Syntaxin 1A Supports Voltage-Dependent Inhibition of α_{1B} Ca^{2+} Channels by G$\beta\gamma$ in Chick Sensory Neurons

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N-type Ca^{2+} channels are modulated by a variety of G-protein-coupled pathways. Some pathways produce a transient, voltage-dependent (VD) inhibition of N channel function and involve direct binding of G-protein subunits; others require the activation of intermediate enzymes and produce a longer-lasting, voltage-independent (VI) form of inhibition. The ratio of VD:VI inhibition differs significantly among cell types, suggesting that the two forms of inhibition play unique physiological roles in the nervous system. In this study, we explored mechanisms capable of altering the balance of VD and VI inhibition in chick dorsal root ganglion neurons. We report that (1) VD:VI inhibition is critically dependent on the G$\beta\gamma$ concentration, with VI inhibition dominant at low G$\beta\gamma$ concentrations, and (2) syntaxin-1A (but not syntaxin-1B) shifts the ratio in favor of VD inhibition by potentiating the VD effects of G$\beta\gamma$. Variations in expression levels of G-proteins and/or syntaxin provide the means to alter over a wide range both the extent and the rate of Ca^{2+} influx through N channels.

Key words: G-protein modulation; dorsal root ganglion neurons; GABA receptors; adrenergic receptors; recombinant channels; presynaptic regulation

The modulation of voltage-dependent Ca^{2+} channels by G-protein-coupled receptors takes many forms and varies as a function not only of the receptor and channel type but also of the cell under study (Dolphin, 1998; Dunlap and Ikeda, 1998; Ikeda and Dunlap, 1999). Among the many Ca^{2+} channel gene products identified, those from class B (or N-type) have been best studied, because they have proven to be most sensitive to G-protein-coupled pathways. Several receptor-dependent pathways have been described to target N channels in native cells. The most ubiquitous is mediated by direct binding between $\beta\gamma$ complexes released from heterotrimeric G-proteins (G$\beta\gamma$) and Ca^{2+} channel α_1 subunits (DeWaard et al., 1997; Qin et al., 1997; Zamponi et al., 1997; Furukawa et al., 1998); such binding produces a voltage-dependent (VD) form of inhibition often characterized by slowed current activation kinetics (Marchetti et al., 1986; Bean, 1989; Elmslie et al., 1990; Arnot et al., 2000). In contrast, other forms of modulation are mediated by more complex signaling pathways involving kinase-dependent phosphorylation and producing long-lasting, voltage-independent (VI) effects (Dunlap and Ikeda, 1998).

In most neurons, both VD and VI forms of G-protein-mediated inhibition coexist. Given their unique biophysical and biochemical profiles and variations in their relative abundance from cell to cell, the two forms of inhibition have been hypothesized to play distinct physiological roles in the nervous system (Brody et al., 1997; Park and Dunlap, 1998; Brody and Yue, 2000). Thus, understanding the molecular mechanisms responsible for these forms of inhibition is of potential physiological significance. Our studies have used dorsal root ganglion (DRG) neurons from embryonic chick, because they prominently express both VD and VI inhibition (Luebke and Dunlap, 1994) that can be activated by a variety of G-protein-coupled receptors. We have, in addition, demonstrated some selectivity between receptors and the pathways to which they couple (Diversé-Pierluissi and Dunlap, 1993; Diversé-Pierluissi et al., 1995), suggesting the possibility of receptor-specific modulation of physiological processes.

Use of function-blocking antibodies directed against G-protein α_o and α_i subunits allowed the demonstration that norepinephrine (NE) and GABA mediate VD inhibition in chick DRG neurons via G_o, whereas NE also produces VI inhibition via G_i (Diversé-Pierluissi et al., 1995). This latter pathway requires the activation of PLC_β and protein kinase C (PKC) (Rane et al., 1989; Diversé-Pierluissi et al., 2000). When purified G$\beta\gamma$ is introduced intracellularly into these cells, only the VI form of inhibition is observed, suggesting that VD inhibition might be mediated by Gα_o. This lack of G$\beta\gamma$-mediated VD inhibition, however, is at odds with many studies of mammalian Ca^{2+} channels demonstrating that G$\beta\gamma$ (and not Gα) produces VD inhibition (Herlitze et al., 1996; Ikeda, 1996) and raises the question of whether structural differences in the chick N channel α_{1B} subunits underlie differences in modulation.

To allow a test of this hypothesis, we first cloned cDNAs encoding the expressed N-type channels from chick DRG cells and identified three variable regions in which the avian channels differ significantly from their mammalian counterparts (Lü and

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Dunlap, 1999). Here we compare Gβγ-induced modulation of these recombinant N channels expressed in tsA-201 cells with those of native N channels in chick DRG neurons. Results demonstrate that the modulation of chick N channels does not differ between the isoforms, either in their native environments or when expressed heterologously, and (as is true for rat N channels) Gβγ mediates VD inhibition. We find that, at low Gβγ concentrations, VI inhibition dominates, but syntaxin 1A promotes a switch to VD inhibition by enhancing the interaction between the Gβγ complex and the Ca^{2+} channel subunit.

MATERIALS AND METHODS

Cell culture and transfection. Dorsal root ganglia were dissected from chicken embryos (11- to 12-d-old for most experiments), incubated for 20 min in 0.005–0.01% collagenase A (Sigma, St. Louis, MO or Boehringer Mannheim, Indianapolis, IN) in a Ca^{2+}–Mg^{2+}-free saline, and dissociated mechanically in tissue culture medium (below) by trituration through a small-bore Pasteur pipette. The culture medium was DMEM supplemented with 10% heat-inactivated horse serum, 1 mM glutamine, 50 U/ml penicillin, 50 μg/ml streptomycin (Life Technologies, Gaithersburg, MD), and an empirically determined amount of male mouse submaxillary gland homogenate (to supply nerve growth factor). Cells were plated in 35 mm tissue culture dishes (Falcon).

Human embryonic kidney cells (clone tsA-201) were cultured and transfected using methods previously described (Lü and Dunlap, 1999). Before transfection, cells were split 1:10, and 24 hr later, they were transfected using the calcium-phosphate method (Dhallan et al., 1990) with a mixture of 10 μg of Ca^{2+} channel α_{1B} subunit cDNA with 10 μg of rat β_{1B} (provided by S. R. Ikeda, Guthrie Institute, Sayre, PA), 8 μg of rat $\alpha_2\delta$ (provided by H. Chin, National Institutes of Health, Bethesda, MD), and 2 μg of large T antigen (provided by D. T. Yue, Johns Hopkins University, Baltimore, MD), all in pcDNA3. The cDNA for rn$\alpha_{1B\text{-}b}$ was kindly supplied by Diane Lipscombe (Brown University, Providence, RI). In some experiments, 4 μg each of cDNAs encoding G-protein β_1 and γ_2 subunits (in pcDNA3; provided by Stephen Ikeda) or syntaxin 1A (in pMT2; Jarvis et al., 2000) were also cotransfected. Currents were recorded 2–4 d after transfection.

