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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ABSTRACT

From its position in presynaptic nerve terminals, the large conductance Ca\(^{2+}\)-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 Ca\(_{\text{v}}\)2.2 (N-type) Ca\(^{2+}\) channel binds to and is modulated by syntaxin-1A and SNAP-25. Furthermore, a close juxtaposition of Slo and Ca\(_{\text{v}}\)2.2 is presumed to be necessary for functional coupling between the two channels, which has been demonstrated in neurons. We report that Slo exhibits a strong association with syntaxin-1A. Robust coimmunoprecipitation 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 Ca\(_{\text{v}}\)2.2, these three proteins do not coimmunoprecipitate in a trimeric complex from transfected HEK293 cells. The Slo-syntaxin-1A coimmunoprecipitation is not significantly influenced by \([\text{Ca}\(^{2+}\)]\). 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 coimmunoprecipitate with syntaxin-1A. The presence of syntaxin-1A leads to reduced Slo channel activity, due to an increased \(V_{1/2}\) for activation in 100 nM, 1 \(\mu\)M and 10 \(\mu\)M Ca\(^{2+}\), reduced voltage-sensitivity in 1 \(\mu\)M Ca\(^{2+}\) and slower rates of activation in 10 \(\mu\)M Ca\(^{2+}\). 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’s binding to the Ca\(_{\text{v}}\)2.2 II-III loop.
INTRODUCTION

The large conductance Ca\(^{2+}\)-activated K\(^+\) (K\(_{\text{Ca}}\)) channel (Slo) is expressed widely in mammalian tissues (Latorre et al., 1989). Both Ca\(^{2+}\) and voltage regulate its gating: elevated intracellular Ca\(^{2+}\) 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 Ca\(^{2+}\) (Ca\(_{\text{v}}\)) 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 Ca\(^{2+}\) sparks produced by ryanodine receptors after depolarization of smooth muscle cells and Ca\(^{2+}\) influx through Ca\(_{\text{v}}\) channels, thereby relaxing smooth muscle tone (Brayden and Nelson, 1992; Nelson et al., 1995).

Functional coupling between K\(_{\text{Ca}}\) and Ca\(_{\text{v}}\) channels, characterized by a dependence of K\(_{\text{Ca}}\) channel activation on Ca\(^{2+}\) influx through Ca\(_{\text{v}}\) channels, has been documented across a variety of neuronal cell types. Ca\(_{\text{v}}\).2.2 (N-type Ca\(^{2+}\) channel)-Slo is one such functionally coupled pair in certain neurons (Wisgirda and Dryer, 1994; Marrion and Tavalin, 1998). Cross-talk between Slo and Ca\(_{\text{v}}\).2.2 may be physiologically important at presynaptic nerve terminals, where the channels are colocalized (Roberts et al., 1990; Robitaille et al., 1993; Issa and Hudspeth, 1994) and where they both regulate synaptic transmission (Hirning et al., 1988; Robitaille and Charlton, 1992). Considerations of the temporal and spatial distributions of Ca\(^{2+}\) that enters a cell through Ca\(_{\text{v}}\) channels (Simon and Llinas, 1985; Roberts, 1993) together with the Ca\(^{2+}\) affinity and kinetics of activation of K\(_{\text{Ca}}\) channels have led to the supposition that the channels must be arranged in close
physical proximity in order for functional coupling to occur. Experimental observations that buttress this argument include the disruption of functional coupling by BAPTA, a fast Ca\(^{2+}\) buffer, but not by EGTA, which acts relatively slowly (Robitaille and Charlton, 1992; Robitaille et al., 1993; Prakriya and Lingle, 2000), 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 Ca\(_{v}2.2\) channels, raises the possibility that Slo interacts physically with Ca\(_{v}2.2\) or other neighboring proteins. Syntaxin-1A, SNAP-25 and synaptotagmin, which are integral components of the neurotransmitter release machinery (Li and Chin, 2003) bind to Ca\(_{v}2.2\) at the synprint site within the intracellular loop connecting domains II and III (II-III loop) (Bennett et al., 1992; Sheng et al., 1994; Sheng et al., 1996; Sheng et al., 1997). These interactions may be necessary for proper functioning of the neurotransmitter release mechanism, as their disruption interferes with neurotransmission (Mochida et al., 1996; Rettig et al., 1997). Syntaxin-1A and SNAP-25 also downregulate Ca\(_{v}2.2\) 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, though in this case, interaction would presumably lead to reduced release. K\(_{v}1.1\), 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 (Scannevin and Trimmer, 1997; Jarvis and Zamponi, 2001b). In this study we report that Slo associates with and is modulated by syntaxin-1A.
MATERIALS AND METHODS

