorylation assay as previously described (7). Phosphorylation of His-DREAM or GST-DREAM was performed in 50 μl of phosphorylation buffer (20 mM Tris-HCl, pH 7.5, 5 mM MgCl2, 1.4 mM EDTA, 1 mM EGTA, 80 μM [γ-32P]ATP (2–3 cpm/fmol)) in the presence of 20 mM pyrophosphate to minimize protein dephosphorylation during the reaction. Reactions contained the concentrations of recombinant GRK2, GRK6, GST-DREAM, or GST-DREAM mutants as indicated in the figure legends. In some experiments 0.8 μM heparin (Sigma) was included in the assay buffer. Reactions were started by exposure to fluorescent laboratory light. After incubation for indicated times at 30 °C, the reactions were terminated by the addition of 25 μl of SDS-sample buffer and heating for 5 min at 95 °C. Samples were resolved by 8–12.5% SDS-PAGE, and phosphorylated proteins were detected by autoradiography. Cerenkov counting of the excised bands was used to quantify the phosphorylation and determine stoichiometry. Dephosphorylation assays were initiated by adding 25 μl of 2× calcineurin buffer (20 mM Tris-HCl, pH 7.5, 200 mM NaCl, 5 mM MgCl2, 2 mM dithiothreitol, 2 mg/ml bovine serum albumin) with 1.6 mM heparin in the presence or absence of 1.5 mM CaCl2 and 6.7 mg/ml calmodulin plus 10 units of calcineurin for 1 h at 30 °C.

Two-dimensional Gel Electrophoresis and Mass Spectrometry Analysis—His-DREAM, 75 μg (analytical gel) or 1.5 mg (preparative gel for mass spectrometry analysis), was incubated with GRK2 (50 nM) for 2–3 h at 30 °C in phosphorylation buffer. Samples were concentrated by acetone precipitation before two-dimensional gel electrophoresis (7). Gels were stained with Coomassie Blue, and phosphorylated proteins were detected by autoradiography. Spots of interest were subjected to in situ trypsin digestion, and digested peptides were analyzed by matrix-assisted laser desorption/ionization time of flight mass spectrometry (31) by the Proteomics Service of Centro de Biología Molecular.

Electrophysiological Procedures—Patch pipettes contained 10 mM NaCl, 40 mM KCl, 1 mM MgCl2, 50 mM K2SO4, 10 mM K-HEPES, pH 7.25. Bath solution contained 140 mM NaCl, 5 mM KCl, 1 mM MgCl2, 2.5 mM CaCl2, 11 mM glucose, 5 mM caffeine, 10 mM Na-HEPES, pH 7.35. Coverslips were washed once with bath solution without caffeine and placed in a 500-μl chamber with bath solution. Whole cell currents were recorded using the nystatin-perforated patch mode, only green fluorescent cells were patched. Patch electrodes had a resistance of 2–4 megohms when filled with pipette solution. Current transients were evoked by applying a family of depolarizing pulses from a holding potential of −80 mV to +60 mV (10-mV steps, 1000-ms length) under voltage-clamp using an EPC-7 amplifier (Heka, Lambrecht, Germany). Data were sampled at 1 kHz, and further analysis was performed using pClamp 9 software (Axon Instruments, Foster City, CA). A similar pulse protocol was applied from a holding potential of −40 mV to subtract the Ik component of the current. All experiments were performed at room temperature.

Size Exclusion Chromatography—Cells were harvested and lysed on ice in Tris/NaCl-buffer, pH 7.5, containing 2% CHAPS, protease, and phosphatase inhibitors (Calbiochem). The cell extracts, 300 μl, were cleared by centrifugation and filtered through 0.45-μm filters before being applied to a Superose 6 analytical column (Amersham Biosciences) equilibrated in Tris/NaCl-buffer. Fractions of 400 μl were acetone-precipitated in the presence of trypsin inhibitor as a carrier. Protein pellets were resuspended in SDS-sample buffer and analyzed by PAGE and immunoblotting using an antibody against Kv4.2 (N-15, Santa Cruz). Blots were developed with chemiluminescence using SuperSignal West Dura (Pierce) or ECL Advance (Amersham Biosciences). The Superose column was calibrated with molecular weight standards (Amersham Biosciences) for estimation of the size of proteins in each fraction.