Cell injection. Proteins were overexpressed in DRG neurons by direct injection (Microinjector 5242; Eppendorf, Westbury, NY) of expression plasmids into the nucleus of cells cultured for 4–30 hr according to methods above. Plasmid concentrations were 50 ng/μl in 125 mM KCl and 5 mM HEPES, pH 7.2. Fluorescein dextran (10 kDa, 2.5 μg/μl; Molecular Probes, Eugene OR) was included to allow later identification of injected cells. Recordings were made 16–24 hr after injection. Approximately 50% of fluorescent cells showed the expected effect of overexpression (alteration of current amplitude and/or time course); in the remaining cells, it is likely that the injection missed the nucleus. Use of green fluorescent protein cDNA as a control for successful nuclear injection was precluded because the protein is toxic to chick DRG neurons, regardless of the expression method (M. S. AtKisson, unpublished observations).

Reverse transcriptase-PCR. Total RNA was purified from whole ganglia or brain dissected from chicks at embryonic days 12, 15, 18, and 21 or from adult chicken or adult Sprague Dawley rats using RNA STAT-60 (Tel-Test B, Friendswood, TX). Poly(A+) RNA was purified from the total RNA using the Oligotex mini kit (Qiagen, Valencia, CA). Reverse transcriptase (RT) reactions were performed with the GeneAmp RNA PCR core kit (PE Biosystems, Foster City, CA) according to the manufacturer's directions, using a ratio of oligo-dT to random hexamers of 3:1. Five microliters of the RT reaction was used for PCR, which was performed using Advantage enzyme (Clontech, Palo Alto, CA). Primers were designed based on alignments of syntaxin 1A sequences from rat, human, and fruit fly (GenBank) accession numbers AF217191, NM_004603, and L37732), and of syntaxin 1B sequences from human, rat, and mouse (GenBank accession numbers NM_003163, M95735, and D29743). Alignments were performed using the ClustalW program maintained on the web by EMBL-European Bioinformatics Institute. Primer designations are for bookkeeping purposes, and do not reflect any information. PCR protocols consisted of a hot start and touchdown (five cycles at 72°C, five cycles at 70°C, and 23 cycles at 68°C), using an Eppendorf MasterGradient thermocycler.

Cloning of rat syntaxin 1B. All reagents and primers were purchased from Life Technologies (Rockville, MD). The forward primer (with a 5′ *Not*I restriction site) was (5′-CGAAGAAGGGGAGGAGGAGCTGCGGCCGCCATGAAGGATCGGACTCAGGAGC-3′), and the reverse primer (with a 5′ *Xho*I restriction site) was (5′GGTCCTGGGCTCGAGAAGGGTAGGGGCCTACAAGCCCAGTGTCCC3′). Rat brain cDNA was kindly provided by Bob Winkfein (University of Calgary, Calgary, Alberta, Canada). The PCR reaction was performed in a volume of 50 μl and included 20 mM Tris-HCl, pH 8.4, 50 mM KCl, dNTPs (0.2 mM each), 3.5 mM $MgCl_2$, 2.5 U of platinum *Taq* DNA polymerase, 20 pmol of each primer, and 50 ng of cDNA. Using a PTC-100HB thermal-cycler (MJ Research, Watertown, MA), the reaction began with a hot start, and was held at 9°C for 2 min. Thirty cycles were conducted, consisting of denaturation for 30 sec at 94°C, annealing for 45 sec at 62°C, and extension for 1.5 min at 72°C. The resultant syntaxin 1B DNA product was run on a 0.8% agarose gel, extracted, and purified using QIAquick Gel Extraction (Qiagen, Mississauga, Ontario, Canada), ligated into a pGEM T-Easy vector (Promega, Madison, WI), and sequenced to rule out PCR errors. The syntaxin 1B-T-Easy construct was then digested by *Not*I and *Xho*I, and the syntaxin 1B fragment was ligated into pMT2sx (Genetics Institute, Andover, MA) for subsequent expression in tsA-201 cells. Rat syntaxin 1A cDNA was also subcloned into pMT2sx, and $G\beta_1$ and $G\gamma_2$ were subcloned into pcDNA3 (Invitrogen, Carlsbad, CA).

Current recording and data analysis. Standard tight-seal, whole-cell recording methods were used to measure Ca^{2+} current through N-type channels using a List Biologic (Campbell, CA) EPC9 amplifier. Internal solution for recording from chick DRG neurons or tsA-201 cells expressing the recombinant chick N channel clones contained 150 mM CsCl, 10 mM HEPES, 5 mM BAPTA, and 5 mM MgATP, pH-adjusted to 7.2 with CsOH; external solution contained 93 mM NaCl, 50 mM tetraethylammonium chloride, 2 mM $CaCl_2$, 25 mM HEPES, 12.5 mM NaOH, 5 mM D-glucose, and 0.3 μ mM tetrodotoxin, pH-adjusted to 7.4 with TEA-OH.

Where noted, some experiments on recombinant chick and rat N channels expressed in tsA-201 cells used an external solution of 20 mM $BaCl_2$, 1 mM $MgCl_2$,10 mM HEPES, 40 mM TEA-Cl, 10 mM glucose, and 65 mM CsCl, pH 7.2 with TEA-OH, and an internal solution containing 108 mM Cs-methanesulfonate, 4 mM $MgCl_2$, 9 mM EGTA, and 9 mM HEPES, pH 7.2. Such solutions enhanced current amplitudes but otherwise did not alter fundamental properties of their modulation by G-protein subunits when compared with the Ca^{2+}-based external solution.

For some experiments, purified bovine brain Gβγ was applied intracellularly via the patch pipette. The Gβγ preparation (kindly provided by John Hildebrandt, University of South Carolina Medical Center, Charleston, SC) was stored at −80°C as a stock solution of ~1 mg/ml in buffer containing 20 mM Tris, pH 8.0, 1 mM EDTA, 1 mM dithiothreitol, 100 mM NaCl, and either 0.7% CHAPS or 0.7% CHAPS and 0.1% polyoxyethylene-9-lauryl ether (Thesit). No differences were observed between the two storage buffers. On the day of the experiments, a sample was diluted into intracellular recording solution to a final concentration of 20 nM and applied by passive diffusion from the pipette to the cytoplasm. Heat-inactivated Gβγ served as negative control.