cDNA constructs

The mbr5 clone (Butler et al., 1993) encoding the mouse Slo K$_{Ca}$ channel in the mammalian expression vector pcDNA3 was used in the present 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 EVEDEC. The S0-S1 loop of Slo with an HA tag on its C-terminus (Slo S0-S1 loop-HA) was constructed by PCR and used in the pcDNA3.1 vector. Seventy amino acids from Slo are encoded by Slo S0-S1 loop-HA, beginning with RTLKYL and ending with QTLTGR. 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 pIRES2-EGFP, a bicistronic vector that permits coexpression of syntaxin-1A and enhanced green fluorescent protein 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 SRα 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 to
264) and syntaxin-3 (amino acids 2 to 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\textsuperscript{2+} channel Ca\textsubscript{v}2.2 was amplified by PCR, then 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.

**Coimmunoprecipitation and Western blotting**

In experiments that tested for associations between proteins with a coimmunoprecipitation strategy, HEK293 cells were transfected with the appropriate cDNAs 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 NaF, 2 DTT, plus 1% CHAPS or 1% Triton X-100 and the protease inhibitors PMSF (0.2 mM), aprotinin, leupeptin and pepstatin A (1 µg/ml each). Lysate was precleared with protein A/G-agarose beads (Santa Cruz Biotechnology), then incubated with the appropriate antibody for at least 2 hr at 4°C. Immune complexes were precipitated with protein A/G-agarose beads by incubation for 1 – 2 hr and washed five times with ≥ 10X bead volume of lysis buffer. After sample loading buffer was added, the sample was heated to 100°C. The eluted proteins were then loaded into gels for Western blotting.

Lysis buffers containing various concentrations of free Ca\textsuperscript{2+} were made in the following way, consistent with the method for adjusting free [Ca\textsuperscript{2+}] in solutions used for electrophysiological recording (below). To the basic buffer (in mM: 20 Tris-Cl (pH 7.5), 150 NaCl, 50 KCl, 50 NaF), 5 mM of Ca\textsuperscript{2+} chelator (EDTA for zero, EGTA for 100 nM free Ca\textsuperscript{2+}, HEDTA for 1 µM and 10 µM free Ca\textsuperscript{2+}, and no chelator for 100 µM and 2 mM free Ca\textsuperscript{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) (downloadable at [http://www.stanford.edu/~cpatton/maxc.html](http://www.stanford.edu/~cpatton/maxc.html)). pH was then adjusted to 7.2. Finally, 1% CHAPS, 2 mM DTT, 0.2 mM PMSF and 1 µg/ml each aprotinin, leupeptin and pepstatin A were added just prior to use. Intracellular [Ca$^{2+}$] was varied pre-lysis by one of two treatments of transfected HEK293 cells: (1) extracellular application of thapsigargin (2 µM) for 10 min, followed by BAPTA-AM (10 µM) for 10 min, each in Ringer’s solution containing 5 mM EGTA and no added Ca$^{2+}$, 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 µM) for approximately 3 min in Ringer’s 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 from Santa Cruz Biotechnology.

For coimmunoprecipitation of native proteins, crude membranes were prepared from mouse brain and mouse pancreas (Pel-Freez Biologicals). The tissues were ground to fine powder in liquid N$_2$, then 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 µg/ml each). The homogenate was centrifuged at 1000 X g for 10 min; supernatant was collected and centrifuged at 150,000 X g for 1 hr. 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 ~ 2 mg/ml with lysis buffer. The sample was then treated as HEK293 cell lysate, above.