RESULTS

DREAM Interacts with GRK6 in a Ca2+- and Leucine-charged Residue-rich Domain (LCD)-dependent Manner—To disclose protein-protein interactions involving the DREAM protein that could reveal new functions in the cell, we screened a pretransformed bone marrow library using the yeast two-hybrid system. Previously, this approach resulted exclusively in His-DREAM, 75 μg (analytical gel) or 1.5 mg (preparative gel for mass spectrometry analysis), was incubated with GRK2 (50 nM) for 2–3 h at 30 °C in phosphorylation buffer. Samples were concentrated by acetone precipitation before two-dimensional gel electrophoresis (7). Gels were stained with Coomassie Blue, and phosphorylated proteins were detected by autoradiography. Spots of interest were subjected to in situ trypsin digestion, and digested peptides were analyzed by matrix-assisted laser desorption/ionization time of flight mass spectrometry (31) by the Proteomics Service of Centro de Biología Molecular.

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and we decided to use a DREAM mutant insensitive to Ca\(^{2+}\) (EFmDREAM) (19) as bait. Several positive interacting clones were selected and fully sequenced. Among them, one interaction, corresponding to a clone encoding an N-terminal truncation of the GRK6 kinase (GRK6\(\Delta N\)), called our attention and was further studied. Direct yeast two-hybrid with GRK6\(\Delta N\) and EFmDREAM confirmed the interaction; however, bait containing wild type DREAM did not result in a positive interaction in the yeast system (data not shown), suggesting that the interaction between DREAM and GRK6\(\Delta N\) might be sensitive to Ca\(^{2+}\). To further substantiate this observation we aimed to reproduce the Ca\(^{2+}\)-dependent interaction in pulldown assays using GST-DREAM and \textit{in vitro} translated \(^{35}\)S-labeled GRK6\(\Delta N\). In these experiments we observed a considerable nonspecific binding of GRK6\(\Delta N\) to GST alone; nevertheless, the binding to the fusion protein GST-DREAM was reproducibly 2-fold above the nonspecific binding in three independent experiments. An example is shown in Fig. 1A. The addition of 2 mM CaCl\(_2\) to the incubation did not affect the nonspecific interaction with GST but substantially reduced the interaction with GST-DREAM to background levels, supporting that the interaction is sensitive to calcium. To validate these results and to get a hint about the domains in GRK6 involved in the interaction with DREAM, we performed coimmunoprecipitation experiments after cotransfection in HEK 293 cells of DREAM and GRK6\(\Delta N\) or GRK6 truncated both at the N and C terminal (GRK6\(\Delta N\Delta C\)) (Fig. 1B). Transient cotransfection of DREAM and GRK6\(\Delta N\) reproduced the interaction previously observed in yeast and pulldown experiments (Fig. 1B); however, deletion of the C-terminal region in truncated GRK6\(\Delta N\Delta C\) blocked its ability to immunoprecipitate DREAM, indicating that the C-terminal region is important for the interaction (Fig. 1B). GRK6\(\Delta N\) did also not immunoprecipitate a mutant DREAM protein (DREAMmLCD\(_{1,2}\)), in which two LCDs, located at positions amino acids 47–52 and 155–159, have been inactivated (Fig. 1B). This result indicates that, as previously shown for the interactions between DREAM and nucleoproteins cAMP-response element-binding protein and \(\alpha\)-AMP-responsive element-modulator (21, 22), the LCDs in DREAM are important for the interaction with GRK6.