RESULTS

Purified Gβγ produces VI but not VD inhibition of N current

Tight-seal, whole-cell methods were used to record macroscopic, N-type Ca^{2+} channel currents from dissociated chick DRG neurons and from tsA-201 cells transfected with N channel clones (Lü and Dunlap, 1999). Currents were evoked by a 15 msec test pulse to 0 mV and were monitored during the intracellular application of purified, bovine brain Gβγ added to the recording pipette solution. In chick DRG neurons, a saturating concentration of Gβγ (20 nM; Diversé-Pierluissi et al., 1995, 2000) produced a maximal 49.7 ± 4.2% (n = 6) inhibition of N current after ~25 min of dialysis with Gβγ-containing solution as compared with control recordings with heat-inactivated Gβγ in the pipette (Fig. 1*A*). A three-pulse voltage protocol—consisting of two test pulses to 0 mV separated by a 15 msec conditioning depolarization to 80 mV—was used to assay for the relief of inhibition that is characteristic of VD inhibition by G-proteins (Elmslie et al., 1990). Gβγ-mediated inhibition was insensitive to the conditioning pulse (i.e., no prepulse-induced facilitation),

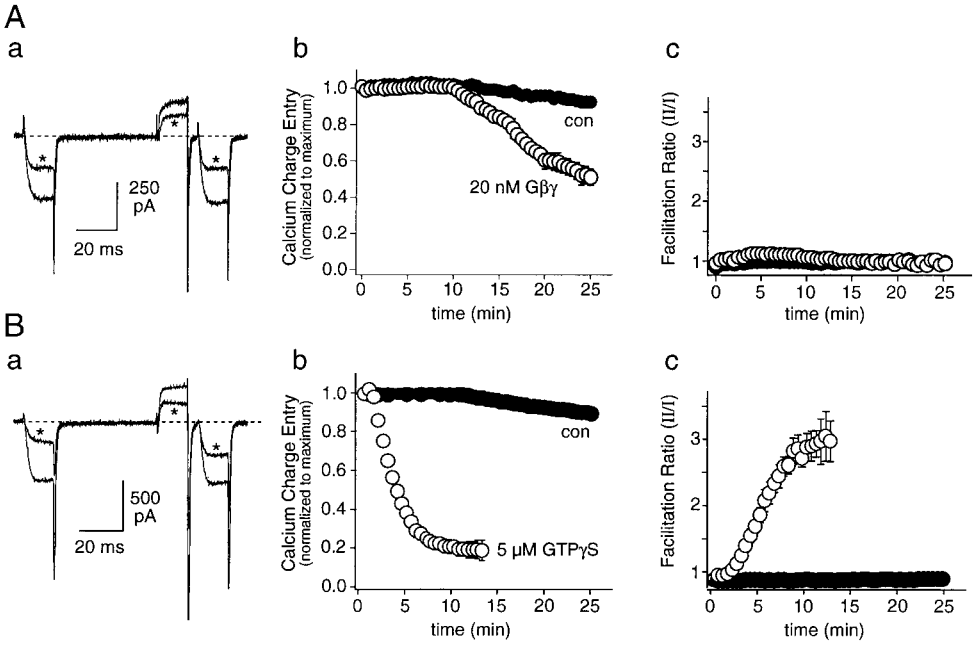

Figure 1. Gβγ produces VI inhibition in chick DRG neurons. Whole-cell Ca^{2+} current evoked by a three-pulse stimulus paradigm was monitored over time during application of 20 nM Gβγ (*A*) or 5 μM GTPγS (*B*) through the patch pipette solution. Controls contained heat-inactivated Gβγ (20 nM). Panels marked *a* each show two superimposed current traces taken at the start of whole-cell recording or after (*) maximal effect of Gβγ or GTPγS (*Aa* and *Ba*, respectively). Panels marked *b* show the mean ± SEM Ca^{2+} charge entry (normalized to the maximum) as a function of time for control cells (*filled circles*) or during application of Gβγ ($n = 6$) or GTPγS ($n = 5$) (*open circles*); panels marked *c* plot the time course of the facilitation ratio produced in the same cells by conditioning depolarization (defined as charge entry during test pulse II divided by charge entry during test pulse I).

indicating a distinct absence of VD inhibition (Fig. 1*A*). In contrast, intracellular application of 5 μM GTPγS (to directly activate endogenous G-protein heterotrimers) inhibited N current even more strongly (82.0 ± 5.1%; $n = 5$; Fig. 1*B*) and produced the slowing of current activation that is characteristic of VD inhibition (Marchetti et al., 1986; Bean, 1989; Grassi and Lux, 1989; Elmslie et al., 1990). Furthermore, in the presence of GTPγS, conditioning depolarizations evoked strong facilitation (Fig. 1*Bc*).

These results suggest that VI inhibition is mediated by Gβγ (confirming previous studies of Diversé-Pierluissi et al., 1995, 2000) but they imply that VD inhibition may be mediated by some other mechanism. Given, however, that VD inhibition of mammalian N channels is well accepted to involve direct binding of Gβγ to Ca^{2+} channel α_1 subunits (Herlitze et al., 1996; Ikeda, 1996; DeWaard et al., 1997; Qin et al., 1997; Zamponi et al., 1997; Furukawa et al., 1998), we sought an explanation for the apparent resistance to Gβγ of the VD pathway in chick DRG cells. Alternatively, (1) the VI pathway could be activated at lower concentrations of Gβγ, (2) the VD pathway could reside in a compartment of the cell that is inaccessible to applied Gβγ, and/or (3) other accessory proteins could be present in the primary cells that promote VI or suppress VD inhibition. To explore between these possibilities, experiments used recombinant Ca^{2+} channels expressed heterologously.

Gβγ-mediated inhibition of recombinant chick N channels

Chick N-type Ca^{2+} channel cDNAs were transiently expressed in tsA-201 cells, and Gβγ was applied via the patch pipette as for the primary cells above. Recombinant rat rn$\alpha_{1B\text{-}b}$ (Lin et al., 1997) was studied for comparison. Four different, full-length α_1 subunit clones from chick (cdB1–4; Lü and Dunlap, 1999) were tested for their sensitivities to Gβγ. The chick channels differed from one another in their putative cytoplasmic linkers between membrane-spanning domains I and II and/or in an alternatively spliced C-terminal domain (Fig. 2*A*)—both regions generally implicated in Gβγ-binding to mammalian Ca^{2+} channel α_1 subunits (Zhang et al., 1996; DeWaard et al., 1997; Qin et al., 1997; Zamponi et al., 1997). When applied for 20–30 min through the patch pipette, 20 nM Gβγ evoked only a small inhibition of the recombinant channel currents (23.2 ± 1.7%, $n = 25$ for chick; 24.8 ± 4.2%, $n = 5$ for rat); a conditioning depolarization relieved a small fraction of this inhibition (8.4%). No differences among any of the four cdB clones or the rat clone were observed (Fig. 2*B*).