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 3 times with TBST then incubated with horseradish peroxidase-conjugated anti-mouse or anti-rabbit IgG secondary antibody (Amersham Biosciences). Proteins were detected using enhanced chemiluminescence (Amersham Biosciences). Each coimmunoprecipitation 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 to 1135 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 Ca\textsubscript{v}2.2: amino acids 821 to 1030 (EPGRD…DLEAI) of the rbB-G clone (Y. Zhou and I.B. Levitan, unpublished results). Monoclonal anti-syntaxin-1A (clone HPC-1) and polyclonal antibodies for syntaxins 1, 2, 3 or 4 were purchased from Sigma-Aldrich. Monoclonal anti-HA antibody was purchased either from Sigma-Aldrich or Roche (HRP-linked). Monoclonal (B-14) and polyclonal (Z-5) antibodies against GST were purchased from Santa Cruz Biotechnology. Control IgG was purchased from
Santa Cruz Biotechnology and anti-green fluorescent protein (GFP) antibody from Molecular Probes.

**Electrophysiological recording**

HEK293 cells were transfected with either Slo plus EGFP cDNAs, in separate vectors, or Slo (in the pcDNA3 vector) plus syntaxin-1A in the pIRES2-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 Corp.) 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, 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 nM bath Ca²⁺, an additional step to +40mV, following the test pulse, was applied for the measurement of deactivation rate, due to rapid channel closing at -80mV in this low concentration of Ca²⁺. Leak subtraction was performed online 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 -80mV (in 1 or 10 µM Ca\textsuperscript{2+}) or +40 mV (in 100 nM Ca\textsuperscript{2+}) was used to generate conductance-voltage (G-V) relationships. G-V curves were then 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 ($V_{1/2}$ and slope for G-V curves and time constants for activation and deactivation) were then 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 coimmunoprecipitate from HEK293 cells cotransfected with cDNAs for mouse Slo (Butler et al., 1993) and human syntaxin-1A (Zhang et al., 1995) (Fig. 1A, B). The result is robust (an intense signal with short exposure of the blot to film), reproducible, is seen whether immunoprecipitation is elicited with 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 (not shown). Coimmunoprecipitation 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, then there was no detectable copurification (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 coimmunoprecipitates 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) demonstrates 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-green fluorescent protein (GFP) (Fig. 2C, 2D). Compared to the Slo coimmunoprecipitated with syntaxin (Fig. 2C, lane 3) and the syntaxin coimmunoprecipitated 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. 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. 2C and D, lane 2). No nonspecific signals of the appropriate molecular weights arose after precipitations from this tissue (Fig. 2C 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 the present 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 demonstrated that syntaxin-3 does not interact with Slo, by the lack of coimmunoprecipitation of native proteins from brain and of cloned versions from an expression system (Ling et al., 2003). But in addition, we show here that a polyclonal anti-syntaxin-1 antibody which 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. 2B and 2D) 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\textsubscript{v,2.2} do not coimmunoprecipitate**

Since the N-type Ca\textsuperscript{2+} channel Ca\textsubscript{v,2.2} is known to bind syntaxin-1A, and syntaxin-1A, Ca\textsubscript{v,2.2} and Slo are colocalized at presynaptic neurotransmitter release sites (Roberts et al., 1990; Robitaille et al., 1993; Issa and Hudspeth 1994), we considered two scenarios: either syntaxin-1A accommodates both Slo and Ca\textsubscript{v,2.2} at once, or one interaction precludes the other. Our strategy was to look for the trimeric complex Slo-syntaxin-1A-Ca\textsubscript{v,2.2} II-III loop in
immunoprecipitates from HEK293 cells transfected with these three constructs and precipitated for Slo or Ca,v,2.2 II-III loop. The II-III loop of Ca,v,2.2 fused to GST, in the pEBG-1 vector, was used rather than the full length Ca,v,2.2 channel because full length Ca,v,2.2 coimmunoprecipitates with Slo from cotransfected HEK293 cells (unpublished results), whereas the II-III loop does not. Cotransfection of syntaxin-1A with Slo and Ca,v,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,v,2.2 II-III loop and syntaxin-1A are readily seen (Fig. 3). From the lysate of cells cotransfected with all three cDNAs, syntaxin-1A copurifies with Ca,v,2.2 II-III loop (using anti-Ca,v,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 (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,v,2.2 II-III loop is not (Fig. 3C, lane 3). From the other half of the same lysate, Ca,v,2.2 II-III loop coimmunoprecipitates 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,v,2.2 simultaneously, which could have important implications for the regulation of neurotransmitter release.

