G Protein Modulation of N-type Calcium Channels Is Facilitated by Physical Interactions between Syntaxin 1A and Gβγ*

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The direct modulation of N-type calcium channels by G protein βγ subunits is considered a key factor in the regulation of neurotransmission. Some of the molecular determinants that govern the binding interaction of N-type channels and Gβγ have recently been identified (see, i.e., Zamponi, G. W., Bourinet, E., Nelson, D., Nargeot, J., and Snutch, T. P. (1997) Nature 385, 442-446); however, little is known about cellular mechanisms that modulate this interaction. Here we report that a protein of the presynaptic vesicle release complex, syntaxin 1A, mediates a crucial role in the tonic inhibition of N-type channels by Gβγ. When syntaxin 1A was coexpressed with (N-type) α1B + α2δ + β1b channels in tsA-201 cells, the channels underwent a 18 mV negative shift in half-activation potential, as well as a pronounced tonic G protein inhibition as assessed by its reversal by strong membrane depolarizations. This tonic inhibition was dramatically attenuated following incubation with botulinum toxin C, indicating that syntaxin 1A expression was indeed responsible for the enhanced G protein modulation. However, when G protein βγ subunits were co-comitantly coexpressed, the toxin became ineffective in removing G protein inhibition, suggesting that syntaxin 1A optimizes, rather than being required for G protein modulation of N-type channels. We also demonstrate that Gβγ physically binds to syntaxin 1A, and that syntaxin 1A can simultaneously interact with Gβγ and the synprint motif of the N-type channel II-III linker. Taken together, our experiments suggest a mechanism by which syntaxin 1A mediates a colocalization of G protein βγ subunits and N-type calcium channels, thus resulting in more effective G protein coupling to, and regulation of, the channel. Thus, the interactions between syntaxin, G proteins, and N-type calcium channels are part of the structural specialization of the presynaptic terminal.

Calcium entry into neurons via voltage-dependent calcium channels mediates a range of responses, including neurotransmitter release, proliferation, and the activation of calcium-dependent enzymes. Most neurons express multiple calcium channel isoforms with distinct functional properties. Molecular cloning has identified genes encoding at least nine different neuronal calcium channel α1 subunits (termed α1A through α1H). Expression studies have revealed that α1A encodes both P/Q-type calcium channels (1-4); α1C defines N-type channels (5-7); α1C, α1B, and α1F comprise L-type calcium channels (8-11); α1E likely encodes a component of the “resistant” (or R-type) current (12, 13); and α1D, α1H, and α1G form T-type calcium channels (14-16). Among these channels, α1A and α1B are predominantly located at presynaptic nerve terminals (17, 18) where they interact with presynaptic vesicle release proteins (19, 20).

The modulation of presynaptic calcium channel activity by intracellular messenger pathways, including protein kinase C, and G protein βγ subunits (21-25), has been well documented. Upon activation of G protein-coupled seven-helix transmembrane receptors, both N-type and P/Q-type channels undergo a pronounced voltage-dependent inhibition (26-30). This inhibition is likely caused by direct binding of G protein βγ subunits to the calcium channel α1 subunit with 1:1 stoichiometry, which results in a stabilization of the deep closed states of the channel (31-33). This effect can be reversed by strong membrane depolarizations, resulting in an apparent facilitation of calcium currents after application of depolarizing prepsules (22, 27, 32). Recent molecular biological evidence indicates that the G protein βγ subunits interact with the calcium channel α1 subunit at multiple points, in particular, the I-II linker and the carboxyl tail regions (34-36). It has been reported that cleavage of the synaptic vesicle release protein syntaxin 1A with botulinum toxin C1 (BTC1) abolishes the ability of N-type channels to undergo G protein modulation (37). Syntaxin is a cytoplasmically oriented membrane protein that is involved in synaptic vesicle release triggered by the influx of calcium through voltage-dependent calcium channels. Proteolysis by BTC1 completely and irreversibly inhibits synaptic release. Because syntaxin 1A is known to physically bind to these channels (19, 20), Stanley and Mirotsznik (37) suggested that syntaxin 1A might facilitate the co-localization of Gβγ and the channel, but no direct evidence for this has been presented. Furthermore, because G protein modulation of transiently expressed N-type channels does not require coexpression with syntaxin 1A (22, 34), it is

