Analysis of the Complex between Ca\(^{2+}\) Channel \(\beta\)-Subunit and the Rem GTPase*

Received for publication, May 19, 2006. Published, JBC Papers in Press, June 21, 2006, DOI 10.1074/jbc.M604867200

Brian S. Finlin, Robert N. Correll, Chunyan Pang, Shawn M. Crump, Jonathan Satin, and Douglas A. Andres

From the Departments of Molecular and Cellular Biochemistry and Physiology, University of Kentucky College of Medicine, Lexington, Kentucky 40536-0509

Voltage-gated calcium channels are multiprotein complexes that regulate calcium influx and are important contributors to cardiac excitability and contractility. The auxiliary \(\beta\)-subunit (Ca\(_{\alpha}\beta\)) binds a conserved domain (the \(\alpha\)-interaction domain (AID)) of the pore-forming Ca\(_{\alpha}\) subunit to modulate channel gating properties and promote cell surface trafficking. Recently, members of the RGK family of small GTPases (Rem, Rem2, Rad, Gem/Kir) have been identified as novel contributors to the regulation of L-type calcium channel activity. Here, we describe the Rem-association domain within Ca\(_{\alpha}\). The Rem interaction module is located in a ~130-residue region within the highly conserved guanylate kinase domain that also directs AID binding. Importantly, Ca\(_{\alpha}\beta\) mutants were identified that lost the ability to bind AID but retained their association with Rem, indicating that the AID and Rem association sites of Ca\(_{\alpha}\beta\) are structurally distinct. In vitro binding studies indicate that the affinity of Rem for Ca\(_{\alpha}\beta\) interaction is lower than that of AID for Ca\(_{\alpha}\beta\). Furthermore, in vitro binding studies indicate that Rem association does not inhibit the interaction of Ca\(_{\alpha}\beta\) with AID. Instead, Ca\(_{\alpha}\beta\) can simultaneously associate with both Rem and Ca\(_{\alpha}\) to form AID. Previous studies had suggested that RGK proteins may regulate Ca\(^{2+}\) channel activity by blocking the association of Ca\(_{\alpha}\beta\) subunits with Ca\(_{\alpha}\), to inhibit plasma membrane trafficking. However, surface biotinylation studies in HIT-T15 cells indicate that Rem can acutely modulate channel function without decreasing the density of L-type channels at the plasma membrane. Together these data suggest that Rem-dependent Ca\(^{2+}\) channel modulation involves formation of a Rem-Ca\(_{\alpha}\beta\)-AID regulatory complex without the need to disrupt Ca\(_{\alpha}\)-Ca\(_{\alpha}\) association or alter Ca\(_{\alpha}\)-expression at the plasma membrane.

Voltage-dependent calcium channels regulate Ca\(^{2+}\) entry to control numerous cellular functions, including muscle contrac-

* This work was supported by Public Health Service Grants HL072936 (to D. A. A.), HL074091 (to J. S.), and P20 RR20171 from the National Center for Research Resources, National Institutes of Health (to D. A. A.), an American Diabetes Association Junior Faculty award (to B. S. F.), and an American Heart Association pre-doctoral fellowship (to R. N. C.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 These authors contributed equally to this work.
2 An Established Investigator of the American Heart Association.
3 To whom correspondence should be addressed: Dept. of Molecular and Cellular Biochemistry, BBSRB Rm. B-179, University of Kentucky College of Medicine, 741 South Limestone St., Lexington, KY 40536-0509. Tel.: 859-257-6775; Fax: 859-323-1037; E-mail: dandres@pop.uky.edu.

4 The abbreviations used are: AID, \(\alpha\)-interaction domain; RGK proteins, Rem, Rem2, Rad, and Gem/Kir GTPases; GK, guanylate kinase domain; SH3, Src homology 3 domain; ABP, \(\alpha\)-subunit binding pocket; GST, glutathione S-transferase; HEK, human embryonic kidney; GFP, green fluorescent protein; BID, \(\beta\)-interaction domain; HA, hemaggglutinin; GTP\(\alpha\)S, guanosine 5’-O-(thiotriphosphate) or guanosine 5’-O-(thiophosphosphate); WT, wild type; pF, picofarad.
inhibit voltage-gated Ca\(^{2+}\) channel activity in a manner that appears to depend upon β-subunit interaction (21–23, 31–33), many issues remain concerning their mechanism of action. Although several recent studies have indicated that the expression of RGK GTPases inhibits the trafficking of co-transfected epitope-tagged Ca\(_{\alpha_1}\)α subunits to the plasma membrane (21, 26, 34), we have shown that expression of Rem2 can modulate both endogenous Ca\(^{2+}\) channel activity and glucose-dependent insulin release in insulinoma cells without obviously altering membrane expression of the endogenous channel (23). Moreover, overexpression of Rem2 had no effect on the surface density of N-type Ca\(^{2+}\) channels stably expressed in tsA201 cells while potently inhibiting channel function (32). Thus, questions concerning the nature of the RGK-β subunit association and its role in RGK-dependent regulation of Ca\(^{2+}\) channel activity and trafficking remain to be addressed.

In the present study we define a region located within the conserved GK domain as being critically involved in the interaction of β subunits with Rem. Biochemical characterization indicates that the Rem-β subunit interaction is of relatively low affinity, with competitive binding studies demonstrating that Rem fails to effectively compete with AID for β-subunit association. Moreover, Rem can enter into a Ca\(_{\alpha_1}\)β\(_{2a}\)-AID complex, establishing that Ca\(_{\alpha_1}\)β-Rem binding is mechanistically and structurally distinct from Ca\(_{\alpha_1}\)β-AID binding. Taken together, these data indicate that Rem does not inhibit channel activity by competing with Ca\(_{\alpha_1}\)α subunits for a limiting intracellular pool of uncomplexed Ca\(_{\alpha_1}\)β subunits. In support of this hypothesis, surface biotinylation studies indicate no change in the number of surface-exposed Ca\(^{2+}\) channels after Rem co-expression at a time when channel activity is greatly inhibited. These studies suggest that Rem-mediated regulation of Ca\(^{2+}\) channel activity involves direct regulation of the plasma membrane-located channel complex and can occur without the need to disrupt the steady-state levels of surface-expressed Ca\(^{2+}\) channels.