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

The syntaxin-1A-Ca,v,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\textsuperscript{2+} -sensitive, especially given that Slo itself is regulated by Ca\textsuperscript{2+}? To address this possibility, we measured the Slo-syntaxin-1A coimmunoprecipitation in a range of Ca\textsuperscript{2+} concentrations: zero, 100 nM, 1 µM, 10 µM and 100 µM. Free [Ca\textsuperscript{2+}] in the lysis buffer was adjusted using 5 mM EGTA for 100 nM Ca\textsuperscript{2+} and 5 mM HEDTA for 1 and 10 µM Ca\textsuperscript{2+}, as for solutions used in electrophysiological recording, below. The nominally zero Ca\textsuperscript{2+} solution contained 5 mM EDTA, and no Ca\textsuperscript{2+} buffer was used for 100 µM free Ca\textsuperscript{2+}. Slo and syntaxin-1A do copurify in all five Ca\textsuperscript{2+} concentrations tested (Fig. 4A). Densitometric analysis of the results from three sets of experiments yielded no significant differences across [Ca\textsuperscript{2+}] for Slo coimmunoprecipitation with syntaxin-1A (Fig. 4B).

To test the possibility that changing [Ca\textsuperscript{2+}] in the lysis buffer is not sufficient to affect the Slo-syntaxin-1A interaction, we additionally elicited changes in intracellular [Ca\textsuperscript{2+}] before lysis. Hi Ca\textsuperscript{2+} was achieved by incubating transfected cells with ionomycin (1 µM) in a standard Ringer’s solution containing 2 mM Ca\textsuperscript{2+} for three minutes and then immediately lysing cells in buffer containing 2 mM free Ca\textsuperscript{2+}. This pre-lysis treatment produces a significant rise in intracellular Ca\textsuperscript{2+} that saturates in three minutes, as measured with the Ca\textsuperscript{2+} indicator fluo-4 in pilot experiments (Fig. 4C). Lo Ca\textsuperscript{2+} was produced by incubating cells with thapsigargin (2 µM) in Ringer’s solution containing 5 mM EGTA and no added Ca\textsuperscript{2+} for 10 minutes, to deplete internal Ca\textsuperscript{2+} stores, followed by incubation with the membrane-permeant version of BAPTA (BAPTA-AM, 10 µM) in Ringer’s solution with no Ca\textsuperscript{2+} for 10 minutes, to buffer intracellular Ca\textsuperscript{2+}. Subsequent lysis of lo Ca\textsuperscript{2+}-treated cells was carried out in nominally zero Ca\textsuperscript{2+} lysis buffer, containing 5 mM EDTA and no added Ca\textsuperscript{2+}. Lo Ca\textsuperscript{2+}-treated cells exhibit no increase in
intracellular Ca\(^{2+}\) in response to application of ionomycin in zero Ca\(^{2+}\) Ringer’s solution, as imaged with fluo-4 (Fig. 4C). Slo coimmunoprecipitates with syntaxin-1A under both of these conditions, where [Ca\(^{2+}\)] was changed before and during lysis (Fig. 4D). Compared to 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 copurified with syntaxin-1A (Fig. 4E).

*The 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 first 12 amino acids after the end of the S6 transmembrane region) exhibits a weak coimmunoprecipitation with syntaxin-1A (Fig. 5A) relative to the full length Slo-syntaxin-1A interaction (Figs. 1-4). A band representing Slo-CT from coimmunoprecipitation 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 coimmunoprecipitation. The small amount of copurified Slo-CT cannot be explained by poor immunprecipitation of syntaxin-1A itself, as 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 coimmunoprecipitates with syntaxin-1A (Fig. 5B, lane 4). In this case, coimmunoprecipitation can be seen after relatively short exposure of film (compared to Slo-CT), though 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 coexpression 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 pIRES2-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). Pipet 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 (Fig. 6). Conductance-voltage (G-V) relationships were constructed by plotting tail currents at +40 mV (in 100 nM Ca$^{2+}$) or -80 mV (in 1 or 10 µM Ca$^{2+}$) against test pulse voltage. Syntaxin-1A coexpression causes a rightward shift in the Slo G-V curve at all three [Ca$^{2+}$] tested (Fig. 7A-C). The mean $V_{1/2}$ (voltage at which $G$ was half-maximal), from individual fits of the G-V relationship for each patch with a Boltzmann function, is significantly different when Slo alone was compared with Slo + syntaxin-1A at each [Ca$^{2+}$], $p < 0.05$ (Fig. 7D). Shifts of ~15 mV (1 µM Ca$^{2+}$), ~9 mV (10 µM Ca$^{2+}$) and ~7 mV (100 nM Ca$^{2+}$) to more positive potentials in the presence of syntaxin-1A were measured. Mean slope, from individual fits of each G-V relationship with a Boltzmann function, was significantly affected by syntaxin-1A only in 1 µM Ca$^{2+}$, $p < 0.05$ (Fig. 7E). The reduced steepness of the G-V curve in the presence of syntaxin-1A (mean slope was changed from 15.4 mV for Slo alone to 19.9 mV for Slo + syntaxin-1A) can also be seen in the average G-V relationships plotted in Fig. 7B and suggests that, at this [Ca$^{2+}$], syntaxin-1A influences the voltage-sensitivity of Slo activation.