Given that Gβγ-mediated VD inhibition has been generally studied under conditions of G-protein overexpression (Ikeda and Dunlap, 1999), we sought to determine whether VD inhibition was promoted when cells were cotransfected with Gβ and Gγ cDNAs (because the biochemical preparation of purified Gβγ did not allow application of concentrations >20 nM). When Gβγ was overexpressed in tsA-201 cells, a robust, tonic VD inhibition was observed (Fig. 3). Ca^{2+} current amplitude was, on average, 62.2% of control cells transfected with channel subunits alone, and currents activated slowly, as expected for VD inhibition. Conditioning depolarizations produced an average 142% facilitation of Ca^{2+} current, because of a relief of the tonic inhibition (Fig. 3*A*), indicating that chick α_{1B} Ca^{2+} channel subunits are inhibited by Gβγ in a manner similar to that of their mammalian counterparts. In addition, all four cdB clones behaved similarly to one another as well as to the rat clone, rn$\alpha_{1B\text{-}b}$, when cotransfected with Gβγ (Fig. 3*B*), further suggesting that the dominance of the VI pathway in native neurons is not because of the expression of a uniquely Gβγ-resistant α_{1B} channel subunit.

Overexpression of Gβγ produces VD inhibition of native N current

To explore whether native N currents in chick DRG neurons, unlike their mammalian counterparts, are refractory to Gβγ, we overexpressed $G\beta_1\gamma_2$ in the cells by nuclear injection of the cDNAs. In 8 of 13 injected cells tested, Ca^{2+} currents activated slowly, peaked at a level 24.9% of currents measured from uninjected control cells, and facilitated in response to a conditioning depolarization (Fig. 4*A*). Such tonic inhibition and prepulse-induced facilitation is not observed in uninjected control cells. The remaining five injected cells were indistinguishable from control. The apparent lack of $G\beta_1\gamma_2$-induced inhibition in these latter cells (Fig. 4*B*) is likely to result from an absence of $G\beta_1\gamma_2$

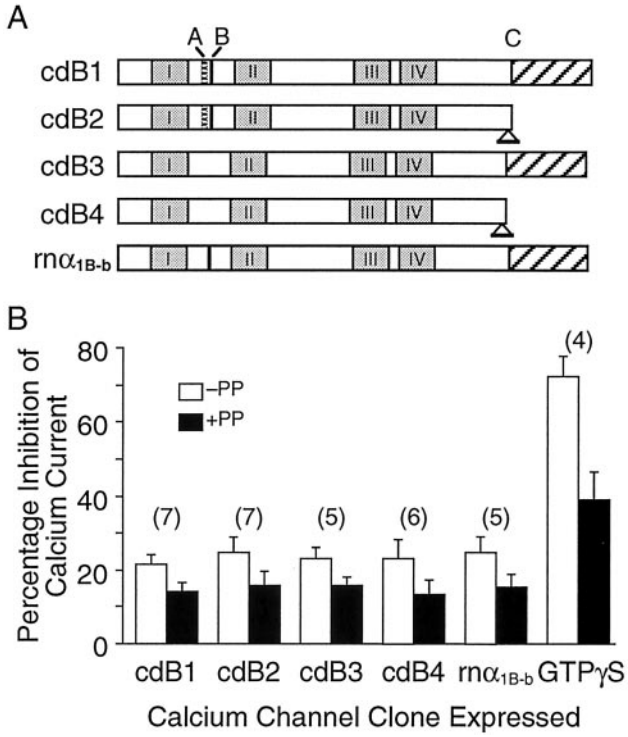

Figure 2. Gβγ-mediated inhibition is similar for all N channel clones tested. *A* contains diagrams summarizing key structural differences between a_{1B} Ca^{2+} channel variants cloned from chick DRG (cdB; Lü and Dunlap, 1999) or rat (Dubel et al., 1992; Lin et al., 1997). The *gray rectangles* marked with *roman numerals* represent the four homologous membrane-spanning repeats; the *white rectangles* represent the putative intracellular domains. *A* is a 75 bp insert contained in some but not all chick clones; *B* is a 33 bp insert contained in all mammalian but not in some chick variants; *C* is a 5 bp insert found in some but not all chick variants (creating a premature stop codon and a channel subunit that is truncated by 175 amino acids in the C-terminal end). *B* is a histogram plotting the percentage inhibition of Ca^{2+} current produced by 20 nM Gβγ in tsA-201 cells transfected with the N channel α_1 subunit clone designated on abscissa, coexpressed with rat β_{1b} and $\alpha_2\delta$ subunits. A three-pulse voltage protocol was used to identify the VD component of inhibition. *White bars* represent total inhibition in the absence of a prepulse (*−PP*); *black bars* denote inhibition after prepulse to +80 mV (*+PP*). Number of cells noted in parentheses.

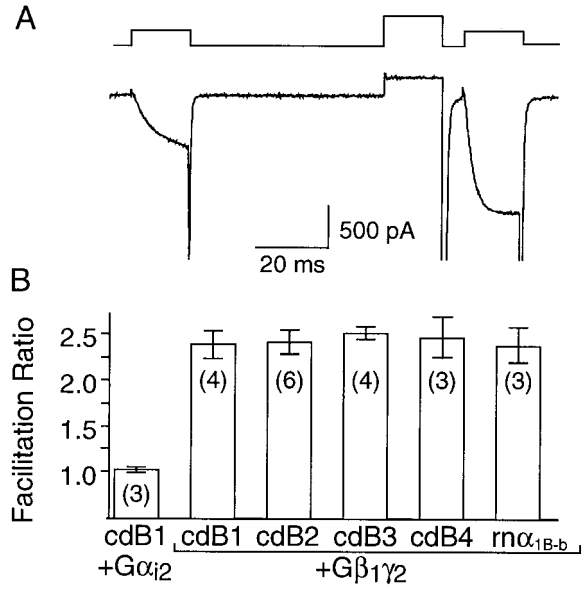

Figure 3. Overexpression of Gβγ enhances VD inhibition of N current. TsA-201 cells were cotransfected with α_1 subunit clones designated on abscissa of *B* and either $G\beta_1\gamma_2$ or $G\alpha_{i2}$ (as marked). *A,* Ca^{2+} current evoked in cell expressing cdB1 by voltage pulse protocol used to estimate VD inhibition (*top panel*). *B,* Prepulse-induced facilitation (average ± SEM) for each of the Ca^{2+} channel clones studied. Numbers of cells in parentheses.

expression (from ineffective injection), because basal Ca^{2+} currents were found to be no different between these cells and uninjected control cells (Fig. 4*C*); were $G\beta_1\gamma_2$ actually overexpressed, robust VI inhibition would be expected, even in the absence of VD inhibition. These results demonstrate that Gβγ can evoke significant VD inhibition of chick DRG N channels (as with mammalian N channels), but apparently only when sufficiently high concentrations (and/or correct targeting of Gβγ) is achieved.