**EXPERIMENTAL PROCEDURES**

**Plasmids**—Mammalian expression vectors for Ca\(_{\alpha_1}\)1.2α-subunit, β\(_{2a}\)-subunit, FLAG epitope-tagged β\(_{2a}\)-subunit, and HA epitope-tagged Rem have been described previously (22). Ca\(_{\alpha_1}\)β\(_{2a}\) was subcloned into pcDNA3 (Novagen) for production of the Single Tube Protein System 3 (STP3) kit (Novagen) according to the manufacturers’ instructions. Ca\(_{\alpha_1}\)β\(_{2a}\) or the indicated Ca\(_{\alpha_1}\)β\(_{2a}\) truncation mutants cloned into the pCITE vector were used as the template. Binding of radio-labeled β\(_{2a}\) to Rem was assessed as follows. All manipulations were carried out at 4 °C. Ten µl glutathione-Sepharose beads (GE Healthcare) were washed 2 times with 500 µl of EDTA buffer (50 mM Tris, pH 7.5, 100 mM NaCl, 0.05% Tween 20, 0.1 mM DTT, 1 mM EDTA). The beads were resuspended in 1 ml of EDTA buffer, and either GST (10 µg) or GST-Rem (10 µg) was added. The beads were incubated for 5 min with end-over-end rotation at 4 °C. The beads were washed 2 times with 1 ml of EDTA buffer and then with 1 ml of EDTA buffer or 1 ml of GDP buffer (50 mM Tris, pH 7.5, 100 mM NaCl, 0.05% Tween 20, 0.1 mM DTT, 10 mM MgCl\(_2\), 20 µM GDP) or 1 ml of GTP buffer (50 mM Tris pH 7.5, 100 mM NaCl, 0.05% Tween 20, 0.1 mM DTT, 10 mM MgCl\(_2\), 20 µM GTP\(_{\gamma}\)S) as indicated to facilitate nucleotide exchange. The buffer was aspirated so that 20 µl of a 50% slurry was allowed to remain in the tube. EDTA, GDP, or GTP buffer (76 µl) was added as indicated, and binding was initiated by the addition of [\(^{35}\)S]-labeled β\(_{2a}\)-subunit (4 µl). The reaction was incubated for 3 h with end-over-end rotation. The beads were then washed three times with the same buffer used in the assay. GST or GST Rem was eluted from the beads with two 20-µl washes of assay buffer supplemented with 25 mM glutathione. The eluted proteins were resolved on 10% SDS-PAGE gels, which were dried and exposed to film for 16–72 h.

**Competition Assays**—The ability of Rem to compete with Ca\(_{\alpha_1}\) subunit intracellular loop I-II (AID) was assayed as follows. First, the minimal amount of GST-AID protein required to bind [\(^{35}\)S]-labeled β\(_{2a}\) was determined by adding the indicated amount of GST-AID or un fused GST to 10 µl of pre-equilibrated glutathione-Sepharose beads (GE Healthcare). GTP buffer (76 µl) was then added, and binding was initiated by the addition of 4 µl of [\(^{35}\)S]-labeled β\(_{2a}\) subunit. The reaction was incubated for 3 h with end-over-end rotation. The beads were then washed 3 times with 500 µl of GTP buffer. Bound protein was eluted from the beads with two 20-µl washes of assay buffer containing 25 mM glutathione. The eluted proteins were resolved on 10% SDS-PAGE gels, which were dried and exposed to film for 16–72 h.

**In Vivo GST Pulldown Assays**—HEK293 cells were transiently transfected with the indicated plasmids as described (35). 48 h post-transfection cells were harvested and lysed in 20
Defining the Rem-Interaction Domain of Ca\(^{2+}\) Channel \(\beta\) Subunits

\[\text{mm Tris, pH 7.5, 250 mM NaCl, 1\% Triton X-100, 0.5 mM DTT, 1\times protease inhibitor mixture (Calbiochem), 10 mM MgCl}_2, \text{and 10 \(\mu\)M GTPyS and centrifuged at 100,000 \(\times\) g for 10 min. The supernatant was harvested, and protein concentration was determined using the Bradford assay. Cleared supernatant (1 mg) was incubated with GST (control) or GST-AID and 10 \(\mu\)l of glutathione-agarose (GE Healthcare) as indicated for 3 h at 4 °C with gentle end-over-end rotation. The beads were then isolated by centrifugation for 10 s at 14000 rpm in a microcentrifuge, and 5 \(\mu\)l of the supernatant was retained for analysis. The beads were washed 3 times with 1 ml of lysis buffer and then eluted with assay buffer containing 25 mM glutathione. The eluted fraction was then analyzed with the supernatant for the presence of FLAG-Ca\(_{\alpha1\beta2a}\), as follows. The supernatant and pellet were boiled in SDS-PAGE buffer and resolved on 10% SDS-PAGE gels. The gels were transferred to nitrocellulose and immunoblotted with either anti-FLAG or anti-HA monoclonal antibodies as indicated.}

