Syntaxin-1A Binds to and Modulates the Slo Calcium-Activated Potassium Channel via an Interaction That Excludes Syntaxin Binding to Calcium Channels

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Submitted 3 August 2004; accepted in final form 18 October 2004

Cibulsky, Susan M., Hong Fei, and Irwin B. Levitan. Syntaxin-1A binds to and modulates the Slo calcium-activated potassium channel via an interaction that excludes syntaxin binding to calcium channels. J Neurophysiol 93: 1393–1405, 2005. First published October 20, 2004; doi:10.1152/jn.00789.2004. From its position in presynaptic nerve terminals, the large conductance Ca2+-activated K+ channel, Slo, regulates neurotransmitter release. Several other ion channels known to control neurotransmitter release have been implicated in physical interactions with the neurotransmitter release machinery. For example, the Cav2.2 (N-type) Ca2+ channel binds to and is modulated by syntaxin-1A and SNAP-25. Furthermore, a close juxtaposition of Slo and Cav2.2 is presumed to be necessary for functional coupling between the two channels, which has been shown in neurons. We report that Slo exhibits a strong association with syntaxin-1A. Robust co-immunoprecipitation of Slo and syntaxin-1A occurs from transfected HEK293 cells as well as from brain. However, despite this strong interaction and the known association between syntaxin-1A and the II–III loop of Cav2.2, these three proteins do not co-immunoprecipitate in a trimeric complex from transfected HEK293 cells. The Slo-syntaxin-1A co-immunoprecipitation is not significantly influenced by [Ca2+]. Multiple relatively weak interactions may sum up to a tight physical coupling of full-length Slo with syntaxin-1A: the C-terminal tail and the S0–S1 loop of Slo each co-immunoprecipitate with syntaxin-1A. The presence of syntaxin-1A leads to reduced Slo channel activity due to an increased V1/2 for activation in 100 nM, 1 µM, and 10 µM Ca2+, reduced voltage-sensitivity in 1 µM Ca2+, and slower rates of activation in 10 µM Ca2+. Potential physiological consequences of the interaction between Slo and syntaxin-1A include enhanced excitability through modulation of Slo channel activity and reduced neurotransmitter release due to disruption of syntaxin-1A binding to the Ca2,2 II–III loop.

Introduction

The large conductance Ca2+-activated K+ (KCa) channel (Slo) is expressed widely in mammalian tissues (Latorre et al. 1989). Both Ca2+ and voltage regulate its gating: elevated intracellular Ca2+ and depolarization cause the channel to open, producing K+ efflux (McManus 1991). In excitable cells, the consequent hyperpolarizing influence on membrane potential opposes action potential firing and urges voltage-gated Ca2+ (Ca0) channels to close. Thus Slo acts as a negative feedback modulator, exercising control over the excitability of nerve and muscle (Jan and Jan 1997). For example, Slo contributes to action potential repolarization in neurons, which modulates neurotransmitter release (Robitaille and Charlton 1992; Robitaille et al. 1993); it also responds to Ca2+ sparks produced by ryanodine receptors after depolarization of smooth muscle cells and Ca2+ influx through Ca0 channels, thereby relaxing smooth muscle tone (Brayden and Nelson 1992; Nelson et al. 1995).

Functional coupling between KCa and Ca0 channels, characterized by a dependence of KCa channel activation on Ca2+ influx through Ca0 channels, has been documented across a variety of neuronal cell types. Ca2,2 (N-type Ca2+ channel)-Slo is one such functionally coupled pair in certain neurons (Marrion and Tavalin 1998; Wisgirda and Dryer 1994). Cross-talk between Slo and Ca2,2 may be physiologically important at presynaptic nerve terminals, where the channels are colocalized (Issa and Hudspeth 1994; Roberts et al. 1990; Robitaille et al. 1993) and where they both regulate synaptic transmission (Robitaille and Charlton 1992). Considerations of the temporal and spatial distributions of Ca2+ that enters a cell through Ca0 channels (Roberts 1993; Simon and Linas 1985) together with the Ca2+ affinity and kinetics of activation of KCa channels have led to the supposition that the channels must be arranged in close physical proximity for functional coupling to occur. Experimental observations that buttress this argument include the disruption of functional coupling by BAPTA, a fast Ca2+ buffer, but not by EGTA, which acts relatively slowly (Pakriya and Lingle 2000; Robitaille and Charlton 1992; Robitaille et al. 1993), and the short time delay between openings of functionally coupled single channels, which is so minimal for one type of pair that even BAPTA is without effect (Marrion and Tavalin 1998).

The putative constraint on localization of presynaptic Slo channels, within short range of Ca2,2 channels, raises the possibility that Slo interacts physically with Ca2,2 or other neighboring proteins. Syntaxin-1A, SNAP-25, and synaptopagin, which are integral components of the neurotransmitter release machinery (Li and Chin 2003), bind to Ca2,2 at the synprint site within the intracellular loop connecting domains II and III (II–III loop) (Bennett et al. 1992; Sheng et al. 1994, 1996, 1997). These interactions may be necessary for proper functioning of the neurotransmitter release mechanism, because their disruption interferes with neurotransmission (Mochida et al. 1996; Retrig et al. 1997). Syntaxin-1A and SNAP-25 also downregulate Ca2,2 channel activity by shifting steady-state inactivation to less depolarized potentials (Bezprozvanny et al. 1995; Jarvis and Zamponi 2001a). This itself could influence neurotransmitter release, although in this

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case, interaction would presumably lead to reduced release.  
K\(_{1.1}\), another K\(^+\) channel that is expressed in presynaptic nerve terminals and regulates neurotransmitter release, is associated with and modulated by syntaxin-1A and SNAP-25 (Fili et al. 2001; Ji et al. 2002). However, the first report of an affiliation between Slo and a synaptic protein was published only recently (Ling et al. 2003). Surprisingly, considering the length of its C-terminal tail, the map of known protein–protein interactions involving Slo is relatively sparsely populated; in contrast, many other ion channels have been implicated in a myriad of interactions (Jarvis and Zamponi 2001b; Scannevin and Trimmer 1997). In this study, we report that Slo associates with and is modulated by syntaxin-1A.