The notion that concentration plays an important role in the selective activation of VI and VD pathways is supported by previous results from our laboratory. Lower concentrations of NE (≤10 μM) preferentially activate the VI pathway, with 10 μM producing a total inhibition of 25 ± 7%, most of which is resistant to depolarizing prepulses. Higher concentrations of NE (100 μM) recruit a VD component to the inhibition, producing a total inhibition of 30%, a third of which is reversed by conditioning depolarizations (Diversé-Pierluissi et al., 1995). It makes further sense that VI inhibition would be preferentially activated by lower concentrations of Gβγ, because Gβγ binds PLC_β at higher affinity (Myung et al., 1999) than that to which it binds the Ca^{2+} channel (DeWaard et al., 1997).

Syntaxin 1A potentiates VD inhibition by Gβγ

The strength of the G-protein–Ca^{2+} channel interaction is known to be modulated by accessory proteins, such as syntaxin 1A (Stanley and Mirotznik, 1997; Jarvis et al., 2000) and Ca^{2+} channel β subunits (Roche et al., 1995; Roche and Triestman, 1998; Meir et al., 2000). We focused our studies on syntaxin 1A to explore whether the predominance of VI inhibition in chick DRG neurons could be influenced by the expression level of this protein, known to directly interact with Ca^{2+} channel α_1 subunits (Sheng et al., 1998; Catterall, 1999). In particular, we tested whether overexpression of syntaxin 1A in chick DRG cells would support VD inhibition by 20 nM applied Gβγ. The cDNA for rat syntaxin 1A was injected into DRG cell nuclei, and Ca^{2+} currents were recorded 16–24 hr later. Among a total of 39 injected cells (identified by the presence of coinjected fluorescent dextran), 18 showed tonic VD inhibition (Fig. 5*A*) in the absence of Gβγ application (facilitation ratio of 1.14 vs 1.02 for control and the other 21 injected cells). Total charge entry for these 18 cells (12.07 ± 1.22 pC) was 70% of that measured from cells showing no tonic inhibition. This result suggests that syntaxin 1A expression increases the sensitivity of N channels to modulation by endogenous free Gβγ. Moreover, whole-cell recordings from 11 of these 18 cells lasted long enough to detect further inhibition by 20 nM Gβγ applied through the recording pipette. Six of the eleven responded with significantly more VD inhibition during exposure to Gβγ (additional inhibition, 46%; final facilitation ratio, 1.38 Fig. 5*B*). Given that uninjected control cells do not respond to 20 nM Gβγ with VD inhibition (Fig. 1), voltage-dependent responses observed in these six injected neurons argue strongly that syntaxin 1A shifts the equilibrium in favor of VD inhibition. In the remaining five cells, Gβγ produced a mean 50 ±

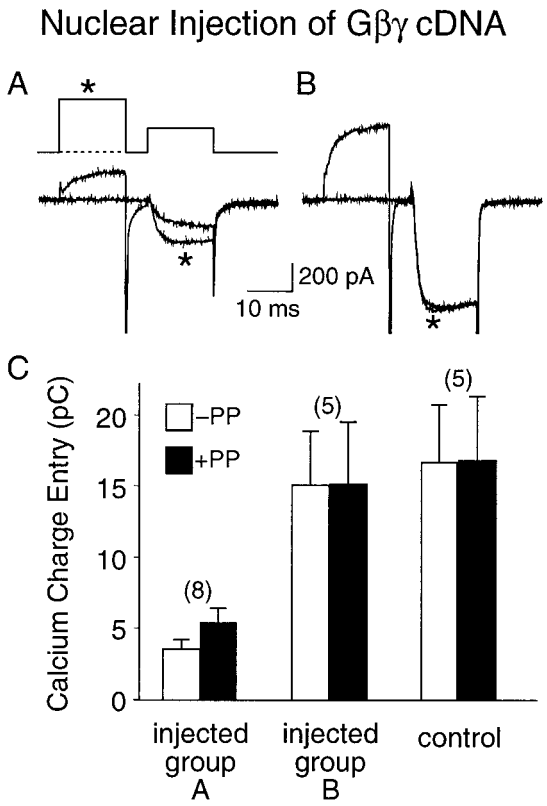

Figure 4. Overexpression of Gβγ enhances VD inhibition in chick DRG neurons. $G\beta_1$ and $G\gamma_2$ cDNAs were injected into chick DRG cell nuclei, and Ca^{2+} currents were studied 18–24 hr later. *A*, *B*, Two superimposed Ca^{2+} currents evoked by test pulses to 0 mV with (*) or without a preceding conditioning pulse to +80 mV. *Traces* in *A* taken from an injected cell with tonic VD inhibition; *traces* in *B* from an injected cell (identified by fluorescence) without tonic inhibition. *C,* Histogram of average total Ca^{2+} charge entry (±SEM) for the sample showing tonic inhibition (group A), the sample of injected cells without tonic inhibition (group B), and control (noninjected) cells. Effect of conditioning depolarization (+*PP*) shown in *black bars*; number of cells shown in parentheses.