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1 The abbreviations used are: BTC1, botulinum toxin C1; EGFP, enhanced green fluorescent protein; GST, glutathione S-transferase; PMSF, phenylmethylsulfonyl fluoride; PAGE, polyacrylamide gel electrophoresis; DMEM, Dulbecco’s modified Eagle’s medium; FBS, phosphate-buffered saline; PBST, phosphate-buffered saline plus Tween 20; PCR, polymerase chain reaction; MOPS, 4-morpholinepropanesulfonic acid; SNARE, soluble N-ethylmaleimide-sensitive factor attachment protein receptor.
uncover why the cleavage of syntaxin 1A in the native neuronal environment would lead to the loss of G protein modulation. Here, we present evidence that syntaxin 1A augments, but is not required per se for G protein modulation of transiently expressed calcium channels. Human embryonic kidney cells do not endogenously express syntaxin 1A; however, coexpression of N-type calcium channels with Gβγ2 resulted in a pronounced inhibition of N-type currents. Upon coexpression of syntaxin 1A, N-type channels underwent a large tonic G protein inhibition even in the absence of exogenous G protein βγ subunits. This inhibition was sensitive to botulinum toxin C1. However, the effect of the toxin was abolished upon overexpression of exogenous Gβγ subunits. Biochemical data show that syntaxin 1A is able to physically interact concomitantly with both Gβγ and the calcium channel. Overall, our data are consistent with a model in which syntaxin 1A physically localizes free endogenous G protein βγ subunits near the vicinity of the N-type calcium channel α1 subunit, thus increasing the effective local Gβγ concentration near the channel and thereby facilitating the interactions between the channel and the G protein. P/Q-type calcium channels regulate syntaxin 1A expression (51), which would in turn modulate tonic inhibition of N-type calcium channels by Gβγ. In that context, our data suggest a potential feedback mechanism by which expression of one calcium channel type regulates the activity of another.

**EXPERIMENTAL PROCEDURES**

**Transient Transfection of HEK Cells**

Human embryonic kidney tsA-201 cells were grown in standard DMEM medium, supplemented with 10% fetal bovine serum and penicillin-streptomycin. The cells were grown to 85% confluence, split with DMEM medium, supplemented with 10% fetal bovine serum and penicillin-streptomycin. The cells were grown to 85% confluence, split with 0.8% EDTA (Life Technologies, Inc.) to wash off and 2 ml of trypsin-EDTA (Life Technologies, Inc.) was added to the culture dish. The cells were transfected, as described above. Following the 2-day incubation at 37°C, the DMEM media was washed off and 2 ml of trypsin-EDTA (Life Technologies, Inc.) added and the cells were incubated for 3 min. 8 ml of PBS buffer (137 mM NaCl, 2.7 mM KCl, 4.3 mM NaHPO4·7H2O, 1.4 mM KH2PO4) was added, and the cells were pelleted by centrifugation at 3000 × g for 5 min. The supernatant was removed, and the cells were resuspended in 3 ml of homogenization buffer (50 mM HEPES-Tris, pH 7.3, 8% sucrose, 100 mM NaCl, 1 mM EDTA, 10 mM diithiothreitol, 0.1% Tween 20, 30 μg of pepstatin, 30 μg of leupeptin, 30 μg of aprotinin, 0.1% Triton X-100), followed by three rapid freeze-thaw cycles between a dry ice-ethanol slurry and 37°C water. The preparation was centrifuged at 3000 × g for 5 min to remove cell debris, and this supernatant was then spun at 100,000 × g for 1 h. The pellet was then treated with 2% Laemmli sample buffer (100 mM Tris-HCl, pH 6.8, 200 mM β-mercaptoethanol, 20% glycerol, 4% SDS, 0.2% bromophenol blue), and shaken for 2 h at 4°C. The proteins were denatured at 95°C for 1 min and loaded for SDS-PAGE.

Proteins were transferred from the gel to Hybond ECL nitrocellulose membrane (Amersham Pharmacia Biotech, Buckinghamshire, United Kingdom) at 30 V for 10 h. Transfer buffer was removed by two washes with PBS, and the membrane was blocked in 5% skim milk powder in PBS (PBS + 0.1% Tween 20) for 2 h. The volume of the blocking solution was reduced to 10 ml, and the primary antibody (mouse anti-syntaxin 1A (Stressgen, Victoria, British Columbia, Canada) or rabbit anti-Gβγ (Calbiochem, La Jolla, CA)) was added at a concentration of 1:1000. After 2 h of incubation, the membrane was washed twice with PBS for 10 min and twice with PBS for 10 min, and subsequently incubated with the secondary antibody (horseradish peroxidase-conjugated goat anti-mouse, or goat anti-rabbit, Amersham Pharmacia Biotech) in 2% skim milk powder in PBS at a concentration of 1:5000 for 2 h. The membrane was again washed twice with PBST for 10 min and twice with PBST for 10 min. Following the final wash, the blot was subjected to chemiluminescence analysis using ECL plus (Amersham Pharmacia Biotech) and detected on film, or via Storm Scan 860.