**METHODS**

**cDNA constructs**

The mbr5 clone (Butler et al. 1993) encoding the mouse Slo K\(_{\text{ca}}\) channel in the mammalian expression vector pcDNA3 was used in this study. In some biochemical experiments, a modified version of this construct, in which a hemagglutinin (HA) epitope tag had been added through PCR to the C-terminal end of the channel, was employed. For expression of the C-terminal tail of Slo absent the membrane-spanning domain (Slo-CT) in HEK293 cells, sequence encoding this tail region was subcloned into the pcDNA3.1-HisC vector, which encodes hexahistidine and Xpress epitope tags upstream of the insert, using a combination of native restriction sites and PCR to add desired sites. The 835 amino acid Slo-CT insert starts with the amino acid sequence YSAVSG, 13 amino acids after the predicted end of S6, and ends with EWEDEC. Human syntaxin-1A cDNA (Zhang et al. 1995) in the pcDNA3 vector was used for biochemical experiments. For electrophysiological recording, the syntaxin-1A cDNA was subcloned into pRES2-EFGP, a bicistronic vector that permits co-expression of syntaxin-1A and enhanced green fluorescent protein (GFP) as separate proteins in the same cell after transfection with this single plasmid. Rat syntaxin-1B cDNA in the pMT2sx vector was obtained from Dr. Gerald Zamponi (University of Calgary). Dr. Andrew Braun (University of Calgary) provided rat syntaxin-3A in the SR\(_{\alpha}\) plasmid. Mouse syntaxin-4A in the pCMV-SPORT6 plasmid from the Mammalian Genome Collection was purchased from American Type Culture Collection. Constructs encoding GST-fusion proteins of the cytoplasmic portions of rat syntaxin-2 (amino acids 2–264) and syntaxin-3 (amino acids 2–264) in the pGEX-KG vector were provided by Dr. Shu-Chan Hsu (Rutgers University), cDNA encoding the entire II–III loop of the Ca\(^{2+}\) channel Ca\(_{\text{v}}\)2.2 was amplified by PCR, digested with the appropriate restriction enzymes, and ligated into the pEBG-1 vector (a gift from Dr. Joseph Avruch, Harvard Medical School), which is designed to express the insert as a GST fusion protein in mammalian cells.

**Co-immunoprecipitation and Western blotting**

In experiments that tested for associations between proteins with a co-immunoprecipitation strategy, HEK293 cells were transfected with the appropriate cDNA using a calcium phosphate protocol. Two days after transfection, cells were lysed in a buffer containing (in mM) 20 Tris-Cl (pH 7.5), 10 EDTA, 150 NaCl, 50 KCl, 50 Na\(_4\)F, and 2 DTT, plus 1% CHAPS or 1% Triton X-100 and the protease inhibitors PMSF (0.2 mM), aprotinin, leupeptin, and pepstatin A (1 \(\mu\)g/ml each). Lysate was precleared with protein A/G-agarose beads (Santa Cruz Biotechnology) and incubated with the appropriate antibody for \(\geq 2\) h at 4°C. Immune complexes were precipitated with protein A/G-agarose heads by incubation for 1–2 h and washed five times with \(\geq 10\) times head volume of lysis buffer. After sample loading buffer was added, the sample was heated to 100°C. The eluted proteins were loaded into gels for Western blotting.

Lysis buffers containing various concentrations of free Ca\(^{2+}\) were made in the following way, consistent with the method for adjusting free Ca\(^{2+}\) in solutions used for electrophysiological recording. To the basic buffer [in mM: 20 Tris-Cl (pH 7.5), 150 NaCl, 50 KCl, 50 Na\(_4\)F], 5 mM of Ca\(^{2+}\) chelator (EDTA for 0, EGTA for 100 nM free Ca\(^{2+}\), HEDTA for 1 \(\mu\)M, and 10 \(\mu\)M free Ca\(^{2+}\), and no chelator for 100 \(\mu\)M and 2 mM free Ca\(^{2+}\)] and the appropriate amount of total CaCl\(_2\) were added, based on calculations made with MaxChelator software version 2.40 (Bers et al. 1994). pH was adjusted to 7.2. Finally, 1% CHAPS, 2 mM DTT, 0.2 mM PMSF, and 1 \(\mu\)g/ml each of aprotinin, leupeptin, and pepstatin A were added just prior to use. Intracellular [Ca\(^{2+}\)] was varied prelysis by one of two treatments of transfected HEK293 cells: 1) extracellular application of thapsigargin (2 \(\mu\)M) for 10 min, followed by BAPTA-AM (10 \(\mu\)M) for 10 min, each in Ringer solution containing 5 mM EGTA and no added Ca\(^{2+}\), and then lysis in standard lysis buffer containing 5 mM EDTA and no added Ca\(^{2+}\) (lo Ca\(^{2+}\) treatment); or 2) extracellular application of ionomycin (1 \(\mu\)M) for \(\sim\)3 min in Ringer solution containing 2 mM Ca\(^{2+}\), followed immediately by lysis in buffer containing 2 mM Ca\(^{2+}\) and no Ca\(^{2+}\) chelator (hi Ca\(^{2+}\) treatment). Thapsigargin and BAPTA-AM were purchased from Calbiochem and ionomycin was from Santa Cruz Biotechnology.