\textbf{DREAM Interacts with GRK2 in an LCD-dependent Manner}—To investigate the physiological relevance of the interaction between DREAM and GRK6, we first intended to reproduce the coimmunoprecipitation results with endogenously expressed proteins. For this, we used rat pheochromocytoma PC12 cells, shown to express high levels of DREAM (22), and several GRKs, including GRK6 and GRK2.\(^4\) In these experiments use of an antibody specific for GRK6 successfully immunoprecipitated endogenous DREAM both from whole cell extracts and membrane preparations (Fig. 2A). Use of an antibody specific for GRK2 also resulted in positive immunoprecipitation of DREAM from PC12 whole cell extract and from a membrane preparation (Fig. 2A). Notably, transient depolarization of PC12 cells with 60 mM extracellular KCl resulted in a reduced GRK2 coimmunoprecipitation with DREAM, supporting the notion of a Ca\(^{2+}\)-sensitive interaction between DREAM and GRK proteins (Fig. 2B). In addition, \(\beta\)-adrenergic receptor stimulation known to trigger \(\beta\)AR-GRK interaction and the internalization of the complex (1) also reduced the ability of GRK2 to coimmunoprecipitate with DREAM (Fig. 2B). The combined exposure to KCl and isoproterenol further reduced the interaction between DREAM and GRK2 as judged by the reduced coimmunoprecipitation (Fig. 2B). Taken together, these results indicate a physiological modulation of the DREAM/GRK2 interaction by calcium and \(\beta\)-adrenoreceptor stimulation.

In previous work from our laboratory we have shown that two LCDs in DREAM are essential for its interaction with \(\alpha\) or \(\varepsilon\)CAMP-responsive element modulator (21), whereas this interaction with cAMP-response element-binding protein that blocks CRE-dependent transcription requires exclusively the N-terminal LCD located between residues 47 and 52 (LCD\(_1\)) in the DREAM protein (22). As shown above, LCDs are important also for the interaction between DREAM and GRK6. To further characterize the DREAM/GRK2 interaction and especially the involvement of the LCD domains in DREAM, we performed pulldown experiments with GST-DREAM, wild type, or

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mutated in each of the two functional LCDs and $^{35}$S-labeled GRK2 (Fig. 2C). GST-DREAM efficiently pulled down GRK2, whereas mutation of the C-terminal LCD (LCD$_2$) in DREAM reduced 5-fold the interaction with GRK2 (Fig. 2C). Mutation of the DREAM N-terminal LCD (LCD$_1$) resulted in a 50% decreased interaction (Fig. 2B). Similar results were observed with GRK6 (data not shown), confirming the results shown in Fig. 1B.

DREAM Is a Substrate for GRK2 and GRK6—DREAM belongs to a family of small neuronal calcium sensors. A member of this family, the retinal protein recoverin, has been reported to interact with and to inhibit GRK1 (16). Therefore, we assessed whether DREAM is an inhibitor of GRK activity toward rhodopsin, a receptor substrate routinely used for determining GRK functionality. GST-DREAM (100 nM) did not inhibit light-dependent phosphorylation of rhodopsin (100 nM) by GRK2 (Fig. 3A); instead we noticed that GST-DREAM clearly became phosphorylated, suggesting that it could be a substrate for GRK2. Consistently, GST-DREAM was phosphorylated also by recombinant GRK2 in the absence of rhodopsin (Fig. 3, A, right lane, and B), and this phosphorylation was abolished in the presence of heparin (Fig. 3B), a GRK inhibitor (32). We then investigated whether DREAM could be phosphorylated also by GRK6. We found that, in our experimental conditions, an ~10-fold higher concentration of GRK6 compared with GRK2 was required to attain similar phosphorylation activity toward DREAM (Fig. 3C) despite the fact that GRK6 efficiently phosphorylated the soluble substrate phosvitin (Fig. 3D). Therefore, we focused our attention on the characterization of DREAM phosphorylation by GRK2.