**Preparation of Rat Hippocampal Homogenate**—Rat hippocampi were transferred from the gel to Hybrid ECL nitrocellulose membrane (Amersham Pharmacia Biotech, Buckinghamshire, United Kingdom) at 30 V for 10 h. Transfer buffer was removed by two washes with PBS, and the membrane was blocked in 5% skim milk powder in PBS (PBS + 0.1% Tween 20) for 2 h. The volume of the blocking solution was reduced to 10 ml, and the primary antibody (mouse anti-syntaxin 1A (Stressgen, Victoria, British Columbia, Canada) or rabbit anti-Gβγ (Calbiochem, La Jolla, CA)) was added at a concentration of 1:1000. After 2 h of incubation, the membrane was washed twice with PBST for 10 min and twice with PBS for 10 min, and subsequently incubated with the secondary antibody (horseradish peroxidase-conjugated goat anti-mouse, or goat anti-rabbit, Amersham Pharmacia Biotech) in 2% skim milk powder in PBS at a concentration of 1:5000 for 2 h. The membrane was again washed twice with PBST for 10 min and twice with PBST for 10 min. Following the final wash, the blot was subjected to chemiluminescence analysis using ECL plus (Amersham Pharmacia Biotech) and detected on film, or via Storm Scan 860.
hand homogenized with a Teflon-coated homogenizer in 0.32 m sucrose, 10 mM HEPES KOH (pH 7.0), 1 mM EGTA, 0.1 mM EDTA, 0.5 mM PMSF, protease inhibitor mixture (Roche Molecular Biochemicals), 1 μM microcin, 1 μM okadaic acid, and 1 mM sodium orthovanadate (2 mM Hippocampus). The homogenate was centrifuged for 10 min at 500 × g and the supernatant collected and subsequently centrifuged for 300 × g at 20,000 × g (4°C). The pellet containing the synaptic proteins was resuspended in 1% Triton X-100, 20 mM MOPS (pH 7.0), 4.5 mM Mg(CH$_3$COO)$_2$, 150 mM KCl, and 0.5 mM PMSF, protease inhibitor mixture (Roche Molecular Biochemicals), 1 μM microcin, 1 μM okadaic acid, 1 mM sodium orthovanadate and incubated for 30 min at 37°C for solubilization, large membrane fragments were removed by centrifugation at 1000 × g for 5 min. The resulting supernatant is a crude hippocampal homogenate containing synaptic proteins. Protein concentration was determined by Bio-Rad protein assay using bovine serum albumin as the standard.

Preparation of SNARE Fusion Proteins—Glutathione S-transferase (GST) fusion proteins of syntaxin 1A and VAMP2 were prepared as described previously (49). Briefly, GST fusion proteins encoding the cytoplasmic portions of syntaxin 1A and VAMP2 were constructed in the vector pGEX-KG (50) and expressed in AB1889 strain of E. coli. After induction of expression with 100 μM isopropyl-β-D-thiogalactopyranoside for 5 h, the bacteria were suspended in PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na$_2$HPO$_4$, 2 mM KH$_2$PO$_4$) supplemented with 0.05% Tween 20, 2 mM EDTA, and 0.1% β-mercaptoethanol and lysed by two passages through a French press (Spectronic Instruments Inc.). The fusion protein was recovered by binding of the GST domain to glutathione-agarose beads (Sigma). The fusion protein beads were washed extensively and finally resuspended in 0.5% Triton X-100, 20 mM MOPS (pH 7.0), 4.5 mM Mg(CH$_3$COO)$_2$, 150 mM KCl, and 0.5 mM PMSF.