Macroscopic kinetics of Slo channel activity were measured by fitting single exponential functions to activating or deactivating currents over a range of test potentials for which currents were well measured and reasonably fit with the single exponential. Rates of channel activation
are significantly slowed at several test potentials, but only in 10 µM Ca\(^{2+}\) (Figs. 6 and 8A, B, C). At +30, +50, +60, +70 and +80 mV, \(\tau_{\text{activation}}\) is significantly greater in the presence of syntaxin-1A than in its absence, \(p < 0.05\) (Fig. 8C). Deactivation rates at -80 mV, in 1 and 10 µM Ca\(^{2+}\), and at +40 mV for 100 nM Ca\(^{2+}\), are not significantly different for Slo channels with and without syntaxin-1A (Figs. 6 and 8D, E, F). Syntaxin-1A coexpression, therefore, inhibits the Slo channel by shifting the G-V relationship to more depolarized potentials in 100 nM, 1 µM and 10 µM intracellular Ca\(^{2+}\), reducing the voltage-dependence of activation in 1 µM Ca\(^{2+}\) and under some circumstances (in 10 µM Ca\(^{2+}\), at some test potentials) by slowing the time course of activation. These effects could presumably influence the excitability of presynaptic nerve terminals and thereby regulate neurotransmitter release.
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. Coexpression 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 $Ca_{v}2.2$.

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, though 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 coimmunoprecipitation of Slo-CT with syntaxin-1A demonstrated 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, as 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 coimmunoprecipitation 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).

This is not the first demonstration of modulation of voltage-gated ion channel activity by syntaxin-1A. N- and Q-type (Ca\(_{V2.2}\) and 2.1) Ca\(^{2+}\) channels have reduced activity in the presence of syntaxin-1A due to a shift in steady-state inactivation to less depolarized potentials (Bezprozvanny et al., 1995) and the L-type Ca\(^{2+}\) channel Ca\(_{V1.2}\) is also inhibited by syntaxin-1A (Wiser et al., 1996). The voltage-gated K\(^{+}\) channel K\(_{V1.1}\), which, like Slo, is expressed in presynaptic nerve terminals and has a role in neurotransmitter release, as well as K\(_{V2.1}\), which regulates insulin secretion from pancreatic \(\beta\) cells, are both inhibited by syntaxin-1A (Fili et al., 2001; Leung et al., 2003).

From our electrophysiological recordings, it appears 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 \(\mu\)M), but not higher (10 and 100 \(\mu\)M). 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 to 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: \(35 \pm 0.5^\circ\text{C}\) by Ling et al., and room temperature (\(~22^\circ\text{C}\)) in the present 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 coinjected 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 copurification 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 Ca\(_V\)2.2 do not bind syntaxin-1A at the same time. This finding raises questions of whether there is competition for syntaxin-1A between Slo and Ca\(_V\)2.2 and what might regulate syntaxin’s preference for one channel over the other. If there is competition, then Slo might disrupt syntaxin1A’s 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, $\text{Ca}_v$2.2 might interfere with the syntaxin-1A’s 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 $K_{\text{Ca}}$ 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_v$1.1 for interaction with syntaxin-1A in the oocyte expression system (Fili et al., 2001). The possibility of competition between Ca$_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; Jarvis and Zamponi, 2001b).

Furthermore, G-protein signaling pathways impinge on the proteins in question: syntaxin-1A facilitates G-protein $\beta\gamma$-mediated inhibition of Ca$_v$2.2 channels (Stanley and Mirotznik, 1997; Jarvis et al., 2000) and G$\beta\gamma$ may be required for syntaxin-1A to regulate $K_v$1.1 (Michaelievski et al., 2002). Phosphorylation of Ca$_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 the present work, 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\textsubscript{v}2.2 II-III loop.