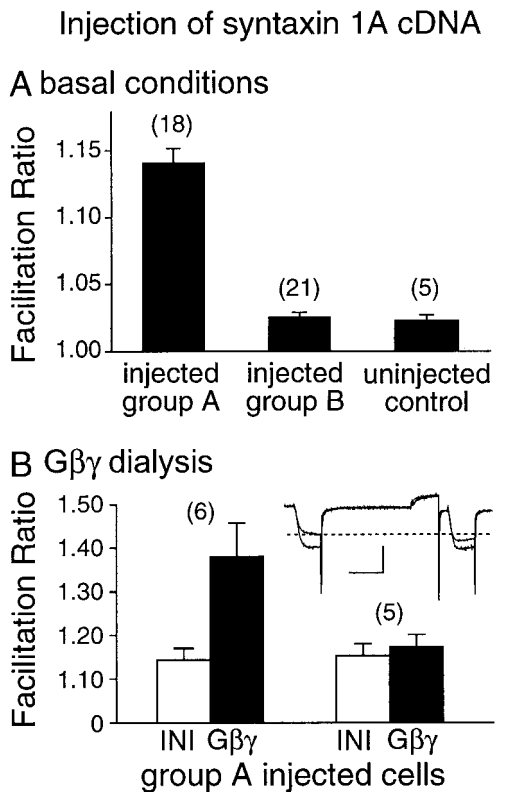

Figure 5. Syntaxin 1A expression in chick DRG neurons enhances VD inhibition by Gβγ. Rat syntaxin 1A cDNA was injected into nuclei of chick DRG neurons, and Ca^{2+} currents were studied 16–24 hr later. Three-pulse voltage protocol was used to measure prepulse-induced facilitation. *A,* Histogram of facilitation induced by prepulse in three groups of cells: group A (injected and demonstrating facilitation), group B (injected but without facilitation), and control (uninjected). *B,* Histogram showing responsiveness of Ca^{2+} currents in group A injected cells to intracellular application of 20 nM Gβγ through the patch pipette. *White bars* represent mean prepulse-induced facilitation seen immediately after achieving whole-cell access; *black bars* represent mean facilitation after ~20 min of dialysis with 20 nM Gβγ. The two data sets are from cells in which additional VD inhibition was observed (*left*) or not (*right*). Number of cells shown in parentheses. *Inset,* Two superimposed current traces before and after application of Gβγ (taken from one of the cells in the left-hand group). *Dotted line* marks level of peak current during first test pulse. Calibration: 1 nA, 20 msec.

6.3% inhibition over 12–27 min of dialysis; the additional inhibition was purely VI, however, showing no prepulse-induced facilitation over that associated with tonic inhibition (Fig. 5*B*). These results confirm recent reports of syntaxin 1A-induced enhancement of Gβγ-mediated inhibition of recombinant rat N channels expressed in tsA-201 cells (Jarvis et al., 2000; Jarvis and Zamponi, 2001) and provide additional data supporting the similarity between avian and mammalian N channels.

Chick DRG neurons do not express syntaxin 1A but do express syntaxin 1B

An absence of syntaxin 1A expression in chick DRG neurons could explain why purified Gβγ does not produce VD inhibition of N currents in these cells. RT-PCR was used to explore this issue, using primers (Table 1) designed against three domains that are tightly conserved among distantly related species (*Drosophila*, rat, and human). Primer pair designated 301/302 consisted of a sense primer specific to syntaxin 1A and an antisense primer across a domain conserved between syntaxins 1A and 1B. A second pair (303/302) consisted of sense and antisense sequences common to syntaxins 1A and 1B. Both primer pairs amplified the expected products from rat and *Drosophila* nervous tissue, but only the primer pair with homology to both syntaxins 1A and 1B amplified a product from chick brain or DRG (Fig. 6), suggesting that syntaxin 1A is not expressed in chick. Sequence analysis of the 303/302 product from chicken revealed homology to syntaxin 1B, which is a separate gene and not a splice variant of syntaxin 1A (Bennett et al., 1993). To confirm that syntaxin 1B was present, separate RT-PCR reactions were performed using specific primers to syntaxin 1B (pair 304/305). This pair amplified the expected product, confirmed as a syntaxin 1B homolog by sequence analysis.

Because rat syntaxin 1A levels in cerebellum (Veeranna and Pant, 1997) and retina (Dhingra et al., 1997) have been shown to be tightly regulated during development, we looked for variation in syntaxin 1A expression during neuronal embryogenesis in chick using RT-PCR. No syntaxin 1A was found in DRG cells at any embryonic stage (days 12, 15, 18, and 21) or in adult chicken brain (data not shown). No products were amplified from syntaxin 1A-specific primers, but primers 303/302 with homology to both syntaxins 1A and 1B amplified a product.

To confirm that this product was amplified from syntaxin 1B

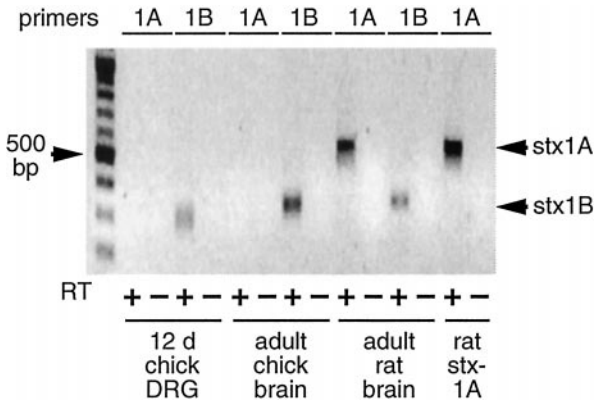

Figure 6. Chick neurons do not express syntaxin 1A. RT-PCR was used to assay for the expression of syntaxin 1A or syntaxin 1B in chick and rat nervous tissue (noted at the *bottom* of the gel). Specificity of primer pairs used and the presence (+) or absence (−) of RT is noted at the *top* and the *bottom* of the gel, respectively. Expected product sizes for syntaxin 1A and syntaxin 1B noted by *arrows* on the *right*. The plasmid carrying rat syntaxin 1A that was used for injection was used here as a positive control (*rat stx-1A*).

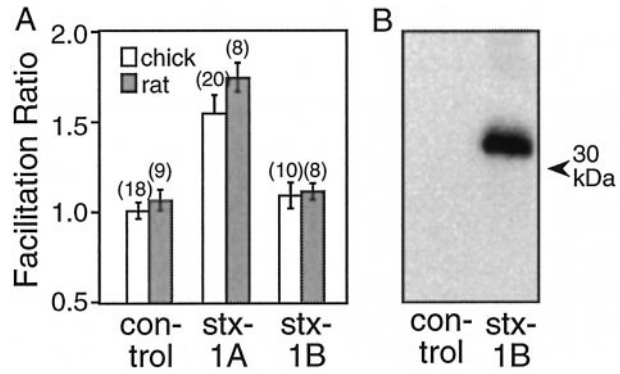

Figure 7. Syntaxin 1A but not syntaxin 1B potentiates VD inhibition by Gβγ. TsA-201 cells were transfected with chick cdB1 (*white bars*) or rat rbBII (*gray bars*) Ca^{2+} channel α_{1B} subunit clones and calcium currents isolated using whole-cell recording. *A,* Histogram of tonic VD inhibition plotted as mean facilitation ratio ± SEM (measured with three-pulse voltage protocol) in control cells or in cells transfected with syntaxin 1A (*stx-1A*) or syntaxin 1B (*stx-1B*), as marked on abscissa. Numbers of cells noted in parentheses. *B,* Western blot of control cells or cells transfected with syntaxin 1B cDNA, probed with anti-syntaxin antibody [methods per Jarvis et al. (2000)].