Surface Biotinylation Studies—HIT-T15 cells were obtained from ATCC and maintained in F12-K media (Invitrogen) supplemented with 50 \(\mu\)g/ml gentamicin, 2.5% fetal bovine serum, and 10% dialyzed horse serum (prepared by dialyzing horse serum in 14,000-kDa cutoff dialysis bags extensively versus 0.15 M NaCl at 4 °C). Ten-cm dishes were seeded with cells the day before infection. Cells were either cultured alone (uninfected control) or incubated for 24 h with CsCl-purified adenovirus-expressing GFP (control) or co-expressing GFP and Rem or GFP and Rem\(^{1-265}\) (10\(^7\) plaque-forming units/ml) (22). This resulted in near complete HIT-T15 cell infection based on the analysis of GFP-positive cells (24 h post-infection). Monolayers were washed three times with ice-cold phosphate-buffered saline (PBS), and surface proteins were biotinylated using 1 mg/ml membrane-impermeant sulfo-NHS-LC-biotin (Pierce) in PBS for 1 h at 4 °C with gentle rocking. Cells were then washed three times with ice-cold phosphate-buffered saline, harvested on ice in 1 ml of Versene (Invitrogen), and pelleted by gentle centrifugation, and the Versene was aspirated. Radioimmune precipitation assay buffer (1% Triton X-100, 1% deoxycholic acid, 0.1% SDS, 50 mM Tris-HCl, pH 7.4, 1 \(\times\) protease inhibitor mixture (Calbiochem)) was added to the cell pellet, which was sonicated twice for 10 s (Kontes). The soluble fraction was isolated after centrifugation at 100,000 \(\times\) g for 10 min. Protein concentrations were determined using the Bio-Rad protein assay kit with bovine serum albumin as a standard. Biotinylated proteins were isolated by adding cleared cell lysates (500 \(\mu\)g) to 100 \(\mu\)l (50% slurry) of streptavidin beads (Pierce) in a total volume of 1 ml of radioimmune precipitation assay buffer. The reaction was gently rotated end over end at 4 °C for 1.5 h, and resin was pelleted by centrifugation, washed once with radioimmune precipitation assay buffer (RIPA) containing 0.3 M NaCl (two times), twice with RIPA containing 0.15 M NaCl, and finally twice with wash buffer containing no detergent (150 mM NaCl, 50 mM Tris-HCl, pH 7.4, 2.5 mM EDTA). The beads were resuspended in 30 \(\mu\)l of 2 \(\times\) SDS loading buffer and boiled for 5 min. The released protein as well as 10 \(\mu\)g of the input was resolved using 6% SDS-PAGE gel electrophoresis, transferred to nitrocellulose, and subjected to immunoblot analysis with affinity-purified L-type calcium channel \(\alpha\)-subunit polyclonal antibody at 2 \(\mu\)g/ml and horseradish peroxidase goat anti-rabbit (Zymed Laboratories Inc.) secondary antibody at a 1:20,000 dilution. Super signal (Pierce) was used as the enhanced chemiluminescent reagent. For inhibition studies, immunoblotting was performed as above, but the \(\alpha\)-subunit polyclonal antibody was preincubated for 1 h at 20 °C with 10 \(\mu\)g/ml GST-fused Ca\(_{\alpha1.2}\) II-III loop protein. To assure that the cells remained intact throughout the surface labeling, biotinylation of glycer-aldehyde-3-phosphate dehydrogenase (GAPDH), a cytosolic protein, was analyzed by immunoblotting with GAPDH monoclonal antibody (Ambion) at 1:2000 dilution.

Electrophysiology—HEK293 cells were transiently transfected with plasmids 48 h before recordings using Effectene (Qiagen) according to the manufacturer’s instructions. tsA201 cells were transiently transfected 48 h before recordings using the calcium phosphate method as described previously (35). Transfected cells were identified by GFP expression. HIT-T15 cells were plated on polylysine-coated coverslips in 24-well tissue culture dishes at 20,000 cells/well. The next day the cells were infected with the indicated adenovirus at 1 \(\times\) 10\(^7\) adenovirus per ml. Adenovirus-infected cells were identified by GFP expression, and recordings were made 22 h post-infection (23). The whole-cell configuration of the patch clamp technique was used to measure ionic current. Patch electrodes with resistances of 2–4 megaohms contained 110 (or 0) mM potassium gluconate, 40 (or 150) mM CsCl, 1 mM MgCl\(_2\), 5 mM Mg-ATP, 3 mM EGTA, 5 mM Hepes, pH 7.36. The bath solution for HEK and tsA201 cells consisted of 112.5 mM CsCl, 30 mM BaCl\(_2\), 1 mM MgCl\(_2\), 10 mM tetraethylammonium chloride, 5 mM glu-cose, 5 mM Hepes, pH 7.4. The bath solution for adenoviral infected HIT-T15 cells consisted of 102.5 mM CsCl, 40 mM BaCl\(_2\), 1 mM MgCl\(_2\), 10 mM tetraethylammonium chloride, and 5 mM Hepes, pH 7.4. Signals were amplified with an Axopatch 200B amplifier and 333-kHz A/D system (Axon Instruments, Union City, CA). Data were analyzed with Clampfit 9 (Axon Instruments) and Origin statistical software (OriginLab Corp., Northampton, MA). All recordings were performed at room temperature (20–22 °C).

RESULTS

Identification of the Rem Binding Domain in \(\beta_{2a}\)—We previously reported that Rem binds to a variety of Ca\(_{\alpha1\beta}\) subunits, including Ca\(_{\alpha1\beta2a}\) (22). Because RGK proteins are capable of associating with all four \(\beta\)-subunit gene products (18, 21, 22) and Ca\(_{\alpha1\beta}\)-subunit binding appears critical to the ability of RGK proteins to disrupt Ca\(_{\alpha1\alpha1}\) function (21–23, 26), we reasoned that RGK binding occurs via a conserved Ca\(_{\alpha1}\)-subunit sequence. Although previous work suggested that Ca\(_{\alpha1}\) binding to RGK GTPases may be GTP-dependent (21), in vitro Ca\(_{\alpha1}\beta_{2a}\) binding to Rem was found to be nucleotide-independent, with both GDP-bound and GTP\(\gamma\)S-bound Rem displaying equivalent Ca\(_{\alpha1}\)-subunit binding (Fig. 1B). To identify the structural domain of Ca\(_{\alpha1}\beta_{2a}\) responsible for Rem association, we used \(^35\)S-labeled, in vivo translated wild-type and truncated Ca\(_{\alpha1}\beta_{2a}\) subunit probes to examine the interaction between the various structural domains of Ca\(_{\alpha1}\beta_{2a}\) and both wild-type Rem and the loop I-II (AID) domain of Ca\(_{\alpha1}\)2 (Fig. 1A). GST-Rem and GST-AID both associated with \(^35\)S-labeled in vitro trans-
Defining the Rem-Interaction Domain of Ca\textsuperscript{2+} Channel \(\beta\) Subunits

FIGURE 1. The Rem interaction site of Ca\textsubscript{\(v\)} \(\beta\textsubscript{2a}\) is localized to amino acids 260–344. A, the indicated \(\textsuperscript{35}S\)-labeled \(\beta\textsubscript{2a}\) subunits were prepared by \textit{in vitro} transcription and translation as described under "Experimental Procedures." The \(\textsuperscript{35}S\)-labeled \(\beta\textsubscript{2a}\) subunits were resolved on 10% SDS-PAGE gels, which were then dried and exposed to film. B, recombinant GST or GST-Rem proteins were preloaded with the indicated nucleotide, incubated with \(\textsuperscript{35}S\)-labeled \(\beta\textsubscript{2a}\) or \(\textsuperscript{35}S\)-labeled \(\beta\textsubscript{2a}\) \textsubscript{1–355}, and the reactions were then pelleted and washed as described under "Experimental Procedures." The bound fractions were subjected to SDS-polyacrylamide gel electrophoresis, and the dried gel was exposed to film. C, the ability of GST-Rem or GST-Ca\textsubscript{\(v\)} loop I-II (AID) fusion proteins to interact with \(\textsuperscript{35}S\)-labeled Ca\textsubscript{\(v\)},\(\beta\textsubscript{2a}\) was determined as described under "Experimental Procedures." D, a schematic diagram of the Ca\textsubscript{\(v\)},\(\beta\textsubscript{2a}\) used in the corresponding interaction assay (C) is presented. The conserved SH3- and GK-related structural domains are indicated.