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FIGURE LEGENDS

Figure 1: Slo coimmunoprecipitates with syntaxin-1A from a heterologous expression system. 
A, B. HEK293 cells were transfected with HA-tagged Slo plus syntaxin-1A (lanes 1 and 3, A; lanes 5 and 7, B), Slo-HA alone (lanes 2 and 4, A) or syntaxin-1A alone (lanes 6 and 8, B). From blots probed for Slo (A), immunoprecipitation with anti-syntaxin antibody pulls down Slo from cells transfected with both Slo and syntaxin-1A (lane 3), but not from cells transfected with HA-Slo alone (lane 4). Blots probed for syntaxin-1A (B) show that anti-Slo antibody immunoprecipitates syntaxin-1A from cells cotransfected with syntaxin-1A and Slo (lane 7) and not from cells transfected with syntaxin-1A alone (lane 8).

Figure 2: Native proteins Slo and syntaxin-1 are associated in brain. A, B, Slo coimmunoprecipitates 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 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, D, IPs from brain with control IgG (lane 5) or anti-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 to 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.

**Figure 3:** Slo, syntaxin-1A and Ca,2.2 II-III loop do not copurify as a trimeric complex. HEK293 cells were transfected with all three constructs (Slo-HA, syntaxin-1A and Ca,2.2 II-III loop) or with only one of the three, as a control. A, Slo is not detectable in immunoprecipitates produced with anti-Ca,2.2 II-III loop from cells transfected with all three constructs (lane 3). The lysates in lanes 1 and 2 were divided in half: one half was immunoprecipitated with anti-Ca,2.2 antibody (lanes 3 and 4) and the other half with anti-syntaxin antibody (lanes 5 and 6). B, syntaxin-1A is present after immunoprecipitation with anti-Ca,2.2 antibody from cells cotransfected with Slo-HA, syntaxin-1A and Ca,2.2 II-III loop. Material in lanes 7 and 9 is from the same sample as in lanes 1 and 3, A. Ca,2.2 II-III loop is also easily seen in the same sample (lanes 11 and 12); the blot in A was stripped and reprobed with anti-Ca,2.2 antibody. C, D, Same organization as in A, B. Samples in lanes 1 and 2 (C) were divided in half and immunoprecipitation elicited with anti-HA (lanes 3 and 4, C) or syntaxin antibody (lanes 5 and 6, C). Samples in lanes 1 and 3 (C) were also examined for the presence of syntaxin-1A (lanes 7 and 9, D). The Ca,2.2 II-III loop blot (C) was stripped and reprobed for Slo (lanes 11 and 12, D).

**Figure 4:** Slo coimmunoprecipitates with syntaxin-1A across a range of [Ca$^{2+}$]. A, HEK293 cells were cotransfected with Slo + syntaxin-1A, except for lane 1, which contains sample from mock-transfected cells as a control. Control lysis and immunoprecipitation were carried out in
zero Ca$^{2+}$. Blots were first probed for Slo, then stripped and reprobed for syntaxin-1A.  

Results of densitometric analysis of Western blots from three separate experiments, of which A is representative. The value listed for each [Ca$^{2+}$] is the ratio of Slo in the precipitate to Slo in the lysate (Slo IP/lysate) divided by syntaxin-1A in the precipitate ((Slo IP/lysate)/syntaxin IP). Data are expressed as mean ± S.E.  

C, Fluo-4 imaging of intracellular Ca$^{2+}$ in HEK293 cells in response to ionomycin (1 µM) in Ringer’s solution with 2 mM Ca$^{2+}$ (hi Ca$^{2+}$) and the response to ionomycin in zero Ca$^{2+}$ Ringer’s of HEK293 cells that had been treated with thapsigargin (2 µM) and BAPTA-AM (10 µM) (lo Ca$^{2+}$). D, HEK293 cells were cotransfected with Slo + syntaxin-1A, except for sample in lane 1, which is from cells transfected with Slo alone. Controls (c) in lane 1 (Slo alone) and lane 2 (Slo + syntaxin-1A) were lysed and immunoprecipitated under standard conditions, with lysis buffer containing zero added Ca$^{2+}$. Cells in lane 3 (lo) had been treated with thapsigargin (2 µM) and BAPTA-AM (10 µM), 10 min each in zero Ca$^{2+}$ Ringer’s solution, then lysed in zero Ca$^{2+}$ buffer. Sample in lane 4 (hi) is from cells incubated with ionomycin (1 µM) in Ringer’s solution with 2 mM Ca$^{2+}$ for 3 min, then lysed in buffer containing 2 mM Ca$^{2+}$. E, Results of densitometric analysis from three experiments of which D is representative, as in B.