For co-immunoprecipitation of native proteins, crude membranes were prepared from mouse brain and mouse pancreas (Pel-Freeze Biologicals). The tissues were ground to fine powder in liquid N\(_2\) and homogenized with five strokes in a glass homogenizer and buffer containing (in mM) 2.5 KCl, 250 sucrose, 25 HEPES, 0.1 EGTA, 0.1 EDTA (pH 7.4) plus DTT (2 mM), and the protease inhibitors PMSF (0.2 mM), aprotinin, leupeptin, and pepstatin A (1 \(\mu\)g/ml each). The homogenate was centrifuged at 1,000g for 10 min; supernatant was collected and centrifuged at 150,000g for 1 h. The pelleted crude membrane fraction was resuspended in lysis buffer containing 1% Triton X-100, 2 mM DTT, and protease inhibitors as above for HEK293 cell lysate. Total protein concentration of the solubilized membrane prep was determined using a DC protein assay (Bio-Rad) and adjusted to \(\sim\)2 mg/ml with lysis buffer. The sample was treated as HEK293 cell lysate.

Polyacrylamide gel electrophoresis was used to separate denatured proteins in cell lysates or immunoprecipitates. Samples were loaded into precast Mini-Gels (Bio-Rad). After separation, proteins were transferred to nitrocellulose membranes using a wet transfer protocol. Blots were blocked with 5% nonfat dry milk in TBST (10 mM Tris-Cl, pH 7.5, 150 mM NaCl, 0.1% Tween 20) before incubation with primary antibody in 5% milk/TBST overnight at 4°C. The next day, blots were washed three times with TBST and incubated with horseradish peroxidase-conjugated anti-mouse or anti-rabbit IgG secondary antibody (Amersham Biosciences). Proteins were detected using enhanced chemiluminescence (Amersham Biosciences). Each co-immunoprecipitation experiment was carried out at least three times, and results representative of the overall trend are displayed in the figures.

For detection of Slo, a polyclonal antibody recognizing amino acids 972–1,135 of mouse Slo (mbr5) was raised in rabbits (H. Wen and I. B. Levitan, unpublished results). Rabbit polyclonal antibody was also produced against sequence in the II–III loop of Cav2.2: amino acids 972–1,135 of mouse Slo (mbr5) was raised in rabbits (H. Wen and I. B. Levitan, unpublished results). Rabbit polyclonal antibody was also produced against sequence in the II–III loop of Cav2.2: amino acids 821–1,030 (EPGRD...DLEAI) of the rbB-G clone (Y. Zhou I. B. Levitan, unpublished results). Rabbit polyclonal antibody was also produced against sequence in the II–III loop of Cav2.2: amino acids 821–1,030 (EPGRD...DLEAI) of the rbB-G clone (Y. Zhou I. B. Levitan, unpublished results). Rabbit polyclonal antibody was also produced against sequence in the II–III loop of Cav2.2: amino acids 821–1,030 (EPGRD...DLEAI) of the rbB-G clone (Y. Zhou I. B. Levitan, unpublished results). Rabbit polyclonal antibody was also produced against sequence in the II–III loop of Cav2.2: amino acids 821–1,030 (EPGRD...DLEAI) of the rbB-G clone (Y. Zhou I. B. Levitan, unpublished results). Rabbit polyclonal antibody was also produced against sequence in the II–III loop of Cav2.2: amino acids 821–1,030 (EPGRD...DLEAI) of the rbB-G clone (Y. Zhou I. B. Levitan, unpublished results). Rabbit polyclonal antibody was also produced against sequence in the II–III loop of Cav2.2: amino acids 821–1,030 (EPGRD...DLEAI) of the rbB-G clone (Y. Zhou I. B. Levitan, unpublished results). Rabbit polyclonal antibody was also produced against sequence in the II–III loop of Cav2.2: amino acids 821–1,030 (EPGRD...DLEAI) of the rbB-G clone (Y. Zhou I. B. Levitan, unpublished results).
chased from Santa Cruz Biotechnology and anti-GFP antibody was from Molecular Probes.

**Electrophysiological recording**

HEK293 cells were transfected with either Slo plus EGFP cDNA, in separate vectors, or Slo (in the pcDNA3 vector) plus syntaxin-1A in the pRES2-EGFP vector using the lipid-based reagent FuGene 6 (Roche) for experiments in which Slo channel currents were to be recorded. Pipets were pulled from borosilicate glass (Jencons-PLS), coated with Sylgard (Dow Corning) and fire polished; pipet resistances were 1.3–3.1 MΩ. Currents were recorded in excised patches using the resistive feedback circuit of an Axopatch 200 amplifier (Axon Instruments). Filtering was performed on-line at 10 kHz using the amplifier’s internal filter, and the sampling interval was 20 μs.

Data were digitized using a Digidata 1322A and pClamp 8.2 software (Axon Instruments). The pipet solution (extracellular) consisted of (in mM) 150 KCl, 0.5 MgCl₂, 10 HEPES, and 5 HEDTA, pH 7.2. All bath solutions (intracellular) contained 150 mM KCl and 10 mM HEPES, and pH was adjusted to 7.2 after addition of Ca²⁺ buffer (5 mM EGTA for 100 nM free Ca²⁺; 5 mM HEDTA for 1 and 10 μM free Ca²⁺) and CaCl₂. The appropriate amount of CaCl₂ to add was calculated using MaxChelator software, version 2.40. Test pulses of 30- or 35-ms duration were applied every second from a holding potential of −80 mV. In 100 mM bath Ca²⁺, an additional step to +40 mV, following the test pulse, was applied for the measurement of deactivation rate, due to rapid channel closing at −80 mV in this low concentration of Ca²⁺. Leak subtraction was performed on-line with a P/4 protocol.

Two or more traces from the same patch were averaged before analysis. Tail current amplitude measured 200 μs after the step from the test potential to −80 (in 1 or 10 μM Ca²⁺) or +40 mV (in 100 mM Ca²⁺) was used to generate conductance-voltage (G-V) relationships. G-V curves were fit with a Boltzmann function. Exponential fits to activation and deactivation rates were performed with pClamp 8.2 software using the Chebyshev method. The results of such fits for individual patches (Vt1/2 and slope for G-V curves, and time constants for activation and deactivation) were compared for Slo ± syntaxin-1A with an unpaired t-test.