Immunoblotting for Syntaxin 1A-G Protein Binding—Proteins were transferred electrophoretically at constant voltage from polyacrylamide gels to nitrocellulose (0.2 μm) in 20 mM Tris, 150 mM glycine, 12% methanol. Transferred proteins were visualized by staining with Pronase S. Nitrocellulose membranes were blocked for nonspecific binding with 5% milk, 0.15% Tween 20, PBS solution (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na$_2$HPO$_4$, 1.4 mM KH$_2$PO$_4$ (pH 7.3)) and incubated overnight with antibody (1:1000). The membranes were washed four times in the above milk/Tween 20/PBS solution and incubated for 30 min with goat anti-rabbit or goat anti-mouse IgG-coupled horseradish peroxidase. Antigen was detected using chemiluminescent horseradish peroxidase substrate (ECL, Amersham Pharmacia Biotech). Immunoreactive bands were visualized following exposure of the membranes to Amersham Hyperfilm-MP.

Alternatively, for the synprint-syntaxin 1A-G$\beta$7 binding assay, bacterial cell lysate of the 6×His-synprint peptide was batch-bound to 50% (v/v) ProBond Ni$^{2+}$-agarose beads (Invitrogen) and washed four times with three buffers of pH levels 7.8, 6.0, and 5.5, containing 20 mM Na$_2$PO$_4$ and 500 mM NaCl to remove nonspecifically bound proteins. The beads were then washed and equilibrated with TBST-Ca (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.1% Triton X-100, 15 μM CaCl$_2$). Bound 6×His-fusion protein from a fraction of the beads was eluted and quantified by a Bradford assay using bovine serum albumin as a standard. The 6×His fusion protein bound to beads was incubated for 2 h at 4°C with 40 μg of rat hippocampal homogenate; bound proteins were subsequently eluted and immunodetected for both G$\beta$7 and syntaxin 1A. Before loading on SDS-PAGE, protein samples were treated with 2× Laemmli sample buffer, rotated end-over-end for 2 h at 4°C, denatured at 95°C for 1 min, and loaded. Transfer from SDS-PAGE to Hybond ECL nitrocellulose (Amersham Pharmacia Biotech) and subsequent immunodetection was performed as described above.

RESULTS

G Protein Modulation of N-type Channel Occurs Independently of Syntaxin 1A—It is well established that N-type calcium channels transiently expressed in human embryonic kidney tsA-201 (HEK) cells are subject to potent inhibition by G protein $\beta$γ subunits upon either activation of seven helix transmembrane receptors (33), cytoplasmic application of purified G$\beta$γ (33, 34), or transient overexpression of G$\beta$γ (38, 39). One characteristic feature of this type of inhibition is its reversal following a strong membrane depolarization (termed facilitation). Such an experiment is illustrated in Fig. 1A. In the absence of exogenous G$\beta$γ, application of a strong depolarizing prepulse (+150 mV, 50 ms) to N-type ($\alpha_{1b}$ + $\beta_{1b}$ + $\alpha_{2b}$-δ) channels has little effect on peak current amplitude, or current waveform. However, upon cotransfection with G$\beta$γ, peak current amplitude is increased by almost 2-fold subsequent to application of the prepulse, thus reflecting relief of the tonic inhibition of the channel induced by G$\beta$γ. The experiments of Stanley and Mirotznik (37) suggested that syntaxin 1A might be a prerequisite for G protein modulation of N-type calcium channels, and if so, this would imply that HEK cells should endogenously express syntaxin 1A. To examine this possibility, we carried out an immunoblot for syntaxin 1A in HEK cells under several different conditions. As seen in Fig. 1B, syntaxin 1A could only be detected when transfected, indicating that syntaxin 1A is not present endogenously in HEK cells. In Fig. 1C, we also see that syntaxin 1A expression is not triggered by overexpression of G$\beta$γ or secondarily by expression of N-type calcium channels as described recently for P/Q-type calcium channels (51). Under the same experimental conditions, we could not detect any expression of nSec-1 or SNAP-25, even following transfection with syntaxin 1A (data not shown), indicating that HEK cells lack many of the proteins responsible for vesicle release, and that the exogenous expression of syntaxin 1A is not responsible for the concomitant expression of other proteins important for vesicle release. Shown in Fig. 1C, syntaxin 1A transfection did not mediate a detectable change in the amount of endogenous G$\beta$γ present in HEK cells. However, this result needs to be viewed cautiously, because high endogenous G$\beta$γ levels could mask small increases in exogenous G$\beta$γ, and Western blot analysis does not evaluate
was independent of the type of calcium channel peak current amplitude. The magnitude of the prepulse effect i.e. coexpressed (..., SNAP-25 and nSec-1, is not required for N-type channels—