lated wild-type Ca\textsubscript{\(v\)},\(\beta\textsubscript{2a}\) and, more importantly, with the Ca\textsubscript{\(v\)},\(\beta\textsubscript{2a}\)\textsubscript{226–604} truncation mutant (Fig. 1C). This localized the Rem interaction site within the highly conserved GK domain (GK) found in all Ca\textsubscript{\(v\)},\(\beta\) subunit genes and known to harbor the ABP binding domain primarily responsible for Ca\textsubscript{\(v\)},\(\beta\)-subunit/AID binding (2). No interaction could be detected with Ca\textsubscript{\(v\)},\(\beta\textsubscript{1–225}\), which comprises the first conserved SH3-related structural domain, or Ca\textsubscript{\(v\)},\(\beta\textsubscript{226–604}\), which included the C-terminal portion of Ca\textsubscript{\(v\)},\(\beta\textsubscript{2a}\) beyond the GK domain. Recent crystallographic studies have resolved the structure of the \(\beta\)-subunit in association with the AID sequence domain of Ca\textsubscript{\(v\)},\(\alpha\textsubscript{1}\) and allowed us to generate mutants predicted to disrupt
Defining the Rem-Interaction Domain of Ca²⁺ Channel β Subunits

FIGURE 2. Rem does not inhibit the Caᵥ1.2-AID-Caᵥβ₂a interaction in vitro. A, the indicated amounts of GST (control), GST-AID, or GST-Rem were used to analyze [³⁵S]-GST interaction as described under “Experimental Procedures.” GST-AID (10 ng) was incubated with [³⁵S]-labeled β₂a(1–355) with the indicated amount of inhibitor, and binding assays were performed as described under “Experimental Procedures.”

the ABP domain (6–8). Consistent with these studies, Caᵥαᵥ1–355 (lacking the α8 helix), Caᵥβ₂a–343 (deletion of both α5 and α8 helices), and Caᵥβ₂a–604 (deletion of the α3 helix) truncation mutants (8), which delete critical portions of the ABP domain, fail to demonstrate binding to the Caᵥ1.2-AID domain, but importantly, all three were capable of binding WT-Rem. Taken together, this analysis indicates that both Rem and Caᵥαᵥ1-association domains falls within the well conserved GK module found in all β subunits and suggest that these domains are separate.

Rem Does Not Disrupt the Interaction between the AID of Caᵥαᵥ1 and β₂a—Although the binding studies indicate that the Rem and AID association domains of β₂a are structurally distinct, we next examined whether these binding domains functionally overlap. As seen in Fig. 1C, more [³⁵S]-labeled β₂a was bound to the AID domain of Caᵥ1.2 than to GST-Rem (see the top panel). To examine the relative binding affinity in more detail, we examined the interaction of in vitro translated [³⁵S]-labeled β₂a with increasing concentrations of GST-Caᵥαᵥ1-AID or GST-Rem. As shown in Fig. 2A, Caᵥαᵥ1-β₂a demonstrates greater interaction with the isolated AID domain than with Rem, with similar amounts of [³⁵S]-labeled Caᵥαᵥ1-β₂a found to associate with 300 pg of GST-AID as with 10 μg of GST-Rem. As expected, no interaction between radiolabeled β₂a and unfused GST protein was seen in control GST pull-down reactions. This affinity difference suggested that Rem may not be capable of competing with the Caᵥαᵥ1-AID for Caᵥβ₂a binding. To test this notion, we next examined the ability of increasing concentrations of untagged wild-type Rem to disrupt the association of GST-AID with [³⁵S]-labeled Caᵥβ₂a. As shown in Fig. 2B, more than a 100-fold molar excess of recombinant Rem protein failed to disrupt GST-AID-[³⁵S]-labeled Caᵥβ₂a association, although the addition of only 25 ng of the untagged Caᵥαᵥ1-AID effectively inhibited the interaction of Caᵥβ₂a with GST-AID. Although Rem failed to efficiently disrupt β₂a association with AID, it inhibited the interaction of GST-Rem with β₂a (Fig. 2C). Taken together, these results indicate that Rem displays much lower binding affinity for Caᵥβ₂a subunits than the previously described high affinity interaction between Caᵥαᵥ1-1loop-I and Caᵥβ₂a subunits and indicate that Rem does not disrupt AID/Caᵥβ₂a-subunit binding.

Contribution of β-Subunit to Rem-mediated Ca²⁺ Channel Regulation—Although it is now well established that RGK GTPases associate with a range of Caᵥαᵥ1-β subunits (21–23), the question of whether the nature of the auxiliary β-subunit modulates Rem-mediated Ca²⁺ channel regulation has not been thoroughly examined. Because the mapping data indicate that Rem binds β subunits near the junction of the highly conserved GK domain and a variable linker domain positioned between the SH3 and GK domains (Fig. 1C), we next examined whether structural differences within distinct β subunits may functionally contribute to Rem-dependent channel regulation. To examine this issue, we assessed the ability of Rem to regulate Caᵥ1.2 channel complexes containing either Caᵥβ₁b or Caᵥβ₂a. These β subunits differ greatly within their variable domains (Fig. 3C), including the domain between the conserved SH3 and GK modules, but demonstrate similar in vitro binding to Rem and Rem2 (22, 23).

As seen in Fig. 3B, HEK293 cells transiently co-transfected with Caᵥ1.2 and Caᵥβ₂a express a >9 pA/pF peak inward current (9.33 ± 1.91 pA/pF; n = 7). As we have described previously (22), co-expression of wild-type Rem with Caᵥβ₁b and Caᵥβ₂a results in a complete absence of detectable ionic current expression (Fig. 3B). Co-expression of Caᵥ1.2 and Caᵥβ₁b in HEK293 cells results in a >5 pA/pF peak inward current (5.85 ± 2.02 pA/pF; n = 10), and importantly, expression of Rem results in a complete blockade of ionic current expression (Fig. 3A). These data appear to rule out a major contribution of the variable regions of different β subunits to Rem-mediated Ca²⁺ channel activity. Taken together these results suggest that Rem association occurs within the well conserved GK domain found within all β subunit proteins but that sequence differences within the variable regions of β subunits are unlikely to significantly modulate Rem-mediated channel inhibition.