**RESULTS**

**Slo and syntaxin-1A are tightly associated both in an expression system and in brain**

Slo and syntaxin-1A co-immunoprecipitate from HEK293 cells cotransfected with cDNAs for mouse Slo (Butler et al. 1993) and human syntaxin-1A (Zhang et al. 1995) (Fig. 1, A and B). The result is robust (an intense signal with short exposure of the blot to film), reproducible, is seen whether immunoprecipitation is elicited by antibody recognizing syntaxin or Slo, and occurs with both HA epitope-tagged Slo (anti-HA antibody used for immunoprecipitation) and untagged Slo. The association is maintained in either 1% CHAPS or 1% Triton X-100 detergent. Negative controls, where Slo is expressed alone and immunoprecipitated with anti-syntaxin antibody (Fig. 1A, lane 4) or syntaxin-1A is expressed alone and immunoprecipitated with anti-Slo antibody (Fig. 1B, lane 8), rule out significant nonspecific recognition of either protein. Mock-transfected cells were also used as negative controls in some experiments and yielded no nonspecific signals (data not shown). Co-immunoprecipitation of Slo and syntaxin-1A required cotransfection of the two cDNAs; if lysates of separate batches of cells transfected with either Slo alone or syntaxin-1A alone were mixed just prior to immunoprecipitation, there was no detectable co-purification (data not shown). Such a phenomenon suggests that the proteins may associate early during biosynthesis.

Likewise, the native proteins are associated with each other. From crude membrane preps of whole mouse brain, where Slo and syntaxin-1A are both richly expressed (Fig. 2A, lane 2; Fig. 2B, lane 7), Slo is present in a precipitate produced with anti-syntaxin-1A antibody (Fig. 2A, lane 4), and syntaxin in turn co-immunoprecipitates with Slo (Fig. 2B, lane 9). Mouse pancreas was used as a negative control in this experiment. Slo was not detected in the pancreas membrane preps with our antibody (Fig. 2A, lane 3), and the lack of signal after immunoprecipitation with anti-syntaxin-1A antibody (Fig. 2A, lane 5) shows that this antibody does not precipitate anything from pancreas that is recognized by anti-Slo antibody. Syntaxin-1A itself is expressed in pancreas (Fig. 2B, lane 8), consistent with published reports (Nagamatsu et al. 1996), and the lack of syntaxin in precipitate produced by anti-Slo antibody (Fig. 2B, lane 10) shows that anti-Slo antibody does not precipitate syntaxin-1A from pancreas. To be sure that Slo and syntaxin-1A are not nonspecifically precipitated from brain under these experimental conditions, we produced precipitates with control IgG and anti-GFP (Fig. 2, C and D). Compared with the Slo co-immunoprecipitated with syntaxin (Fig. 2C, lane 3) and the syntaxin co-immunoprecipitated with Slo (Fig. 2D, lane 3) in the same experiment, neither control IgG nor anti-GFP yielded significant labeling of proteins recognized as Slo (Fig. 2A).
FIG. 2. Native proteins Slo and syntaxin-1 are associated in brain. A and B: Slo co-immunoprecipitates with syntaxin (A) from crude membranes of brain (lane 4), but not pancreas (lane 5), where Slo is not detected in the membrane fraction (lane 3). Syntaxin is present in Slo immunoprecipitate (B) from crude brain membranes (lane 9), but not pancreas membranes (lane 10), even though syntaxin is expressed in pancreas (lane 8). Lanes 2 and 3 contain the starting material (solubilized membrane prep) for the immunoprecipitates (IPs) shown in lanes 4 and 5; likewise, lanes 7 and 8 contain the starting material for the IPs in lanes 9 and 10. Lysates from HEK293 cells transfected with the appropriate construct are shown in lanes 1 and 6 for comparison. 

C and D: IPs from brain with control IgG (lane 5) or anti-green fluorescent protein (GFP; lane 6) or from liver (lane 4) with anti-syntaxin-1A or anti-Slo show no significant background labeling on a Slo blot (C) and a syntaxin blot (D). 

E: Western blots of lysates from HEK293 cells transfected with syntaxin-1A (lane 1), -1B (lane 2), -3 (lane 3), or -4 (lane 4) and lysates from bacteria expressing a GST-fusion protein of the cytosolic region of syntaxin-2 (GST-syntaxin-2, amino acids 4 to 264; lane 5) or syntaxin-3 (GST-syntaxin-3, amino acids 4–264; lane 6) were probed with the indicated anti-syntaxin antibodies. F: as in D, but Western blot was probed with polyclonal anti-syntaxin-1 antibody.
2C, lanes 5 and 6) or syntaxin (Fig. 2D, lanes 5 and 6). In addition, immunoprecipitations were performed with liver, where neither Slo nor syntaxin-1A is detected in membrane preps (Fig. 2, C and D, lane 2). No nonspecific signals of the appropriate molecular weights arose after precipitations from this tissue (Fig. 2, C and D, lane 4).

Because 15 syntaxin genes have been identified in the human genome, many of which are expressed in brain and some of which are closely related to syntaxin-1A, we tested the specificity of the monoclonal anti-syntaxin-1A used in this study. Western blots of lysates from HEK293 cells transfected with syntaxin-1A, -1B, -3, or -4 and from bacteria transformed with a GST-fusion protein of the cytoplasmic portion of syntaxin-2 or -3 were probed with several different anti-syntaxin antibodies (Fig. 2E). Monoclonal anti-syntaxin-1A avidly recognizes cloned syntaxin-1A (Fig. 2E, lane 1) and -1B (Fig. 2E, lane 2), identifies syntaxin-3 (Fig. 2E, lanes 3 and 6) to a much lesser extent, and does not recognize syntaxins-2 (Fig. 2E, lane 5) and -4 (Fig. 2E, lane 4). Syntaxins-1A and -1B are >80% identical in amino acid sequence, and therefore antibody cross-reactivity to these two proteins is to be expected. Previous work has shown that syntaxin-3 does not interact with Slo by the lack of co-immunoprecipitation of native proteins from brain and of cloned versions from an expression system (Ling et al. 2003). However, in addition, we showed here that a polyclonal anti-syntaxin-1 antibody that does not significantly label syntaxin-3 (Fig. 2E, lane 3, bottom) yields results consistent with those obtained with monoclonal anti-syntaxin-1A, when used to probe Western blots after immunoprecipitations from native tissue (Fig. 2F). The weak labeling by polyclonal anti-syntaxin-1 (Fig. 2F) relative to monoclonal anti-syntaxin-1A (Fig. 2, B and D) is consistent with weaker labeling of cloned syntaxins (Fig. 2F, compare top and bottom blots). Finally, after immunoprecipitation from brain with anti-Slo or monoclonal anti-syntaxin-1A, Western blots probed with antibodies against syntaxin-2, -3, or -4 provide no evidence of specific labeling of syntaxin (data not shown). Our results are consistent with an association between Slo and syntaxin-1A in brain. The possibility that Slo also interacts with syntaxin-1B cannot be ruled out at this point and merits further study.