Both GST-DREAM and His-DREAM were good substrates for GRK2, with slightly different stoichiometries, ~0.5 and ~0.86 mol Pi/mol protein, respectively. Phosphorylation of His-DREAM by GRK2 proceeded rapidly ($t_{1/2} = 5$ min) to a stoichiometry of ~0.8 when both proteins were present at a concentration of 25–50 nM (Fig. 4A). Double-reciprocal plot analysis revealed that GRK2 displayed an apparent $K_m$ for DREAM of $4.6 \pm 0.11$ nM, with a $V_{max}$ of $78.43 \pm 20$ nmol of Pi min$^{-1}$mg$^{-1}$ of protein (Fig. 4A). Comparison of these data with those reported for other GRK2 substrates (33, 34, 5, 7, 35, 6) strongly suggests that DREAM may constitute a good target for GRK2 in vivo. The interaction with GRK2 involves the LCDs domains of DREAM (see Figs. 1 and 2). Consistently, mutation
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FIGURE 4. Kinetics of DREAM phosphorylation by GRK2. A, time-course of His-DREAM phosphorylation by GRK2. His-DREAM (50 nM) was incubated with 25 nM recombinant GRK2 for the indicated times. Double-reciprocal plot analysis of GST-DREAM (B) and the double mutant GST-DREAM-LCD1,2 (C) phosphorylation by GRK2. Different concentrations of GST-DREAM wild type or mutant were incubated with 25 nM GRK2, and phosphorylation was analyzed and quantified. Data are the mean of three independent experiments.

FIGURE 5. Mutation of Ser-95 abolishes DREAM phosphorylation by GRK2. A, schematic representation of the DREAM protein showing the phosphorylation site for GRK2. B, indicated concentrations of GST-DREAM wild type or mutants in which serine 95 was substituted by alanine (GST-DREAM-S95A) or by aspartic acid (GST-DREAM-S95D) were incubated with 25 nM GRK2, and phosphorylation was analyzed. Results are representative of three independent experiments. wt, wild type. C, the purity of the fusion protein preparations was tested by gel electrophoresis.

of one or both LCD domains also affected the ability of GRK2 to phosphorylate DREAM (Fig. 4, B and C). Phosphorylation of the double LCD mutant was decreased by ~50% compared with wild type DREAM (data not shown), consistent with the reduction in the $V_{\text{max}}$ of the phosphorylation of GST-DREAM-LCD1,2 (0.86 pmol/min mg of protein) by GRK2 compared with wild type DREAM (2.16 pmol/min mg protein) (Fig. 4, B and C).

Identification of the GRK2 Phosphorylation Site in DREAM—To address the potential functional consequences of DREAM phosphorylation by GRK2, we next attempted to localize the phosphorylation site(s). In agreement with the stoichiometric data, two-dimensional gel electrophoresis analysis of DREAM phosphorylated by GRK2 suggested that the major part of DREAM incorporated a single phosphate (data not shown). To identify this preferential phosphorylation site, recombinant His-DREAM was incubated with recombinant GRK2, and mass spectrometry analysis identified a specific phospho-tryptic peptide, ELQS-LYR, that contained the serine residue at position 95 (Fig. 5A). An acidic residue is present in the vicinity of the suggested phosphorylation site, as has been observed in both plasma membrane receptors and cytoplasmic substrates of GRK2. The serine at position 95 as well as the acidic residue in its vicinity are conserved in the DREAM protein from several mammalian species. Mutation of this single serine residue to an alanine (GST-DREAM-S95A) or to an aspartic acid (GST-DREAM-S95D) substantially reduced the ability of GRK2 to phosphorylate DREAM over a wide range of DREAM and kinase concentrations (Fig. 5B and C). In conclusion, our data indicate that Ser-95 is the critical site for GRK2-mediated DREAM phosphorylation.