Rem Does Not Disrupt β-Subunit/AID Domain Association—It is known that Caᵥαᵥ1-β subunits regulate Ca²⁺ channel activity in part through interaction with the AID domain in Caᵥαᵥ1 subunits (2). In addition, it has been suggested that the Rem-related GTPase regulates Ca²⁺ channel function by inhibiting the trafficking of newly synthesized Caᵥαᵥ1 subunits to the plasma membrane by sequestering cellular β subunits (21, 34). The in vitro binding data presented in Fig. 2 indicate that Rem cannot effectively compete with Caᵥαᵥ1 for cellular Caᵥβ₂a subunits; however, these studies may lack endogenous cellular factors that promote Rem-β subunit complex formation. To further examine the ability of Rem to interfere with Caᵥαᵥ1-β-subunit association in a cellular context, HEK293 cells were transfected with FLAG-tagged wild-type Caᵥβ₂a, and empty vector or Flag-
Defining the Rem-Interaction Domain of Ca\(^{2+}\) Channel \(\beta\) Subunits

\[\beta_2a\] was co-transfected with either HA-tagged wild-type Rem or HA-Rem\(^{1\text{--}265}\), a previously characterized C-terminal Rem truncation mutant that does not bind \(\text{Ca}_\text{v}/\beta_2\) subunits (22). Cell lysates were then incubated with recombinant GST-AID, the bound complexes were isolated with glutathione-Sepharose, and Rem association was analyzed by anti-HA immunoblotting. As seen in Fig. 4B, HA-Rem was found associated with GST-AID, but not with unfused GST, in a \(\text{FLAG-Ca}_\text{v}/\beta_2a\)-dependent manner. These findings indicate that \(\beta_2a\) bound to Rem is still capable of associating with the AID domain. To confirm that \(\beta\)-subunit association was necessary for Rem association with the GST-AID complex, the ability of Rem\(^{1\text{--}265}\) to enter the complex was examined. The binding of HA-Rem\(^{1\text{--}265}\) to the GST-AID complex was significantly reduced compared with that of wild-type Rem (Fig. 4B), consistent with its reduced \(\text{Ca}_\text{v}/\beta\)-binding affinity (22). These results suggest that Rem does not compete with \(\text{Ca}_\text{v}/\alpha_1\)-AID for \(\beta\)-subunit association but, instead, that \(\text{Ca}_\text{v}/\beta\) functions as a necessary scaffold, mediating independent interactions with both Rem and \(\text{Ca}_\text{v}/\alpha_1\) to promote a higher order complex.

\section*{Rem Inhibits \(\text{Ca}_\text{v}\) Channel Function in HIT-T15 Cells without Reduction in Membrane Localization—Previous studies have suggested that the association of Gem and Rem2 with \(\beta\) subunits prevents the trafficking of \(\text{Ca}_\text{v}/\alpha_1\) subunits to the plasma membrane, resulting in down-regulation of channel activity (21, 26, 34). In contrast, two recent reports indicate that Rem2 expression inhibits voltage-gated \(\text{Ca}_\text{v}\) channel current without affecting channel surface density (23, 32). Thus, the nature of RGK-mediated \(\text{Ca}_\text{v}\) channel regulation remains controversial. To begin to explore the mechanism of Rem-mediated channel regulation, we next determined whether the number of surface-exposed \(\text{Ca}_\text{v}\) channels in HIT-T15 cells was altered after adenoviral-mediated Rem expression. Surface proteins were biotinylated with membrane-impermanent sulfo-NHS-LC-biotin 24 h after adenoviral infection, the same time that patch clamp analysis demonstrated Rem-mediated inhibition of \(\text{Ca}_\text{v}\) channel function in parallel cultures (Fig. 5A). Biotinylated proteins were isolated by incubation with streptavidin resin and subjected to Western blot analysis using an anti-\(\text{Ca}_\text{v}/\alpha_1\) antibody. As seen in Fig. 5B, the surface expression of endogenous \(\text{Ca}_\text{v}/\alpha_1\) subunits was not detectably altered by Rem expression when compared with either uninfected or control Ad-GFP-infected HIT-T15 cell cultures. As an added control, expression of the Rem\(^{1\text{--}265}\), which displays diminished \(\text{Ca}_\text{v}/\beta\)-subunit binding and fails to regulate \(\text{Ca}_\text{v}\) channel function (22), did not alter \(\text{Ca}_\text{v}\) channel surface expression. Endogenous \(\text{Ca}_\text{v}/\alpha_1\) protein expression was unaffected by Rem (Fig.
Regulation, we examined the effect of mutant lacks an SH3 domain and does not bind CaV

AUGUST 18, 2006 • VOLUME 281 • NUMBER 33

FIGURE 4. Rem does not inhibit CaV, aAID-CaV,β2a interaction in vivo. A, HEK293 cells were transiently transfected with the indicated plasmids as described under “Experimental Procedures.” The ability of FLAG-tagged CaV,β2a to interact with either GST (control) or GST-AID was determined by immunoblotting as described under “Experimental Procedures.” The bound protein fractions were resolved on 10% SDS-PAGE gels and immunoblotted with anti-HA antibody to detect the Rem.

650–604 was unable to modulate CaV,α1 activity, having no effect on current amplitude or inactivation kinetics when co-expressed with either CaV,1.2 alone (Fig. 6B, closed squares, 0.953 ± 0.970 pA/pF, n = 8) or with a wild-type CaV,α1/CaV,β channel (Fig. 6D, open triangles, 18.2 ± 5.44 pA/pF, n = 16). As we have described previously (22), co-expression of wild-type Rem with CaV,1.2 and β2a results in a complete absence of detectable ionic current expression (Fig. 6D, closed squares, 0.136 ± 0.157 pA/pF, n = 16), and importantly, co-expression of β2a (650–604) was able to partially relieve the Rem blockade of ionic current expression (Fig. 6D). In these experiments, β2a (650–604) expression increased peak current amplitude to >3 pA/pF peak inward current (3.84 ± 1.77 pA/pF; n = 15). Taken together, these data indicate that CaV,β association plays a critical role in Rem-mediated channel regulation and suggests that formation of a CaV,α1/CaV,β/Rem scaffolding complex may be required Rem-dependent channel regulation.