Syntaxin-1A, Slo, and the II–III loop of Ca\(^{2+}\) do not co-immunoprecipitate

Since the N-type Ca\(^{2+}\) channel Ca\(^{2+}\),2.2 is known to bind presynaptic neurotransmitter release sites (Issa and Hudspeth 1994; Roberts et al. 1990; Robitaille et al. 1993), we considered two scenarios: either syntaxin-1A accommodates both Slo and Ca\(^{2+}\),2.2 at once, or one interaction precludes the other. Our strategy was to look for the trimeric complex Slo-syntaxin-1A-Ca\(^{2+}\),2.2 II–III loop in immunoprecipitates from HEK293 cells transfected with these three constructs and precipitated for Slo or Ca\(^{2+}\),2.2 II–III loop. The II–III loop of Ca\(^{2+}\),2.2 fused to GST, in the pEBG-1 vector, was used rather than the full-length Ca\(^{2+}\),2.2 channel because full-length Ca\(^{2+}\),2.2 co-immunoprecipitates with Slo from cotransfected HEK293 cells (unpublished data), whereas the II–III loop does not. Cotransfection of syntaxin-1A with Slo and Ca\(^{2+}\),2.2 II–III loop does not lead to formation of a trimeric complex, even though associations between Slo and syntaxin-1A and between Ca\(^{2+}\),2.2 II–III loop and syntaxin-1A are readily seen (Fig. 3). From the lysates of cells cotransfected with all three cDNAs, syntaxin-1A co-purifies with Ca\(^{2+}\),2.2 II–III loop (using anti-Ca\(^{2+}\),2.2 antibody for immunoprecipitation; Fig. 3B, lane 9), but Slo is not detected in the same precipitate (Fig. 3A, lane 3), even after long exposure of the film (data not shown). The lysate in Fig. 3A, lane 1, and Fig. 3B, lane 7, was divided in half before immunoprecipitations; after incubation of the other half of this lysate with anti-syntaxin-1A antibody, precipitates contain large amounts of Slo (Fig. 3A, lane 5). The result is the same when immunoprecipitation is performed with anti-HA antibody (which recognizes HA-tagged Slo): syntaxin-1A is present in the precipitate (Fig. 3D, lane 9), but Ca\(^{2+}\),2.2 II–III loop is not (Fig. 3C, lane 3). From the other half of the same lysate, Ca\(^{2+}\),2.2 II–III loop co-immunoprecipitates with syntaxin-1A (Fig. 3C, lane 5). The results of these experiments suggest that syntaxin-1A cannot accommodate both Slo and the II–III loop of Ca\(^{2+}\),2.2 simultaneously, which could have important implications for the regulation of neurotransmitter release.

Co-immunoprecipitation of Slo and syntaxin-1A over a range of [Ca\(^{2+}\)]

The syntaxin-1A-Ca\(^{2+}\),2.2 II–III loop interaction apparently depends on free [Ca\(^{2+}\)], such that binding between the two proteins is stronger in 10–20 μM Ca\(^{2+}\) than in lower or higher Ca\(^{2+}\) concentrations (Sheng et al. 1996). This raises the question: is the Slo-syntaxin-1A interaction Ca\(^{2+}\)-sensitive, especially given that Slo itself is regulated by Ca\(^{2+}\)? To address this possibility, we measured the Slo-syntaxin-1A co-immunoprecipitation in a range of Ca\(^{2+}\) concentrations: 0, 100 nM, 1 μM, 10 μM, and 100 μM. Free [Ca\(^{2+}\)] in the lysis buffer was adjusted using 5 mM EGTA for 100 nM Ca\(^{2+}\) and 5 mM HEDTA for 1 and 10 μM Ca\(^{2+}\), as for the solutions used in electrophysiological recording. The nominally 0 Ca\(^{2+}\) solution contained 5 mM EDTA, and no Ca\(^{2+}\) buffer was used for 100 μM free Ca\(^{2+}\). Slo and syntaxin-1A do co-purify in all five Ca\(^{2+}\) concentrations tested (Fig. 4A). Densitometric analysis of the results from three sets of experiments yielded no significant differences across [Ca\(^{2+}\)] for Slo co-immunoprecipitation with syntaxin-1A (Fig. 4B).