Phosphorylation of DREAM by GRK2 Does Not Affect Its Transcriptional Repressor Activity but Regulates Kv4 Potassium Channel Function—To understand the functional significance of GRK2-mediated DREAM phosphorylation at serine 95, we assayed S95A and S95D DREAM mutants for their capability to repress downstream regulatory element-dependent transcription and/or to regulate Kv4 channel conductance. Nuclear localization of several GRK family members has been described (36), although their role in the nucleus has not been clarified. We performed reporter assays after transient transfection with wild type DREAM or each of the Ser-95 mutants and compared their capability to repress basal expression of a DRE-containing reporter plasmid. No difference in repressor activity was observed between wild type and mutant DREAM (data not shown), indicating that phosphorylation at Ser-95 is not required for, and does not affect DREAM repressor function.

To analyze the effect of GRK-mediated DREAM phosphorylation on outward $I_h$ currents mediated by Kv4 potassium channels, we used transiently transfected HEK 293 cells. Cotransfection of wild type DREAM and Kv4.2 channels markedly increased peak current density from 0.25 ± 0.05 to 0.75 ± 0.1 nA/picofarad ($n = 9$) and slowed the inactivation time constant from 92.2 ± 12. to 340.4 ± 97.8 ms at 50 mV pipette potentials (Fig. 6A), in agreement with previously published data (20). Similar to wild type DREAM, coexpression of Kv4.2 channels with the S95A DREAM mutant resulted in a significant increase in peak current density; 0.43 ± 0.05 nA/picofarad ($n = 6$) and a slowdown of the inactivation time constant, 273.9 ± 66.5 ms ($n = 9$), as compared with channel alone (Fig. 6, B and C). Interestingly, cotransfection with the DREAM-S95D mutant, which incorporates a permanent negative charge at position 95, decreased the peak current density by 50%, 0.31 ± 0.07 nA/picofarad ($n = 6$) compared with Kv4.2 channel alone, whereas the inactivation time constant of the channels incorporated into the membrane, 140 ± 54.8 ms, was not different compared with values from Kv4.2 channel alone (Fig. 6, B and C), suggest-
ing that DREAM phosphorylated at Ser-95 lacks the property to modify Kv4.2 channel gating. Cotransfection of Kv4.2 channels with a palmitoylation-deficient mutant DREAMC45,46 S, known to induce a poor incorporation of Kv4 channels in the plasma membrane (24), reduced the net conductance through Kv4.2 channels but did not differ from wild type DREAM in the regulation of channel gating (data not shown), as previously described (24). Taken together, these data suggest that phosphorylation at Ser-95 interferes with the cell surface expression of Kv4.2 channels. In addition, our results with the S95D mutant may suggest that endogenous DREAM, phosphorylated at Ser-95, needs to be dephosphorylated to modulate channel gating.

**Phosphorylation of DREAM by GRK2 Regulates Trafficking of Kv4 Channels to the Plasma Membrane** —Increased current expression of Kv4 channels at the plasma membrane after cotransfection with KChIP auxiliary proteins is a complex process involving enhanced channel assembly, enhanced folding of the assembled channels, and increased surface expression of the channel complex (37, 38, 24, 39, 40).

To investigate whether the reduced net conductance observed after cotransfection with S95D DREAM mutant could be related to a deficit of channel expression at the plasma membrane, we analyzed the subcellular localization of DREAM-EGFP fusion proteins in the absence or in the presence of Kv4.2. Overexpression of wild type or Ser-95 DREAM-EGFP mutants displayed a randomly distributed fluorescence signal throughout the cell body (Fig. 7). After cotransfection with Kv4.2 and wild type DREAM-EGFP, the signal was accumulated at the plasma membrane (Fig. 7), a pattern similar to what has been previously described (24, 39). Importantly, cotransfection of the DREAM-S95D-EGFP with Kv4.2 did not result in translocation of the fluorescence signal to the plasma membrane (Fig. 7). These results support the notion that GRK2-mediated phosphorylation of Ser-95 in DREAM prevents membrane localization of Kv4.2 and that the negative charge in the DREAM-S95D mutant mimics this activity. Cotransfection with the DREAMS95A-EGFP mutant or the negative control DREAMC42S,C43S-EGFP did not result in an enrichment of the fluorescent signal in the membrane, although these two mutants increased current density compared with channel alone (Figs. 6 and 7).