**Figure 5**: The Slo C-terminal tail and S0-S1 loop each associate weakly with syntaxin-1A.  

A, HEK293 cells transfected with Slo-CT + syntaxin-1A, Slo-CT alone or no DNA (mock-transfected) were treated with anti-syntaxin-1A antibody for immunoprecipitation. A small amount of Slo-CT is detected in precipitates from Slo-CT + syntaxin-1A cotransfected cells (lane 5) but not from controls (lanes 4 and 6), using anti-Slo antibody. The syntaxin blot shows that syntaxin-1A precipitated well from the same sample (lanes 7 and 8); therefore, a general problem
with the immunoprecipitation does not account for the weak Slo-CT signal. B, HA-tagged S0-S1 loop from Slo is visible after immunoprecipitation with anti-syntaxin-1A from cells cotransfected with Slo S0-S1 loop-HA + syntaxin-1A (lane 4) but not from those transfected with Slo S0-S1 loop-HA alone (lane 3) or mock transfected (lane 5). Syntaxin-1A was expressed and precipitated well from the sample (lanes 6 and 7).

**Figure 6:** Representative families of current traces recorded from excised patches of HEK293 cells transfected either with Slo alone or Slo + syntaxin-1A in symmetrical 150 mM K⁺ solutions and various bath [Ca²⁺]. Holding potential was -80 mV in all cases. Currents during test pulses of +70 to +200 mV, followed by tail currents during a step to +40 mV, are shown for 100 nM bath Ca²⁺. For 1 µM bath Ca²⁺, currents during test pulses of +50 to +200 mV and tail currents in response to a step back to the holding potential of -80 mV are displayed. Currents produced by test pulses of -50 to +80 mV, then a step back to -80 mV, are shown for 10 µM Ca²⁺.

**Figure 7:** Syntaxin-1A modulates Slo function. A, B, C, Average G-V relationships, plotted as mean ± S.E. (n = 5 - 7) for each test pulse voltage and fit with a Boltzmann function. Fit parameters: \( V_{1/2} = 119.9, \text{ slope} = 16.3 \text{ mV for Slo alone}, V_{1/2} = 128.0, \text{ slope} = 18.6 \text{ mV for Slo + syntaxin-1A in 100 nM Ca}^{2+} \) (A); \( V_{1/2} = 99.5, \text{ slope} = 16.0 \text{ mV for Slo alone}, V_{1/2} = 114.3, \text{ slope} = 20.9 \text{ mV for Slo + syntaxin-1A in 1 µM Ca}^{2+} \) (B); \( V_{1/2} = 7.5, \text{ slope} = 15.0 \text{ mV for Slo alone}, V_{1/2} = 16.9, \text{ slope} = 15.3 \text{ mV for Slo + syntaxin-1A in 10 µM Ca}^{2+} \) (C). D, Average \( V_{1/2} \) vs. bath [Ca²⁺] from individual Boltzmann fits to the G-V curve for each patch, plotted as mean ± S.E. (n = 5 – 7). \( V_{1/2} \) is significantly different (p < 0.05) at each [Ca²⁺]: 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 ± S.E., 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).

Figure 8: Slo activation rates in 10 µM Ca\(^{2+}\) are significantly slowed in the presence of syntaxin-1A. A, B, C, Time constants (mean ± S.E.) from single exponential fits to time course of macroscopic Slo current activation with or without syntaxin-1A coexpression (n = 5 – 7 excised patches from transfected HEK293 cells). \(\tau_{\text{activation}}\) was significantly greater in the presence of syntaxin-1A than in its absence with 10 µM free Ca\(^{2+}\) in the bath (4.04 ± 0.57, 7.10 ± 0.52 ms at +30 mV, 2.64 ± 0.36, 3.89 ± 0.38 ms at +50 mV, 2.07 ± 0.26, 3.29 ± 0.29 ms at +60 mV, 1.57 ± 0.24, 2.58 ± 0.22 ms at +70 mV, 1.36 ± 0.25, 2.14 ± 0.16 ms at +80 mV, for Slo and Slo + syntaxin-1A, respectively), p < 0.05 (C). D, E, F, Time constants (mean ± S.E.) from single exponential fits to time course of macroscopic Slo current deactivation at +40 mV (for 100 nM Ca\(^{2+}\), D) or -80 mV (for 1 and 10 µM Ca\(^{2+}\), E, F). No significant differences were measured between excised patches from HEK293 cells expressing Slo alone and patches expressing Slo + syntaxin-1A (n = 5 – 7).
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Figure 1