RNA, PCR was used with a new primer pair (304/305) based on regions homologous among syntaxin 1B clones (mouse, human, rat), but differing from syntaxin 1A. This primer pair amplified the expected length product, which was confirmed as a syntaxin 1B homolog by sequence analysis. Thus, chick DRG cells express syntaxin 1B but not syntaxin 1A. To evaluate whether this could explain the lack of VD inhibition by applied Gβγ, we compared effects of syntaxin 1B with those of syntaxin 1A on inhibition of N channels by Gβγ in tsA-201 cells.

Syntaxin 1B does not support VD inhibition of recombinant N channels

A full-length syntaxin 1B cDNA was cloned from rat mRNA using RT-PCR (see Materials and Methods), subcloned into pMT2sx, and expressed in tsA-201 cells along with N channel α_1 subunits from rat (rbBII) or chick (cdB1) and with rat β_{1b}, and α_2-δ. Comparisons were made between cells transfected with syntaxin 1A or syntaxin 1B cDNAs. Overexpression of syntaxin 1A promoted tonic, VD inhibition in these cells expressing the rat and chick N channels, exhibiting facilitation ratios of 1.75 ± 0.08 (rbBII; $n = 8$) and 1.56 ± 0.11 (cdB1; $n = 20$). In such experiments, the VD pathway is saturated by basal concentrations of Gβγ; additional Gβγ (through overexpression) produces no additional effect on Ca^{2+} current (Jarvis et al., 2000). In contrast, N currents in cells transfected with syntaxin 1B showed little or no tonic inhibition, with facilitation ratios near 1 (Fig. 7*A*). Western blot analysis of the syntaxin 1B-transfected cells using an antibody that recognizes both forms of syntaxin demonstrated the expression of syntaxin 1B protein (Fig. 7*B*). Furthermore, when cells were cotransfected with syntaxin 1B and syntaxin 1A (at 1:1), the facilitation ratio was reduced from 1.75 ± 0.08 to 1.2 ± 0.05 (rbBII; $n = 8$), confirming that syntaxin 1B was expressed in the transfected cells and suggesting that it binds the synprint motif in the II-III linker of the N channel α_1 subunit. This dominant-negative effect of syntaxin 1B is likely to be specific, because overexpression of other proteins (e.g., the II-III linker from α_{1C} Ca^{2+} channels) does not alter the ability of syntaxin 1A to promote VD inhibition by Gβγ (Magga et al., 2000). Thus, overexpression of a second protein does not, per se, reduce syntaxin 1A expression. These results suggest that, despite their significant sequence identities, the two syntaxins differ from one another in their abilities to support VD inhibition. Low levels of syntaxin 1A expression are likely to be one reason why low concentrations of Gβγ (either applied or produced by submaximal activation of G-protein-coupled receptors), do not naturally promote VD inhibition in chick DRG cells.

DISCUSSION

Results presented here provide an explanation for the observation that both the form and extent of Gβγ-mediated inhibition of N channels vary among preparations and/or with different experimental approaches. By comparing the inhibition of recombinant and native N channels produced by purified or overexpressed Gβγ, we have shown that VI inhibition is preferentially evoked in chick DRG neurons with direct applications of low Gβγ concentrations, whereas VD inhibition is evoked strongly only when Gβγ is either overexpressed or when syntaxin 1A (but not syntaxin 1B) is present. Our results further demonstrate that four α_{1B} channel splice variants cloned from chick DRG cells are all modulated similarly to one another as well as to rat α_{1B} channels. Thus, these data argue that cell-to-cell variations in the ratio of VI to VD inhibition of N channels are more likely to result from variations in concentration and targeting of the signaling molecules than from differences in primary structure of the α_{1B} subunits present.

That being said, however, it should be noted that saturating concentrations of NE and/or overexpression of Gβγ in chick DRG neurons produce less VD inhibition (as assayed by prepulse-induced facilitation) than that typically reported, for example, in superior cervical ganglion cell somata (Hille, 1994; Ikeda and Dunlap, 1999; Delmas et al., 1999). That is, Gβγ-mediated inhibition of N channels in chick DRG neurons appears to be less sensitive to voltage, even under conditions optimized to produce VD inhibition. Side-by-side comparisons of Gβγ-mediated inhibition of recombinant chick α_{1B} subunit variants with the rnα_{1Bb} variant showed no quantitative differences in tsA-201 cells, however, so the differences in voltage dependence of Gβγ action on native chick and rat neurons may be the result of differences in other accessory proteins expressed by the cells.

Table 1. Pan-species primer sequences for syntaxins 1A and 1B

Primer	Direction	Gene	Sequence
301	Sense	*stx-1A*	AAGAGCATCGAGCAG(AT)(ATC)CATCGAGCA
302	Antisense	*stx-1A/1B*	CTCTGGTACTTGACGGCCTTCTTGG
303	Sense	*stx-1A/1B*	ATGTTCATGGACATGGCCATGCT
304	Antisense	*stx-1B*	GTCATCTGCGAGTCCATTTTGAT
305	Sense	*stx-1B*	CCAAGTTGAAAGC(GC)AT(AC)GAGCA(AG)AGCATT

Results of others demonstrate that the extent of Ca^{2+} current inhibition by G-proteins can be regulated by the type of Ca^{2+} channel β subunit associated with the channel (Roche et al., 1995; Roche and Triestman, 1998; Meir et al., 2000), and we demonstrate here that syntaxin 1A can enhance the ability of Gβγ to produce VD inhibition as well. In addition, Delmas et al. (2000) have identified N channels in somata and dendrites of sympathetic neurons that are differentially sensitive to Gβγ, suggesting that, *in vivo*, the N channel family might exhibit a range of responsiveness to Gβγ.

Given the observation by Jarvis et al. (2000), that syntaxin 1A binds both N channel α_1 subunits and Gβγ, a reasonable interpretation of the results reported here is that syntaxin 1A promotes Gβγ-mediated VD inhibition by increasing the local Gβγ concentration near its binding site or sites on α_{1B} subunits. When syntaxin 1A concentration is low, therefore, VI inhibition predominates, as we see in chick DRG neurons. By contrast, in neurons expressing syntaxin 1A, VD inhibition would be expected to dominate. The targeting, by syntaxin 1A, of Gβγ to its binding sites on Ca^{2+} channels may be the reason why some cells (e.g., rat sympathetic neurons) (1) exhibit prominent basal (or tonic) inhibition and (2) show preferential VD inhibition when stimulated by even very low concentrations of transmitter (Delmas et al., 1999). Thus, the differential expression of such regulatory molecules may promote unique functional states for neurons.