**GRK2-dependent Phosphorylation of DREAM Does Not Affect Kv4 Channel Tetramerization** —Because the S95D mutant reduced the current density with respect to Kv4.2 channel transfected alone, it is tempting to speculate that this
mutant plays an active role, different from the S95A mutant, blocking membrane expression of the channel. Thus, we hypothesized that the negative charge at position 95 that is provided by phosphorylation would be needed at some step during the trafficking of the channel from the endoplasmic reticulum and later must be removed to allow for the correct expression of the KChIP-channel complex in the plasma membrane. To explore this possibility, we used a Kv4.2 tetramerization mutant (ZnB3) unable to incorporate into the plasma membrane because of its incapacity to multimerize within the endoplasmic reticulum, the first event before the channel can be carried toward the plasma membrane (40). Coexpression of DREAM with the Kv4.2-ZnB3 mutant, however, rescues the tetramerization of the channel (25). Thus, we coexpressed the mutant channel together with Ser-95 DREAM mutants and analyzed the formation of tetrameric complexes. Coexpression of wild type DREAM or the Ser-95 mutants rescued tetramerization of mutant channel (Fig. 8 and data not shown), suggesting that the phosphorylation of DREAM by GRK2 interferes with membrane trafficking of Kv4 channels at a later stage.

Calcineurin Regulates DREAM-mediated Kv4 Trafficking—Because the effect on channel activity is a Ca2+-dependent function of DREAM (20) and our results suggest that DREAM has to be dephosphorylated for the proper membrane expression of the channel, we analyzed the involvement of the Ca2+-dependent phosphatase calcineurin in this process. We exposed transfected cells to a mixture of the calcineurin inhibitors cyclosporin A (10 μM) and FK506 (0.1 μM) and measured current density as an index of membrane expression of the channel. Treatment with calcineurin inhibitors blocked the increase in current density induced by DREAM without modifying the current density of the channel alone (Fig. 9A). The effect of calcineurin inhibitors was specific for current density since neither the action of DREAM on channel gating was affected (data not shown) nor was the rescue by DREAM of Kv4 tetramerization mutant modified (Fig. 8B). Analysis of membrane translocation of DREAM-EGFP in the presence of calcineurin inhibitors showed a reduction of the signal at the membrane and a patchy and irregular distribution at the submembranous space (Fig. 9C).

A similar patchy distribution at the submembranous space was shown for the calcium, insensitive KChIP-1 mutant after coexpression with Kv4 channels (41), and since the DREAM-GRK2 interaction is sensitive to calcium, we hypothesized that the low membrane expression of the channel could be related to the inability of DREAM to unbind from the kinase resulting in permanent phosphorylation. Coexpression of the calcineurin insensitive DREAM mutant (EFmDREAM) and the Kv4.2 channel resulted in a diffuse intracellular distribution (Fig. 10A) that reproduced the lack of membrane localization described for the KChIP-1 mutant (41). Overexpression of calcineurin, however, rescued the membrane expression of the calcium insensitive mutant (Fig. 10A), supporting the idea that a coupled phosphorylation-dephosphorylation step controls the trafficking of Kv4 channels to the membrane. Furthermore, recombinant calcineurin efficiently dephosphorylated GRK2-mediated DREAM phosphorylation (Fig 10B).