FIGURE 5. Rem prevents endogenous Ca2+ channel current expression in HIT-T15 β-islet cells without altering Ca2+ channel trafficking. A, current voltage relationships for HIT-T15 cells infected with control virus (filled squares, n = 7) or Rem-expressing virus (open circles, n = 8) 21.25 h after adenoviral infection. B, adenoviral-infected HIT-T15 cells were surface-biotinylated with sulfo-NHS-LC-biotin, and biotinylated proteins were isolated using streptavidin resin. The entire biotinylated eluted protein fraction (500 µg of whole cell lysate) (Pulldown) and 10 µg of input lysate (Input) were resolved on 6% SDS-PAGE gels, transferred to nitrocellulose, and immunoblotted with anti-CaV,α1 antibody to determine relative surface expression of CaV,α1 channels. Membranes were subsequently re-blotted using anti-glycer-aldehyde-3-phosphate dehydrogenase (GAPDH) antibody as a control for cell integrity. Input lysate (10 µg) was resolved on a 10% SDS-PAGE gel, transferred to nitrocellulose, and immunoblotted with anti-Ren antibody as a control for Rem protein expression.

5B), indicating that Rem signaling does not markedly reduce overall channel stability. Taken together, these data indicate that Rem regulates channel activity in HIT-T15 cells without reducing the surface density of L-type Ca2+ channels.

CaV,β Association Is Important for Rem-mediated Channel Regulation—To explore the significance of β-subunit association, we examined the effect of β2a (650–604) mutation expression on Rem-dependent channel regulation. Because the β2a (650–604) mutant lacks an SH3 domain and does not bind CaV,α1-AID but retains the ability to bind Rem (Fig. 1C), we reasoned that the mutant would not modulate CaV,α1 function but might compete with wild-type CaV,β2a for Rem association. In agreement with our initial studies, FLAG-tagged β2a (650–604) retained the ability to associate with Rem (Fig. 6C) but failed to bind GST-AID (Fig. 6A). Cells transiently co-transfected CaV,1.2 and CaV,β2a expressed >16 pA/pF peak inward current (Fig. 6B, open circles, 16.9 ± 3.76 pA/pF; n = 8). As expected for a β-subunit mutant unable to bind AID and lacking its SH3-related domain, β2a (650–604) was unable to modulate CaV,α1 activity, having no effect on current amplitude or inactivation kinetics when co-expressed with either CaV,1.2 alone (Fig. 6B, closed squares, 0.953 ± 0.970 pA/pF, n = 8) or with a wild-type CaV,α1/CaV,β channel (Fig. 6D, open triangles, 18.2 ± 5.44 pA/pF, n = 16). As we have described previously (22), co-expression of wild-type Rem with CaV,1.2 and β2a results in a complete absence of detectable ionic current expression (Fig. 6D, closed squares, 0.136 ± 0.157 pA/pF, n = 16), and importantly, co-expression of β2a (650–604) was able to partially relieve the Rem blockade of ionic current expression (Fig. 6D). In these experiments, β2a (650–604) expression increased peak current amplitude to >3 pA/pF peak inward current (3.84 ± 1.77 pA/pF; n = 15). Taken together, these data indicate that CaV,β association plays a critical role in Rem-mediated channel regulation and suggests that formation of a CaV,α1/CaV,β/Rem scaffolding complex may be required Rem-dependent channel regulation.
Defining the Rem-Interaction Domain of Ca^{2+} Channel β Subunits

It is now clear that all members of the RGK family of GTP-binding proteins interact with auxiliary β subunits to regulate L-type Ca^{2+} channel activity (18, 21, 23, 31–33). In the present study we provide new insight into the mechanism of RGK-mediated Ca^{2+} channel regulation through the identification of the Rem interaction site on Ca_{v}β_{2a}. The most interesting result of this study is that although both the Rem and AID association motifs are located within the conserved Ca_{v}β-subunit GK domain, Rem binding does not inhibit AID binding. Instead, these studies suggest that Ca_{v}β subunits serve as molecular scaffolds to promote a novel regulatory interaction between Rem and the AID domain of Ca_{v}α_{1}. In addition, surface biotinylation studies indicate that this Ca_{v}β-subunit-mediated regulatory complex inhibits Ca^{2+} channel activity without down-regulating surface-expressed channel. Based upon these findings we propose that Rem-dependent inhibition of Ca^{2+} channel activity involves β-subunit-dependent regulation of the membrane-localized channel complex rather than disruption of plasma membrane channel trafficking.

Auxiliary Ca_{v}β subunits share a conserved domain structure with three variable regions separated by conserved SH3-like and GK-like domains (Fig. 3C) (2). In agreement with recent crystallographic studies (6–8), in vitro deletion analysis demonstrates that the high affinity Ca_{v}α_{1} subunit interaction region is located within the ABP region of the GK domain. Although we did not directly examine the effect of truncation on the folding of the SH3/GK core of Ca_{v}β_{2a}, deletion of helices α8, α5, and α3, which contribute to the ABP domain (6–8), disrupted association with Ca_{v}α_{1}-AID (Fig. 1). More importantly, β^{1-355} (lacking α8), Ca_{v}β_{261–604} (deletion of the α3 helix), and β^{1-343} (deletion of both α5 and α8) (8) GK domain mutants that fail to bind AID are each capable of associating with Rem (Fig. 1C). Moreover, deletion of the entire N-terminal domain of Ca_{v}β_{2a}, including the conserved SH3-like domain, failed to disrupt either Rem or AID association. These results suggest that Rem and perhaps all other RGK GTPases will demonstrate uniform binding with all Ca_{v}β subunits containing the conserved GK module. Indeed, extensive in vitro binding analysis supports this conclusion (21–23, 26, 34). Moreover, these data indicate that the nature of the auxiliary Ca_{v}β-subunit, as long as it con-