**Fig. 2. Effect of syntaxin 1A expression on transiently expressed N-type channels.** A, current records obtained with α1a + β2βd + α2-δ as described in Fig. 1. Expression of syntaxin 1A mediates a tonic G protein inhibition of the channels as defined by the prepulse relief. The effect of syntaxin 1A is removed upon cleavage with botulinum toxin C1 (12-h incubation with 1 μg of BTC1/ml of DMEM). Overexpression of exogenous G protein βγ subunits diminishes the effect of the toxin. B, bar graphs reflecting the degree of peak current amplitude enhancement following a depolarizing prepulse as a measure of tonic G protein inhibition. The values reflected in bars 2, 3, 5, and 6 did not differ significantly from each other (p > 0.6).

how much Gγ is complexed with Gα subunits in the cell. Overall, the data shown in Fig. 1 indicate that the presence of syntaxin 1A, or SNAP-25 and nSec-1, is not required for N-type channels to undergo G protein inhibition.

**Syntaxin 1A Promotes G Protein Inhibition of N-type Channels**—Although syntaxin 1A is not a prerequisite for G protein modulation of N-type channels, it is possible that syntaxin 1A does modulate the effects of Gγ on N-type calcium channels. To examine this possibility, we cloned syntaxin 1A from rat brain and investigated its effect on transiently expressed N-type channels. N-type calcium channels coexpressed with syntaxin 1A underwent a hyperpolarizing shift in steady state inactivation from −44.2 mV (n = 11) to −61.9 mV (n = 7), similar to what has been previously reported for N-type channels expressed in Xenopus oocytes (40) and thereby confirming functional syntaxin 1A expression in our experiments. However, as evident upon examination of the current records in Fig. 2A (top left), an additional effect of syntaxin 1A was that the current waveform exhibited dramatically slowed activation and inactivation kinetics, and the average peak current amplitude decreased by nearly 1 order of magnitude. Qualitatively, the current waveform in the presence of syntaxin 1A was reminiscent of that obtained upon coexpression of the channel with exogenous Gγ (compare with Fig. 1A), suggesting that syntaxin 1A might secondarily mediate a tonic G protein modulation of the channel.

Consistent with this idea, application of a strong depolarizing prepulse resulted in a 2.2 ± 0.2-fold (n = 10) increase in peak current amplitude. The magnitude of the prepulse effect was independent of the type of calcium channel β subunit coexpressed (i.e. β1d, β2d, or β3) and of the presence of the ancillary α2-δ complex (data not shown). The prepulse facilitation was reduced to a 1.2 ± 0.04-fold (n = 12) enhancement when cells were incubated with BTC1 for 12 h prior to recording, indicating that our observations were due to the presence of the syntaxin 1A protein rather than the preceding transcription events. To ensure that BTC1 did not directly interfere with G protein modulation of the channel, we investigated the effects of syntaxin 1A on N-type channels coexpressed with both syntaxin 1A and Gβγδγ. As seen from the current records in Fig. 2A (bottom left), the effects of syntaxin 1A and Gβγδγ were not additive, and more importantly, BTC1 was ineffective in removing the tonic G protein inhibition (bottom right). This is also reflected in Fig. 2B in form of bar graphs. Coexpression of the N-type channels with syntaxin 1A or Gβγδγ plus syntaxin 1A resulted in a degree of G protein inhibition that did not differ significantly from that obtained upon coexpression of Gβγδγ alone, and BTC1 selectively removed the tonic G protein inhibition induced by syntaxin 1A overexpression. Overall, these data are consistent with a mechanism by which syntaxin 1A promotes tonic G protein modulation of N-type calcium channels.