DISCUSSION

Specific regulation of distinct functions in the context of a multifunctional protein might be achieved by highly selective posttranslational modifications to disclose one or another particular property in a specific cell compartment. Here we show that phosphorylation of DREAM at Ser-95 reduces the membrane expression of Kv4 potassium channels, an effect that is mimicked by exposure to inhibitors of the Ca2+-dependent phosphatase calcineurin. In addition, our in vitro data indicate that calcineurin directly dephosphorylates Ser-95 phospho-DREAM whether calcineurin also acts on different residues in DREAM or in other targets related to Kv4 channel trafficking in vivo is presently not characterized. Calcineurin inhibitors, however, did not modify membrane expression and channel gating of Kv4.2 channels expressed alone, suggesting that the reduc-
Calcineurin dephosphorylates DREAM and regulates cell surface localization. A, confocal microscopy showing the subcellular localization of EGFP-DREAM, wild-type, and the calcium-insensitive mutant (EFm-DREAM) proteins. Membrane expression of EFmDREAM in HEK 293 cells is only observed in the presence of calcineurin. The experiment was repeated three times. B, in vitro dephosphorylation by calcineurin of GRK2 phosphorylated DREAM is potentiated in the presence of calcium/CaM.

FIGURE 10. Calcineurin dephosphorylates DREAM and regulates cell surface localization. A, confocal microscopy showing the subcellular localization of EGFP-DREAM, wild-type, and the calcium-insensitive mutant (EFm-DREAM) proteins. Membrane expression of EFmDREAM in HEK 293 cells is only observed in the presence of calcineurin. The experiment was repeated three times. B, in vitro dephosphorylation by calcineurin of GRK2 phosphorylated DREAM is potentiated in the presence of calcium/CaM.

Current experimental evidence indicates that Kv4.2 is the channel forming primary subunit that underlies transient outward A-type K⁺ currents in neurons (42, 43) and in cardiac myocytes (44, 45). Previous studies have shown that protein kinase A, protein kinase C, and extracellular signal-regulated kinase/mitogen-activated protein kinase modulate transient outward A-type K⁺ currents in dendrites of hippocampal CA1 pyramidal neurons (46–52). Experiments performed after transfection in heterologous systems have demonstrated that kinase-mediated regulatory events are dependent on the presence of KChIP proteins (53, 54). Of the kinases that modify A-type currents, it has been shown that protein kinase A phosphorylates DREAM in vitro at serines 11 and 14. The physiological relevance of this phosphorylation, however, is not clear since single S14A or double S11A,S14A mutants had little or no effect on the steady state of inactivation and did not change the activation voltage when the mutant DREAM was coexpressed with Kv4.2 (53). More importantly, mutation of these residues did not block the effect of protein kinase A activation on the modulation of the properties of the Kv4.2/DREAM complex (53), indicating that phosphorylation at these residues in vivo does not mediate the protein kinase A effect on channel function. In contrast, phosphorylation of Kv4.2 channels at Ser-552 by protein kinase A was essential for forskolin-induced channel modulation (53). Moreover, phosphorylation of Kv4.2 channels by extracellular signal-regulated kinase at Thr-607 caused a rightward shift in the activation voltage and slowed the rate of recovery from inactivation, as shown by the expression of the T607D mutant in which a negative charge from the aspartate residue resembles Kv4.2 phosphorylation (54). Noteworthy, in all cases phosphorylation of the channel by protein kinase A or extracellular signal-regulated kinase/mitogen-activated protein kinase specifically modified channel gating properties without affecting trafficking or current expression of the channel at the plasma membrane (53, 54). These results further support the notion that the effect of calcineurin on current density is not related to an action on the channel protein itself.

Phosphorylation of DREAM by a kinase downstream from phosphoinositide 3-kinase/Akt has been shown to regulate binding of DREAM to DNA and DREAM nuclear repressor activity of the expression of the hrk gene (55). GRK2-mediated phosphorylation at serine 95 in DREAM, however, does not modify its repressor action. The nuclear and cytosolic functions of the DREAM protein are, thus, specifically regulated by at least these two independent kinases.