To test the possibility that changing [Ca\(^{2+}\)] in the lysis buffer is not sufficient to affect the Slo-syntaxin-1A interaction, we additionally elicited changes in intracellular [Ca\(^{2+}\)] before lysis. Hi Ca\(^{2+}\) was achieved by incubating transfected cells with ionomycin (1 μM) in a standard Ringer solution containing 2 mM Ca\(^{2+}\) for 3 min and immediately lysing cells in buffer containing 2 mM free Ca\(^{2+}\). This prelysis treatment produces a significant rise in intracellular Ca\(^{2+}\) that saturates in 3 min, as measured with the Ca\(^{2+}\) indicator fluo-4 in pilot experiments (Fig. 4C). Lo Ca\(^{2+}\) was produced by incubating cells with thapsigargin (2 μM) in Ringer solution containing 5 mM EGTA and no added Ca\(^{2+}\) for 10 min, to deplete internal Ca\(^{2+}\) stores, followed by incubation with the membrane-permeant version of BAPTA (BAPTA-AM, 10 μM) in Ringer solution with no Ca\(^{2+}\) for 10 min, to buffer intracellular Ca\(^{2+}\). Subsequent lysis of lo Ca\(^{2+}\)-treated cells was carried out in nominally 0 Ca\(^{2+}\) lysis buffer, containing 5 mM EDTA and no added Ca\(^{2+}\). Lo Ca\(^{2+}\)-treated cells exhibited no increase in
intracellular Ca^{2+} in response to application of ionomycin in 0 Ca^{2+} Ringer solution, as imaged with fluo-4 (Fig. 4C). Slo co-immunoprecipitates with syntaxin-1A under both of these conditions, where [Ca^{2+}] was changed before and during lysis (Fig. 4D). Compared with the control condition, in which cells were treated according to the standard lysis protocol, lo and hi Ca^{2+} did not significantly affect the amount of Slo protein that co-purified with syntaxin-1A (Fig. 4E).
Slo–syntaxin-1A interaction involves the Slo C-terminal tail and S0–S1 loop

In an attempt to delineate the binding site(s) for syntaxin-1A on Slo, we made constructs of isolated regions of the channel. The Slo C-terminal tail in the pcDNA 3.1-HisC vector (Slo-CT; the entire tail except for the 1st 12 amino acids after the end of the S6 transmembrane region) exhibits a weak co-immunoprecipitation with syntaxin-1A (Fig. 5A) relative to the full length Slo-syntaxin-1A interaction (Figs. 1–4). A band representing Slo-CT from co-immunoprecipitation with anti-syntaxin-1A antibody is seen only after relatively long exposure of film (Fig. 5, lane 5). However, neither negative control on the same blot (mock-transfected or Slo-CT alone) yielded a signal after the same long exposure (Fig. 5, lanes 4 and 6), thereby validating the Slo-CT-syntaxin-1A co-immunoprecipitation. The small amount of copurified Slo-CT cannot be explained by poor immunoprecipitation of syntaxin-1A itself, because robust signals for syntaxin-1A are seen before and after precipitation (Fig. 5, lanes 7 and 8). Possible explanations for this result are that there truly is a weak interaction between syntaxin-1A and Slo-CT and/or that improper folding of the tail in the absence of the channel core leads to diminished ability to bind syntaxin-1A. If there is only a weak interaction between syntaxin-1A and Slo-CT in situ, this leads to the inference that there must be an additional interaction site(s) elsewhere on the channel to account for the robust interaction between the full-length proteins. With this idea in mind, we made an HA-tagged (C-terminus) construct of the Slo S0–S1 loop, the only cytosolically disposed piece of Slo of significant length (70 amino acids) besides Slo-CT. Slo S0–S1 loop-HA protein is expressed well in transfected HEK293 cells (Fig. 5B, lanes 1 and 2) and it co-immunoprecipitates with syntaxin-1A.
In this case, co-immunoprecipitation can be seen after relatively short exposure of film (compared with Slo-CT), although the intense result observed with full-length Slo and syntaxin-1A is not fully reproduced. The results point out the possibility that the strong association of Slo with syntaxin-1A comprises multiple relatively weak interactions, involving at least the S0–S1 loop and the C-terminal tail of Slo. Additional sites have not been ruled out, including transmembrane regions. It also seems possible that the isolated C-terminal tail and S0–S1 loop might not fold properly without the remainder of the channel or that the epitope tags interfere with proper formation of a syntaxin-1A binding site. Slo-CT and shorter pieces of the tail do not express nearly as well as the full-length channel. These limitations prevented us from further narrowing the search for a syntaxin-1A interaction site on the Slo C-terminal tail.

**Syntaxin-1A negatively regulates Slo channel activity in an expression system**

To examine the functional effect of syntaxin-1A co-expression on Slo channel activity, we recorded macroscopic currents, using symmetrical 150 mM KCl solutions, in excised patches from HEK293 cells transfected with Slo ± syntaxin-1A (Fig. 6). Control cells were transfected with Slo and EGFP in separate vectors. The experimental cells were transfected with Slo in the pcDNA3 vector plus syntaxin-1A in the bicistronic pIRE2-EGFP vector. This ensures that a GFP-
expressing cell also expresses syntaxin-1A, yet the syntaxin-1A protein itself is not tagged with GFP. Patches producing at least ~1 nA of current during a test pulse to +200 mV were included in the analysis. Average outward current at maximal conductance (G_{\text{max}}) was 5.02 ± 0.85 nA in patches expressing Slo without syntaxin-1A and 5.29 ± 1.21 nA in patches with syntaxin-1A (not significantly different). Pipette resistance also did not differ significantly between groups. Inspection of the traces suggests, and we confirmed by quantitative analysis, that Slo channel activation is inhibited by syntaxin-1A only in 1 or 10 μM Ca^{2+} dependence of activation in G-expressing Slo without syntaxin-1A and 5.29 syntaxin-1A (Fig. 6). Qualitative analysis, that Slo channel activation is inhibited by InsP_{3} resistance also did not differ significantly between groups.

The reduced steepness of the G-V curve in 1 μM Ca^{2+} and by slowing the rate of activation in 10 μM Ca^{2+}. The functional effects of syntaxin-1A on Slo channel activity argue that, although the two proteins may associate early in biosynthesis, interaction must be maintained after the mature proteins are in place at the plasma membrane. Our work also suggests that syntaxin-1A may not bind Slo and the N-type Ca^{2+} channel Ca_{v}2.2 II–III loop simultaneously, which raises questions such as whether there is competition for syntaxin-1A between Slo and Ca_{v}2.2 and what regulates syntaxin’s preferential binding of one channel over the other. This relationship between syntaxin-1A and Slo could potentially have two important roles in signal transduction: a direct effect on neuronal excitability, through inhibition of Slo channel activity, and an indirect effect on neurotransmitter release, by interfering with the binding of syntaxin-1A to Ca_{v}2.2.