**Syntaxin 1A Physically Interacts with Gβγ**—One possible explanation for our observations is a mechanism by which syntaxin 1A binding to the channel increases the sensitivity of the channel to free Gγ subunits, i.e. those that are not part of the Gγ trimer associated with seven helix transmembrane receptors. In this scenario, the cleavage of the syntaxin 1A protein would attenuate the enhancing effect, and the overexpression of exogenous Gγ would mask the effect. In principle, there are two possible mechanisms by which this could occur. Binding of syntaxin 1A to the synprint site on the channel (19) could allosterically enhance G protein binding to the channel. Alternatively, syntaxin 1A might serve as an anchoring mechanism by which G protein γ subunits are confined to the vicinity of their site of action on the channel protein. To discriminate between these two possibilities, we carried out a binding assay involving a GST-syntaxin 1A fusion protein. As shown in Fig. 3A, upon incubation with rat hippocampal homogenate, the syntaxin 1A fusion proteins were able to precipitate G protein γ subunits in a syntaxin 1A concentration-dependent manner. Similarly, increasing amounts of nSec-1, a protein known to tightly associate with syntaxin 1A, were precipitated from the homogenate as the GST-syntaxin 1A concentration was increased. On the other hand, GST-VAMP-2 beads were unable to associate with either of Gγ or nSec-1, indicating that nonspecific binding of Gγ to the GST beads did not occur. While these data are consistent with a direct interaction between syntaxin 1A and the G protein, this experiment does not permit us to rule out the possibility that Gγ interacts with syntaxin 1A indirectly via one or more additional proteins. To investigate this possibility, we examined the ability of GST-syntaxin 1A to interact with purified Gγ subunits in the absence of other proteins. As shown in Fig. 3B, the recombinant GST-syntaxin 1A was able to bind the purified Gγ subunits, indicating that there is indeed a direct physical interaction between Gγ and syntaxin 1A, independent of any intermediary proteins. In summary, our data suggest a mechanism by which syntaxin 1A physically binds to Gγ, bringing Gγ into close vicinity of its target site on N-type calcium channel α1 subunits and thereby optimizing Gγ modulation of channel activity.

If our hypothesis is correct, then syntaxin 1A should be able to concomitantly bind to the N-type channel and to Gγ. To confirm this, we carried out an assay examining the interactions of syntaxin and Gγ with fusion proteins directed against the synprint site. As seen in Fig. 3C, the 6×His-synprint site bound syntaxin 1A from rat hippocampal homogenate, but...
more importantly, Gβγ was also detected and reflected the increase in syntaxin 1A binding as the amount of homogenate was increased. We have previously shown that Gβγ does not bind directly to the N-type channel domain II-III linker (34) consistent with the idea that the Gβγ detected in Fig. 3C is coupled to the synprint site via syntaxin. Overall, these data further support the idea that syntaxin 1A may mediate a physical role in enhancing tonic G protein modulation of N-type calcium channels, and it is likely that distinct regions on the syntaxin 1A molecule interact with the calcium channel and Gβγ.

DISCUSSION

It has been known for almost two decades that N-type calcium channels are subject to potent inhibition upon activation of several helix transmembrane receptors (27, 30, 41, 42). Recently, it has been shown that this inhibition is due to direct binding of G protein βγ subunits to the N-type channel domain I-II linker (34) and syntaxin 1A (35, 38, 39), although several other regions of the channel have also been implicated (43–45). The key characteristics of this type of inhibition are its reversal upon application of strong depolarizing pre pulses, as well as an apparent slowing of activation and inactivation kinetics, which can be attributed to an increase in first latency to opening (30–32). G protein inhibition of N-type channels is subject to modulation by a number of factors, including the calcium channel β subunit (22, 46) and protein kinase C (24, 25, 33, 34). More recently, Stanley and Mirotznik (37) suggested that G protein modulation of calcium channels in chick ciliary ganglion might require the presence of syntaxin 1A; however, the underlying molecular mechanisms for this observation remained unknown. Here, we have presented novel evidence that syntaxin 1A may serve to optimize tonic G protein modulation of N-type calcium channels, but is not required.

Syntaxin 1A does not appear to be a requirement for G protein modulation of N-type calcium channels transiently expressed in tsA-201 cells, as these cells lack endogenous syntaxin 1A, and yet, the channels are subject to G protein inhibition either upon transient overexpression of Gβγ, or upon activation of endogenous somatostatin receptors (33). Nonetheless, in our system, syntaxin 1A appeared to modulate the G protein-mediated inhibition of the channel. In the absence of exogenous G protein βγ subunit, the transient expression of syntaxin 1A affected N-type channels in two ways. First, consistent with previous observations by Bezprozvanny et al. (40), syntaxin 1A mediated an 18-mV negative shift in half-inactivation potential, which is likely mediated by direct binding of syntaxin 1A to the synprint site of the channel (19). Second, upon coexpression with syntaxin 1A, the current waveform exhibited slowed activation and inactivation kinetics, and the peak current amplitude could be increased 2-fold upon application of depolarizing pre pulse. While it is possible that these effects are directly due to simple binding of syntaxin 1A to the channel, the notion that such effects were not observed in the Xenopus oocyte expression system (40), and the lack of additivity of the effects of exogenously expressed Gβγ and syntaxin 1A would argue against a direct syntaxin 1A effect. Instead, the observed effects exhibited the key characteristics of Gβγ-mediated inhibition of N-type channels, suggesting that syntaxin 1A expression more likely results in a tonic inhibition of the channel by Gβγ subunits in addition to the previously reported effects of syntaxin 1A on steady state inactivation (40).