**FIGURE 6.** Co-expression of Ca_{v}β_{260–604} partially recovers Ca_{v}α_{1}2 current from Rem-mediated regulation. A, Ca_{v}β_{260–604} does not bind the AID domain of Ca_{v}α_{1}2. FLAG-tagged Ca_{v}β_{260–604} (arrow), FLAG-tagged Ca_{v}β_{2a} WT (arrow head), or pCMV7/F2 (FLAG empty vector) were expressed in tsA-201 cells using the calcium phosphate transfection method. 48 h post-transfection cells were harvested, and lysates were subjected to GST pulldown using either recombinant GST AID protein or GST. Pulldowns were immunoblotted with anti-FLAG antibody. B, Ca_{v}β_{260–604} does not support current through Ca_{v}α_{1}2. Current-voltage curve for tsA-201 cells co-transfected with either Ca_{v}α_{1}2 + Ca_{v}β_{2a} WT + GFP (open circles, n = 8) or Ca_{v}α_{1}2 + Ca_{v}β_{260–604} + GFP (filled squares, n = 8). C, Rem associates with both Ca_{v}β_{2a} WT and Ca_{v}β_{260–604}. 3xHA-tagged Rem was co-expressed in tsA-201 cells with either FLAG-Ca_{v}β_{2a} WT, FLAG-Ca_{v}β_{260–604}, or pCMV7/F2 (FLAG empty vector) using the calcium phosphate transfection method. 48 h post-transfection cells were harvested, and lysates were subjected to FLAG immunoprecipitation (IP) to isolate Ca_{v}β_{2a} complexes. Immunoprecipitates were blotted for Ca_{v}β_{2a} using anti-FLAG antibody and for Rem using biotinylated HA antibody. D, co-expression of Ca_{v}β_{260–604} partially relieves Rem-mediated channel blockade. Current-voltage curve for tsA-201 cells co-transfected with Ca_{v}α_{1}2 + Ca_{v}β_{2a} WT + Ca_{v}β_{260–604} + GFP (open triangles, n = 16), Ca_{v}α_{1}2 + Ca_{v}β_{2a} WT + FLAG vector + GFP-Rem (filled squares, n = 16), or Ca_{v}α_{1}2 + Ca_{v}β_{2a} WT + Ca_{v}β_{260–604} + GFP-Rem (open circles, n = 15).
Defining the Rem-Interaction Domain of Ca\(^{2+}\) Channel \(\beta\) Subunits

tains an intact GK module, is unlikely to significantly modulate RGK-dependent channel regulation. This is in agreement with the ability of Rem to completely inhibit ionic current expression from heterologously expressed Ca\(_{\alpha1.2}\) and Ca\(_{\alpha1.2}\beta\_subunits (Fig. 3) and data from a variety of native and heterologous expression studies (21–23, 26, 31–34). Finally, these studies suggest a novel role for a series of exotic Ca\(_{\alpha}\)-subunit splice variants that have recently been described (9, 36–38). These Ca\(_{\alpha}\)-subunits fail to express a GK module due to alternative splicing and are unable to promote channel trafficking but are capable of modulating single channel gating properties (9, 10). It is likely that these short Ca\(_{\alpha}\)-subunits will escape Rem-mediated Ca\(^{2+}\) channel regulation and may, therefore, play a particularly prominent role in shaping the functional properties of channels in the presence of active Rem-mediated regulation. Studies to address these questions are currently under way.

The AID domain represents a high affinity binding site for all Ca\(_{\alpha}\)-subunits, with the affinity of Ca\(_{\alpha}\)-AID binding reported to be in the low nanomolar range (2). Although recent work has suggested that RGK proteins compete with Ca\(_{\alpha}\)-AID, for available Ca\(_{\alpha}\), the in vitro binding studies presented here indicate that Rem binds \(\beta\) subunits with an apparent affinity much lower than that for the association of Ca\(_{\alpha}\)-beta subunit interaction with Ca\(_{\alpha}\)-AID and the AID domain (Fig. 2). More importantly, these assays show that Rem and AID can simultaneously associate with Ca\(_{\alpha}\)-\(\beta\_2a\) (Fig. 4), indicating that Rem and AID interaction sites are distinct, without obvious structural or functional overlap. Although the lack of effective Rem antibody reagents has thwarted analysis of the putative interaction of endogenous Rem with the native Ca\(^{2+}\) channel complex, these data suggest that Rem association will depend upon the formation of the AID-\(\beta\)-subunit interaction, with Ca\(_{\alpha}\)-beta serving as a scaffold to recruit Rem to the channel complex. Support for this model comes from studies using the \(\beta_{2a}^{660-604}\) mutant (Fig. 6). As expected, overexpression studies found that this mutant was unable to modulate channel activity when co-expressed with Ca\(_{\alpha}\)-AID or alter wild-type Ca\(_{\alpha}\)-\(\beta_{2a}\) channel activity. Interestingly, \(\beta_{2a}^{660-604}\) co-expression can partially relieve Rem-mediated channel inhibition (Fig. 6). These data further support a crucial role for \(\beta\)-subunit association in Rem-mediated channel regulation. Also, Rem, in the absence of \(\beta\)-subunit overexpression, partially blocks Ca\(_{\alpha}\)-AID current, but \(\beta_{2a}\) co-expression yields a complete blockade (41). Thus, it will be important to determine whether association with the Ca\(_{\alpha}\)-\(\beta\)_subunit or other components of the larger membrane-associated channel complex may influence Rem function.

Our results differ from those of Sasaki et al. (34), who recently reported that the BID region, first reported to serve as the Ca\(_{\alpha}\)-beta region involved in direct interaction with the Ca\(_{\alpha}\)-AID, is required for association of the Rem-related Gem protein with \(\beta\) subunits (34). However, the notion that the BID region is required for AID association has been challenged by recent structural studies that indicate that the BID domain is located within the \(\beta\)-subunit core (6–8). Thus, deletion of the BID domain is likely to result in structural changes to the \(\beta\)-subunit, suggesting that the effects of BID mutants on Gem binding may result from alterations to protein folding or stability rather than any specific disruption to a Gem interaction site. Further studies are needed to fully define the structural interactions between RGK proteins and Ca\(_{\alpha}\)-\(\beta\) subunits and to examine the possibility that RGK proteins may directly interact with Ca\(_{\alpha}\)-\(\gamma\_1\) subunits.