A plethora of ion channels, of various types, participate in protein–protein interactions with signaling molecules that in many cases modulate channel activity. These interactions often occur at intracellularly located channel regions. Slo has an extremely long C-terminal tail (>800 amino acids) that is thought to reside mostly in the cytoplasm, although a proximal portion, the RCK domain, may be closely associated with the transmembrane core of the channel and play a key role in gating (Jiang et al. 2002). Relatively few proteins, however, have been found to associate with this tail. The weak co-immunoprecipitation of Slo-CT with syntaxin-1A shown here does not reproduce or fully account for the apparently strong association between full-length Slo and syntaxin-1A, but nonetheless is suggestive of a role for Slo-CT. Weak involvement of Slo-CT is consistent with our finding of another binding site for syntaxin-1A in Slo’s S0–S1 loop. Together, multiple low affinity interactions might add up to a high affinity relationship between proteins. Additional syntaxin binding sites may exist on Slo, including transmembrane regions, because syntaxin-1A itself is inserted into the plasma membrane.

The lack of unequivocal identification of syntaxin-1A binding site(s) notwithstanding, we have shown here a robust, reproducible association between Slo and syntaxin-1A that significantly influences the activity of Slo. The voltage required for half-maximal activation of Slo is right-shifted by syntaxin-1A at all three Ca^{2+} concentrations studied, yet the slope of the G-V curve is significantly affected only at the intermediate concentration of 1 μM. In our assays, the binding of syntaxin-1A to Slo is not Ca^{2+}-dependent. Therefore an explanation for such a phenomenon would seem to require interaction of syntaxin-1A with multiple types of sites on the channel, each of which has a distinct role in gating Slo with Ca^{2+} and/or voltage. For example, in 1 μM Ca^{2+}, syntaxin’s influence on voltage sensitivity, possibly through one particular site, is more prominent than in lower or higher [Ca^{2+}]. The identification of several Ca^{2+} sensor sites per subunit, one of which is in the S0–S1 loop (Braun and Sy 2001), and the remainder of which reside in the long C-terminal tail (Magleby 2003), is consistent with this idea and is corroborated by our demonstration of co-immunoprecipitation of syntaxin-1A with both the S0–S1 loop and the C-terminal tail. Furthermore, although the Ca^{2+} and voltage sensors affect gating largely independently, Slo channel gating is complex, and there is evidence for weak interactions between Ca^{2+} sensors and voltage sensors (Magleby 2003).

**Discussion**

Syntaxin-1A and the large conductance K_{Ca} channel, Slo, exhibit a robust interaction in transfected HEK293 cells and mouse brain that involves the S0–S1 loop and the C-terminal tail of Slo. They associate across a range of Ca^{2+} concentrations. Co-expression of syntaxin-1A with Slo in HEK293 cells leads to inhibition of Slo activity by increasing the voltage necessary to open the channel in the physiologically relevant Ca^{2+} concentrations of 100 nM, 1 μM, and 10 μM, by reducing the steepness of the G-V curve in 1 μM Ca^{2+} and by slowing the rate of activation in 10 μM Ca^{2+}. The functional effects of syntaxin-1A on Slo channel activity argue that, although the two proteins may associate early in biosynthesis, interaction must be maintained after the mature proteins are in place at the plasma membrane. Our work also suggests that syntaxin-1A may not bind Slo and the N-type Ca^{2+} channel Ca_{v}2.2 II–III loop simultaneously, which raises questions such as whether there is competition for syntaxin-1A between Slo and Ca_{v}2.2 and what regulates syntaxin’s preferential binding of one channel over the other. This relationship between syntaxin-1A and Slo could potentially have two important roles in signal transduction: a direct effect on neuronal excitability, through inhibition of Slo channel activity, and an indirect effect on neurotransmitter release, by interfering with the binding of syntaxin-1A to Ca_{v}2.2.
FIG. 7. Syntaxin-1A modulates Slo function. A–C: average conductance-voltage (G-V) relationships, plotted as mean ± SE (n = 5–7) for each test pulse voltage and fit with a Boltzmann function. Fit parameters: $V_{1/2}$ = 119.9, slope = 16.3 mV for Slo alone, $V_{1/2}$ = 128.0, slope = 18.6 mV for Slo + syntaxin-1A in 100 nM Ca$^{2+}$ (A); $V_{1/2}$ = 99.5, slope = 16.0 mV for Slo alone, $V_{1/2}$ = 114.3, slope = 20.9 mV for Slo + syntaxin-1A in 1 μM Ca$^{2+}$ (B); $V_{1/2}$ = 7.5, slope = 15.0 mV for Slo alone, $V_{1/2}$ = 16.9, slope = 15.3 mV for Slo + syntaxin-1A in 10 μM Ca$^{2+}$ (C). D: average $V_{1/2}$ vs. bath [Ca$^{2+}$] from individual Boltzmann fits to the G-V curve for each patch, plotted as mean ± SE (n = 5–7). $V_{1/2}$ is significantly different (P < 0.05) at each [Ca$^{2+}$]; 120.9 ± 2.2 mV for Slo alone (n = 5), 128.3 ± 1.9 mV for Slo + syntaxin-1A (n = 5) at 100 nM Ca$^{2+}$; 99.8 ± 3.0 mV for Slo alone (n = 7), 115.1 ± 4.8 mV for Slo + syntaxin-1A (n = 6) at 1 μM Ca$^{2+}$; 8.0 ± 3.7 mV for Slo alone (n = 5), 17.4 ± 2.2 mV for Slo + syntaxin-1A (n = 7) at 10 μM Ca$^{2+}$. E: average slope vs. bath [Ca$^{2+}$] for individual Boltzmann fits to the G-V curve for each patch (mean ± SE, n = 5–7). Slope is significantly different (P < 0.05) in 1 μM Ca$^{2+}$ (15.4 ± 1.3 for Slo, 19.9 ± 1.8 mV for Slo + syntaxin-1A), but not 100 nM Ca$^{2+}$ (16.7 ± 1.3 for Slo, 18.6 ± 0.9 mV for Slo + syntaxin-1A) or 10 μM Ca$^{2+}$ (14.7 ± 1.1 for Slo, 15.5 ± 1.1 mV for Slo + syntaxin-1A).
pressed in presynaptic nerve terminals and has a role in neurotransmitter release, as well as K_{v}2.1, which regulates insulin secretion from pancreatic β cells, are both inhibited by syntaxin-1A (Fili et al. 2001; Leung et al. 2003).