The issue of targeting is an important one and could complicate the interpretation of our results. Molecules applied to the bulk cytoplasm may not have full access to signaling complexes, particularly if such complexes are sequestered in membrane compartments. Because of this, our results do not eliminate the possibility that spatial inaccessibility accounts, in part, for the inability of Gβγ to promote VD inhibition. Incomplete access may also provide an explanation for our observation that activation of G-proteins via intracellular application of GTPγS evokes stronger VD inhibition of N channels than does direct application of Gβγ. That is, should N channels exist in a complex with G-protein heterotrimers, Gβγ released during activation of the heterotrimer may have more ready access to the channel than would Gβγ applied to the cytoplasm. Because Gβγ evokes VD inhibition in chick DRG neurons when syntaxin 1A is expressed, however, a putative spatially restricted signaling compartment must be amenable to alteration by syntaxin 1A.

Our results raise the possibility that variations in syntaxin expression might underlie differences in N channel modulation among cells or as a result of changes in physiological stimuli. Although our RT-PCR results demonstrate that syntaxin 1A expression is absent from chick DRGs at all stages of development and absent from adult chicken brain, rat syntaxin 1A levels in both cerebellum (Veeranna and Pant, 1997) and retina (Dhingra et al., 1997) have been shown to be low at birth and increase substantially during development. Furthermore, after long-term potentiation-inducing stimulation in the hippocampus, syntaxin 1B expression is upregulated, and the ratio of splice variants syntaxin 3A-to-3B is reversed (Helme-Guizon et al., 1998; Rodger et al., 1998). Additionally, a recent study has demonstrated that syntaxin 1A gene expression is controlled by Ca^{2+} influx through α_{1A} (or P-type) Ca^{2+} channels (Sutton et al., 1999). Thus, differential expression of P-type Ca^{2+} channels is likely to lead to differences in syntaxin 1A content in tissues. It is interesting in this regard that embryonic chick DRG neurons do not express P-type channels at the ages studied (Cox and Dunlap, 1992) (AtKisson, unpublished observations), suggesting an explanation for their low syntaxin 1A levels.

Results reported here are the first to suggest that syntaxins 1A and 1B are functionally distinct. These two proteins, products of separate genes (Bennett et al., 1993), are 82% identical over the 288 amino acid residues constituting the full-length proteins, with only 10 residues (scattered throughout the proteins) representing nonconservative differences. It is, thus, not surprising that both serve as substrates for botulinum toxin C1, and competitive interactions (such as those reported here) would be expected. That syntaxin 1B cannot substitute for syntaxin 1A to enhance Gβγ-mediated VD inhibition offers a powerful tool for identifying residues critical for the enhancement of Gβγ action by syntaxin 1A.

Given that syntaxin 1A is an integral component of the secretory apparatus and is thought to interact directly with exocytotic Ca^{2+} channels at sites of transmitter release (Sheng et al., 1998; Catterall, 1999), VD inhibition of Ca^{2+} channels would be predicted to dominate in nerve terminals. This is supported by direct recordings from presynaptic calyces innervating chick ciliary ganglion neurons, where intracellular application of GTPγS evokes VD inhibition of Ca^{2+} currents—an effect abrogated by proteolysis of syntaxin with botulinum toxin C1 (Stanley and Mirotznik, 1997). This latter result is at odds, however, with the findings presented here in that (1) chick nervous tissue expresses syntaxin 1B, but not syntaxin 1A, and the former cannot support VD inhibition of somatic N currents by Gβγ, and (2) even in the absence of syntaxin 1A, GTPγS evokes VD inhibition in chick neurons lacking syntaxin 1A. Although we do not have an explanation for this discrepancy, it is possible that nerve terminal N channels differ from their somatic counterparts in their regulation by Gβγ (Delmas et al., 2000). Our RT-PCR results demonstrate that, in addition to syntaxin 1B, for example, chick nervous tissue expresses syntaxin 3 (data not shown). Because this latter isoform is also a substrate for botulinum toxin C1 (Schiavo et al., 1995), it might play a role similar to that of syntaxin 1A to potentiate VD inhibition in ciliary ganglion calyx terminals. Alternatively, chick ciliary neurons might express an N channel variant different from those we have tested in our studies. It is interesting in this regard that biophysical studies of the calyx N channel demonstrate cur-

rents that inactivate more slowly than their somatic counterparts (Stanley and Goping, 1991; Stanley and Mirotznik, 1997). In addition, the ability of syntaxin 1A to enhance voltage-dependent inactivation of some N channel types (Bezprozvanny et al., 1995; Degtiar et al., 2000; Jarvis and Zamponi, 2001) is not observed for the calyx N channels (Stanley and Mirotznik, 1997), arguing for their uniqueness. That different N channel types might be differentially regulated by Gβγ is an idea that has received support recently. Delmas et al. (2000) identified an N-type Ca^{2+} channel (uniquely targeted to the dendrites of sympathetic neurons) that is more susceptible to Gβγ-mediated VD inhibition than are somatic N channels in the same cells.

In addition to potentiating receptor-mediated inhibition of Ca^{2+} current, syntaxin 1A clearly also plays a role in tonic modulation of Ca^{2+} channels under basal conditions, as demonstrated by Jarvis et al. (2000) and confirmed here. Many Ca^{2+} channels are tonically modulated under basal conditions (Ikeda, 1991; Kasai, 1991). The VD component of such tonic inhibition can be reversed by a conditioning prepulse (Elmslie et al., 1990; Ikeda, 1991) or (under more natural physiological conditions) during action potential trains (Brody et al., 1997; Patil et al., 1998; Park and Dunlap, 1998; Brody and Yue, 2000). In addition, tonic inhibition can be reversed by activation of protein kinase C (PKC) through a mechanism thought to involve PKC-dependent phosphorylation of the Ca^{2+} channel α_1 subunit, thereby preventing the binding of Gβγ (Swartz, 1993; Yang and Tsien, 1993; Hamid et al., 1999; Cooper et al., 2001). Thus, syntaxin 1A and PKC are antagonistic regulators of VD inhibition in mammalian neurons. By contrast, in cells that lack syntaxin 1A (such as the embryonic chick DRG neurons studied here), PKC may be freed to play a different modulatory role. These neurons do not show tonic inhibition under normal circumstances, and it has long been recognized that activators of PKC produce strong VI inhibition of chick N current (Rane et al., 1989; Diversé-Pierluissi et al., 1995). Syntaxin 1A expression levels might, thus, set the ratio of VD-to-VI inhibition differentially among cell types. In addition, given that Ca^{2+} influx plays a number of physiological and/or biochemical roles in neurons, targeting of syntaxin 1A to particular cellular domains might allow differential or region-specific regulation of Ca^{2+} influx.

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