Although these studies have begun to define the motifs involved in Rem-\(\beta\)-subunit association, the question of how the binding of Rem to \(\beta\)-subunit results in the inhibition of functional Ca\(^{2+}\) channel activity remains to be fully characterized. Previous studies have suggested that co-expressed Gem and Rem2 GTPases regulate channel function at least in part by reducing the density of membrane-localized L-type Ca\(^{2+}\) channels through selective blockade of Ca\(_{\alpha}\)-\(\alpha\_1\)-subunit membrane trafficking (21, 26, 34, 39). Indeed, Gem overexpression in cardiomyocytes results in a reduction in L-type Ca\(^{2+}\) channel gating currents, consistent with inhibition of functional channel expression at the plasma membrane (33). However, we observe potent Ca\(^{2+}\) channel inhibition within 24 h of adenoviral-mediated Rem expression in HIT-T15 cells without a corresponding reduction in the density of plasma membrane Ca\(_{\alpha}\)-\(\alpha\_1\)-subunits (Fig. 5). In support of this finding, we reported similar properties for the Rem2 protein (23), and Chen et al. (32), using a ligand binding assay, have shown that Ca\(_{\alpha}\)-\(\alpha\_2.2\)-subunit surface expression is not reduced by Rem2 overexpression at a time when N-type channel activity is potently inhibited. These data indicate that both Rem and Rem2 allow for acute channel regulation without disrupting channel trafficking or significantly reducing Ca\(_{\alpha}\)-\(\alpha\_1\)-subunit stability at the cell surface. Taken together, these studies suggest that Rem, Rem2, and perhaps other RGK proteins may regulate Ca\(^{2+}\) channel activity using two complementary mechanisms. The first signaling pathway, which has been proposed to operate for Gem and Rem2, would allow for chronic regulation of the surface density of Ca\(^{2+}\) channels by disrupting Ca\(_{\alpha}\)-\(\alpha\_1\)-subunit trafficking. The second regulatory mechanism, now described for both Rem and Rem2, allows for the acute regulation of Ca\(^{2+}\) channels found within the plasma membrane.

Although these studies contradict the hypothesis that RGK binding disrupts the interaction of Ca\(_{\alpha}\)-\(\beta\) with Ca\(_{\alpha}\)-AID, thereby altering the trafficking of newly synthesized channels or generating a population of Ca\(_{\alpha}\)-\(\beta\)-less channels at the cell surface, the data presented here do not address how the Rem/Ca\(_{\alpha}\)-beta complex might regulate channel function. Within the Ca\(_{\alpha}\)-\(\beta\) proteins a structural interaction is known to exist between the SH3 and GK domains (2). Structural studies and biochemical studies have found that the GK domain is responsible for high affinity Ca\(_{\alpha}\)-\(\alpha\_1\)-subunit binding (2). However, the isolated SH3 domain can modulate channel inactivation kinetics, and recent work (2,9–11,13) suggests that low affinity interactions involving both the SH3 domain and the larger SH3/GK core are critical for the regulation of channel activity. In this context the recruitment of Rem to the domain interface between SH3 and GK is particularly intriguing. Whether the association of Rem with \(\beta\)-subunit may affect the intramolecular interaction or orientation of the SH3 and GK domains, promote or disrupt interactions with the Ca\(_{\alpha}\)-\(\alpha\_1\)-subunit, or serve to recruit additional regulatory factors to the channel complex has not been addressed.

Recent studies have suggested that RGK-mediated Ca\(^{2+}\) channel regulation and \(\beta\)-subunit binding are GTP-dependent, indicating that the effector domain of RGK proteins contributes critical contacts to \(\beta\)-subunit association (21, 31). This...
result is surprising, since the effector domain of Ras-related GTP-binding proteins is predicted to be the primary interaction site for cellular binding proteins, and this region within the RGK proteins is highly variable (25). Thus, sequence comparisons suggest that RGK proteins are unlikely to share common effector domain binding targets, although both CaVαβ association and regulation of L-type Ca\(^{2+}\) channel currents are common functions of all members of this GTPase subfamily (17, 18). Indeed, at least using in vitro binding assays, Rem association with CaVαβ subunits is nucleotide-independent (Fig. 1B). Alternatively, it is possible that RGK proteins adopt a unique interaction face with β subunits that does not involve the classical “effector loop” region (25). Recently, we have found that the conserved and extended C-terminal domain of RGK proteins might contribute to in vivo β subunit association, since deletion of the terminal 32 residues from either Rem or Rad proteins destabilizes their βα subunit binding (22). Finally, it is possible that GTP binding might result in a conformational change that recruits additional cellular factors that promote in vivo β subunit association. Resolution of these questions is likely to require structural analysis of a RGK family member-β subunit complex. The recent report of preliminary crystallographic data for the Rad GTPase (40) represents an important first step in this process.

In summary, it is well established that the association of ion channel subunits with intracellular binding proteins is involved in the regulation of channel activity (1). The present studies provide insights into the mechanism of Rem-mediated control of Ca\(^{2+}\) channel activity by defining the Rem interaction domain on CaVαβ subunits. Our results suggest a new context for considering the association of Rem and β subunits because they indicate that CaVαβ provides a scaffold for the recruitment of the Rem GTPase into the plasma membrane Ca\(^{2+}\) channel complex to allow for acute regulation of calcium currents. This novel control mechanism is likely integrated with other Ca\(^{2+}\) channel regulatory processes. Indeed, we have recently found that Rem-mediated channel regulation can be modulated by cellular kinase pathways (41). Clearly, additional studies will be needed to clarify the cellular signaling pathways that control Rem activity, the molecular mechanism of Rem-CaVαβ-mediated regulation of Ca\(^{2+}\) channel function, and whether CaVαβ scaffolding is a general feature of RGK protein channel control including the long-term modulation of Ca\(^{2+}\) channel surface density.

Acknowledgment—We thank Dr. Timothy Kamp (Univ. of Wisconsin, Madison, WI) for CaVα1.2 and CaVαβ2α expression vectors.

REFERENCES

1. Catterall, W. A. (2000) Annu. Rev. Cell Dev. Biol. 16, 521–555
2. Richards, M. W., Butcher, A. J., and Dolphin, A. C. (2004) Trends Pharmacol. Sci. 25, 626–632
3. Pragnell, M., De Waard, M., Mori, Y., Tanabe, T., Snutch, T. P., and Campbell, K. P. (1994) Nature 368, 67–70
4. Bichet, D., Cornet, V., Geib, S., Carlier, E., Voisen, S., Hoshi, T., Mori, Y., and De Waard, M. (2000) Neuron 25, 177–190
5. De Waard, M., Pragnell, M., and Campbell, K. P. (1994) Neuron 13, 495–503
6. Chen, Y. H., Li, M. H., Zhang, Y., He, L. L., Yamada, Y., Fitzmaurice, A., Shen, Y., Zhang, H., Tong, L., and Yang, J. (2004) Nature 429, 675–680