From our electrophysiological recordings, it seems that syntaxin-1A modulates Slo in a way that would dampen the effect of Slo as a negative feedback regulator of neuronal activity. Increased neurotransmitter release from the presynaptic terminal could result. Recently, another group has also described an association between Slo and syntaxin-1A in transfected HEK293 cells and hippocampus, consistent with our results (Ling et al. 2003). However, they also report that the association enhances Slo activity: G-V relations were shifted to the left so that less depolarization was required to open channels, the time course of activation was faster, and deactivation occurred more slowly, all in low Ca^{2+} (−10 nM and 1 μM), but not higher (10 and 100 μM). Our findings are in disagreement, and an explanation is not easily at hand. One difference between the studies is the isoform of Slo used for heterologous expression: Ling et al. (2003) studied a variant that contains a 27 amino acid insert just N-terminal to the Ca^{2+} bowl compared with the clone used here. This insert influences gating behavior such that the rate of activation in certain Ca^{2+} concentrations and Ca^{2+} sensitivity at certain voltages is significantly different from that in channels lacking the insert (Ha et al. 2000). It is conceivable that this influential modulatory region could also shape the channel’s functional response to the binding of syntaxin-1A. A second difference between studies is the temperature at which Slo currents were recorded: 35 ± 0.5°C by Ling et al. and room temperature (≈22°C) in this study. Temperature can have significant effects on protein function and could presumably account for the different results. Another possibility is that dose matters. The specific functional effects of syntaxin-1A on K_{v}1.1 have been shown to be dependent on the amount of syntaxin-1A cRNA cojected into oocytes: a high concentration caused a decrease in current amplitude, due at least in part to fewer channels at the cell surface, whereas a lower concentration enhanced current amplitude without an effect on surface expression (Fili et al. 2001). Levels of protein expression were not carefully controlled by us or Ling et al. (2003), and therefore it seems possible that levels of syntaxin-1A relative to Slo may have differed, and in turn, distinct effects were manifested.

The lack of detectable co-purification of Slo, syntaxin-1A, and Ca_{v}2.2 II–III loop in a trimeric complex is a preliminary indication that Slo and the II–III loop of Cav2.2 do not bind syntaxin-1A at the same time. This finding raises questions of whether there is competition for syntaxin-1A between Slo and Cav2.2 and what might regulate syntaxin’s preference for one channel over the other. If there is competition, Slo might disrupt the syntaxin-1A interaction with the synprint site on the Ca_{v}2.2 II–III loop, which could lead to inhibition of neurotransmitter release (Mochida et al. 1996; Rettig et al. 1997). However, the exact role(s) of synprint may not be completely understood (Spafford and Zamponi 2003), and thus the full consequences of interference with binding at this site are difficult to predict (Bezprozvanny et al. 2000; Jarvis and Zamponi 2001a). Likewise, Ca_{v}2.2 might interfere with the syntaxin-1A association with Slo, leading to relief of the
inhibition of Slo and allowing it an expanded role in regulating excitability. Estimates of distances between functionally coupled $K_{Ca}$ and $Ca_{v}$ channels would seem to allow for such a relationship among the three proteins on a physiologically relevant time scale (Marrion and Tavalin 1998; Prakriya and Lingle 2000). Furthermore, synprint peptide successfully competes with $K_{I.1}$ for interaction with syntaxin-1A in the oocyte expression system (Fili et al. 2001). The possibility of competition between $Ca_{v}.2.2$ and Slo for syntaxin-1A should be examined further.

The Slo–syntaxin-1A interaction must be placed in the context of an already complex network of known contacts among proteins in presynaptic terminals, which will inevitably grow busier. One of the next challenges is to refine the picture by adding the regulatory signals that might constrain when and where interactions occur. Many such signals are known for the area under consideration here. For example, other components of the vesicle fusion machinery influence syntaxin’s modulatory effect (Jarvis and Zamponi 2001a,b). Furthermore, G protein–signaling pathways impinge on the proteins in question: syntaxin-1A facilitates G protein $\beta y$–mediated inhibition of $Ca_{v}.2.2$ channels (Jarvis et al. 2000; Stanley and Mirotsou 1997), and $\beta y$ may be required for syntaxin-1A to regulate $K_{I.1}$ (Michaelenski et al. 2002).

Phosphorylation of $Ca_{v}.2.2$ synprint regulates its ability to bind synaptic proteins (Yokoyama et al. 1997). The ability of syntaxin-1A to interact with $Ca_{v}.2.2$ is also regulated through its own conformational state, which is influenced by other synaptic proteins (Jarvis et al. 2002). Of particular relevance to this study, it will be important to identify the factors that regulate the Slo–syntaxin-1A interaction and that might determine whether syntaxin-1A chooses to associate with Slo or the $Ca_{v}.2.2$ II–III loop.

**Acknowledgments**

We thank E. Oancea and L. Krapivsky for expert technical assistance. Present address of S. M. Cibulsky: Cardiovascular Research, 1309 Enders, Children’s Hospital, 320 Longwood Ave., Boston, MA 02115.

**Grants**

This work was supported by a grant to I. B. Levitan and a fellowship to S. M. Cibulsky from the National Institutes of Health.

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