In principle, the expression of syntaxin 1A could boost the endogenous levels of free Gβγ, thereby resulting in a tonic level of G protein inhibition. We could not observe such an increase in Gβγ levels upon transfection of tsA-201 cells with syntaxin 1A. However, we have no way of knowing if syntaxin 1A expression alters the relative proportion between endogenous Gα and Gβγ subunits, thereby perhaps mediating an increase in free Gβγ that would be able to inhibit the channel. Furthermore, despite the notion that exogenous expression of Gβγ mediated a potent inhibition of N-type currents, we only detected a ~50% increase in total Gβγ levels following transfection with Gβγ, and thus it is possible that we may not be able to resolve any putative syntaxin 1A-mediated increases in free endogenous Gβγ. A more convincing argument can be based on our observation with botulinum toxin C1, which cleaves the syntaxin 1A protein. If syntaxin 1A were to mediate an up-regulation of endogenous Gβγ levels, the increased levels should be still be maintained after the 12-h incubation with BTC1 (particularly because syntaxin 1A is continuously being expressed during that period); yet, after application of BTC1, the tonic G protein inhibition became dramatically attenuated. Together with our observations that syntaxin 1A and Gβγ have the propensity to form a physical complex, we favor a mechanism by which syntaxin 1A facilitates coupling of Gβγ to the channel protein.

Overall, our data are consistent with a mechanism by which syntaxin 1A binding to the channel enhances its susceptibility to G protein modulation. While we cannot completely rule out the possibility that syntaxin 1A binding allosterically increases the affinity of the channel for Gβγ, the existence of a physical interaction between Gβγ and syntaxin 1A leads us to favor a mechanism in which syntaxin 1A mediates a co-localization of...
of the cytoplasmic region connecting domains II and III of the N-type channel partially overlaps with the calcium channel to the cytoplasmic linker between domains I and II at a region that Syntaxin 1A is hypothesized to facilitate G protein modulation of the light blue (19, 20). The interaction of G protein subunits physically couples to the syntaxin 1A/synprint complex, thereby encouraging a more effective interaction with G protein and syntaxin 1A binding to the channel. Via this interaction, G protein inhibits N-type channel activity by binding to the cytoplasmic linker between domains I and II at a region that partially overlaps with the calcium channel β subunit interaction site. Syntaxin 1A is hypothesized to facilitate G protein modulation of the channel by aiding the targeting of Gβγ to its binding site on the channel.

G protein βγ subunits and the N-type calcium channel. Figure 4 illustrates how this might occur. Syntaxin 1A is known to bind at the synprint site of the N-type channel domain II-III linker (19, 20). The interaction of Gβγ subunits on the N-type channel α1 subunit occurs at the domain I-II linker (33, 34, 35, 38) as well as the carboxyl-terminal region (43, 45). We hypothesize that Gβγ subunits physically couple to the syntaxin 1A/synprint complex, thereby encouraging a more effective interaction between Gβγ and its target region on the channel. Via this mechanism, the endogenous levels of free Gβγ would become effective in modulating channel activity when syntaxin 1A is present. BTC1 treatment results in the loss of this effect due to cleavage and removal of the syntaxin 1A protein. Upon exogenous expression of Gβγ or by activation of seven helix transmembrane receptors, the concentration of free Gβγ would become sufficiently high to modulate the channel independently of the enhancing effect of syntaxin 1A, thus rendering BTC1 ineffective in removing G protein inhibition. The observation that recombiant syntaxin 1A was able to interact with Gβγ from rat hippocampal homogenate, together with the observations of Stanley and Mirotznik (37) in chick ciliary ganglion from rat hippocampal homogenate, together with the observations of Stanley and Mirotznik (37) in chick ciliary ganglion (37) and neurotransmission.

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