Increased current expression of Kv4 channels at the plasma membrane after cotransfection with KChIP auxiliary proteins is a complex process involving enhanced channel tetramerization at the endoplasmic reticulum, enhanced folding of the assembled channels, and increased surface expression of the channel complex (24, 37–40). The ability of wild type DREAM to rescue channel assembly of Kv4 tetramerization mutants (25) is conserved in the two serine 95 mutants, suggesting that Ser-95 phosphorylation occurs later or, more generally, does not interfere with the tetramerization process. Notably, calcineurin inhibition by exposure to FK506 and cyclosporin A did not affect the ability of wild type DREAM to rescue channel tetramer formation, again suggesting that GRK2 phosphorylation of serine 95 and calcineurin inhibition might share a common downstream mechanism to block membrane expression of the Kv4.2/DREAM complex. In vitro dephosphorylation of Ser-95 phospho-DREAM by calcineurin supports this notion. Alternatively, it could be hypothesized that calcineurin also acts on a separate protein participating at any of the steps necessary to guide and integrate the Kv4.2/KChIP complex in the membrane. Whether this is the case and whether some of the many other interacting proteins known to modulate Kv4.2 channel function could be the target for calcineurin remains to be investigated.

Mutation of the palmitoylation sites in DREAM and in KChIP2 induce a poor current expression of the channel at the plasma membrane (24). Thus, palmitoylation and Ser-95 phosphorylation have different effects on channels trafficking, indicating that membrane expression of Kv4 channels is a finely tuned process. In addition, it has been recently shown that KChIP-1 interacts with Kv4 potassium channels outside the endoplasmic reticulum at some stage during endoplasmic reticulum-to-Golgi or intra-Golgi traffic and that a correct incorporation in the Golgi complex requires a Ca²⁺-dependent conformational change through the functional EF-hands in the KChIP-1 protein (41). Mutation at the EF-hands in KChIP-1, thus, blocks the transport of Kv4 into the Golgi and directs the

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KChIP-channel complex into intracellular organelles of the endoplasmic reticulum-Golgi intermediate compartment that never progresses toward the plasma membrane (41). Whether Ser-95 phospho-DREAM is temporarily retained in the same intermediate compartment or in a different one is currently not characterized. Nevertheless, overexpression of calcineurin rescues the ability of EFM-DREAM to direct channel expression to the plasma membrane, suggesting that a phosphorylation-related mechanism is mediating the retention of the calcium insensitive mutant in intracellular organelles.

It is known that neuronal calcium sensors share the ability to interact with GRK proteins and to modulate their kinase activity (10, 11). Our results show, however, that in the case of DREAM the interaction results in a specific phosphorylation of DREAM without an apparent effect on the GRK kinase activity. The interaction and subsequent phosphorylation of DREAM by GRKs may provide an additional mechanism for the control of Kv4.2 channel activity by extracellular signals. In addition, we demonstrate that in PC12 cells, by modulating Ca\(^{2+}\) levels or by promoting the recruitment of GRKs by receptor stimulation, the GRK/DREAM association is altered, thus modifying its regulation of Kv4.2 channels. In this regard, it is worth noting that GRK2 and DREAM are expressed in tissues where Kv4.2 channels play a relevant physiological role. GRK2 is highly expressed in nerve terminals, including a peripheral association to the plasma membrane and the endoplasmic reticulum (28). The precise subcellular compartment where GRK2 phosphorylates DREAM and the identification of the receptors and signaling pathways that promote this reaction are critical issues that remain to be established.

Sequence analysis of the other members of the DREAM/KChIP subfamily of calcium sensors did not reveal the existence of an equivalent serine residue in any of the other KChIPs, suggesting that GRKs, especially GRK2, specifically phosphorylates DREAM, modulating its action on Kv4.2 channel function. Whether GRKs can phosphorylate other KChIPs on serine or threonine residues different from Ser-95 in DREAM and regulate their modulation of Kv4 channels in a similar way remains to be investigated. Similarly, future experiments using knock-in mice in which wild type DREAM is replaced by S95D DREAM will unequivocally help to understand the in vivo functional consequences of GRK-mediated DREAM phosphorylation and if this results in a permanent block of the membrane expression of Kv4.2 channels in different cells and organs.

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