**A**

Lysates
Blot: Slo

IP: syntaxin
Blot: Slo

Transfections:
Slo-HA

**B**

Lysates
Blot: syntaxin

IP: HA
Blot: syntaxin

Transfections:
Slo-HA

Syntaxin-1A
Figure 2

**Figure 2**

- **A**: Blot: Slo
  - Solubilized membrane preps
  - Tissue: HEK 293
  - IP: syntaxin
  - Blot: Slo

- **B**: Blot: syntaxin
  - Solubilized membrane preps
  - Tissue: brain, pancreas

- **C**: Blot: syntaxin
  - Solubilized membrane preps
  - IP: syntaxin
  - Blot: Slo

- **D**: Blot: syntaxin
  - Solubilized membrane preps
  - IP: Slo
  - Blot: syntaxin

- **E**: Blot: monoclonal syntaxin-1A (clone HPC-1)
  - Blot: syntaxin-3
  - Blot: syntaxin-4
  - Blot: syntaxin-2
  - Blot: polyclonal syntaxin-1

- **F**: Blot: polyclonal syntaxin-1
  - IP: Slo
  - IP: control IgG
  - IP: GFP

- **Figure 2**: Lysate: syntaxin-1A, syntaxin-1B, syntaxin-3, GST-synxin-2 (H-269), GST-synxin-3 (H-269)
Figure 3

A

Lysates
Blot: Slo

IP: Cav2.2 II-III loop
Blot: Slo

IP: syntaxin
Blot: Slo

HEK 293 Transfections:
Slo-HA + syntaxin + Cav2.2 II-III loop

B

Lysates
Blot: syntaxin

IP: Cav2.2 II-III loop
Blot: syntaxin

IP: Cav2.2 II-III loop
Blot: Cav2.2 II-III loop

Transfections:
Slo-HA + syntaxin + Cav2.2 II-III loop

C

Lysates
Blot: Cav2.2 II-III loop

IP: HA
Blot: Cav2.2 II-III loop

IP: syntaxin
Blot: Cav2.2 II-III loop

Transfections:
Slo-HA + syntaxin + Cav2.2 II-III loop

D

Lysates
Blot: syntaxin

IP: HA
Blot: syntaxin

IP: HA
Blot: Slo

Transfections:
Slo-HA + syntaxin + Cav2.2 II-III loop
Figure 5

A

Lysates
Blot: Slo-CT 80 kD
IP: syntaxin
Blot: Slo-CT 80 kD

Slo-CT

111 kD

1 2 3

Transfections:
mock Slo-CT + syntaxin-1A Slo-CT

Slo-CT

B

Lysates
Blot: HA

Slo S0-S1 loop-HA

14 kD

12

6 kD

3 4 5

Transfections:
Slo S0-S1 loop-HA Slo S0-S1 loop-HA + mock

Slo S0-S1 loop-HA

27 kD

6

27 kD

7

syntaxin
Figure 6

A  
Slo alone  
Slo + syntaxin-1A  
100 nM Ca^{2+}  
2 nA

B  
1 μM Ca^{2+}  
2 nA  
10 ms

C  
10 μM Ca^{2+}  
2 nA  
-80 mV
Figure 7

(A) Relative conductance (G/Gmax) for 100 nM Ca^{2+}

(B) Relative conductance (G/Gmax) for 1 μM Ca^{2+}

(C) Relative conductance (G/Gmax) for 10 μM Ca^{2+}

(D) Voltage at half-maximal activation (V_{1/2}) for different Ca^{2+} concentrations.

(E) Slope of the activation curve for different Ca^{2+} concentrations.

Legend:
- Black circle: Slo alone
- White square: Slo + syntaxin-1A