Recently we suggested that direct interactions between voltage-gated K\(^+\) channels and proteins of the exocytotic machinery, such as those observed between the Kv1.1/Kv\(\beta\) channel, syntaxin 1A, and SNAP-25 may be involved in neurotransmitter release. Furthermore, we demonstrated that the direct interaction with syntaxin 1A enhances the fast inactivation of Kv1.1/Kv\(\beta\)1.1 in oocytes. Here we show that G-protein \(\beta\)\(\gamma\) subunits play a crucial role in the enhancement of inactivation by syntaxin 1A. The effect caused by overexpression of syntaxin 1A is eliminated upon knock-down of endogenous syntaxin or concomitantly eliminated in the presence of chelators of syntaxin 1A. Taken together, we suggest a mechanism whereby antisomal and recombinant syntaxin 1A and concomitantly SNAP-25 are reminiscent of the finding that presynaptic N- and L-type voltage-gated Ca\(^{2+}\) channels interact directly with proteins of the exocytotic apparatus in neurons, and that their interaction with syntaxin 1A and SNAP-25 causes feedback effects on the channel function in oocytes (reviewed in Ref. 7) and in synaptosomes (8). Recent studies have shown that disruption of the interaction with syntaxin 1A in neurons has functional implications for transmitter release, reducing the efficacy of both Ca\(^{2+}\)-dependent (7, 9) and Ca\(^{2+}\)-independent (10) release.

Voltage-gated K\(^+\) (Kv)\(^3\) channels participate in a host of cellular processes, from setting the resting membrane potential and shaping action potential wave-form and frequency to controlling synaptic strength (1). Recently, we challenged the commonly accepted concept that presynaptic Kv channels participate in neurotransmitter release simply by virtue of their ability to shape action potentials that invade nerve terminals (2, 3), and suggested that the fine tuning of transmitter release might be attributable to direct interaction between Kv channels and proteins of the exocytotic machinery (4). We demonstrated that the Kv channel composed of the pore forming Kv.1.1 and auxiliary Kv\(\beta\) subunits interact in fresh brain synaptosomes with syntaxin 1A, SNAP-25, and synaptotagmin, and this interaction is relieved following triggering of transmitter release. Furthermore, in insulinoma HIT-T15 \(\beta\) cells the activity of Kv.1.1 channel was inhibited by SNAP-25 (5). Also, we showed, in Xenopus oocytes, that the direct interaction of the Kv1.1/Kv\(\beta\)1.1 (\(\alpha\)\(\beta\)) channel with syntaxin 1A enhances the fast inactivation of the channel (4) that is conferred by the N-terminal part of \(\beta\), in a mechanism termed “ball and chain” inactivation (6). The reciprocal effects of \(\alpha\), syntaxin 1A, and SNAP-25 are reminiscent of the finding that presynaptic N- and L-type voltage-gated Ca\(^{2+}\) channels interact directly with proteins of the exocytotic apparatus in neurons, and that their interaction with syntaxin 1A and SNAP-25 causes feedback effects on the channel function in oocytes (reviewed in Ref. 7) and in synaptosomes (8). Recent studies have shown that disruption of the interaction with syntaxin 1A in neurons has functional implications for transmitter release, reducing the efficacy of both Ca\(^{2+}\)-dependent (7, 9) and Ca\(^{2+}\)-independent (10) release. Previous studies by our group have shown that the extent of inactivation depends on cellular mechanisms leading to phosphorylation (11) and dephosphorylation (12) of Kv.1.1 and on the interaction of the channel with microfilaments (11). Furthermore, we identified G protein \(\beta\)\(\gamma\) subunits (G\(\beta\)\(\gamma\)) as the main regulators of the interaction with microfilaments and consequently as regulators of the extent of inactivation (13).

Here we report that the effect of syntaxin on the inactivation of the \(\alpha\)\(\beta\) channel requires the presence of G\(\beta\)\(\gamma\). Intriguingly, G-protein modulation of Ca\(^{2+}\) currents in nerve terminals was eliminated by cleavage of syntaxin (14). Also, a major part of syntaxin-induced inhibition of N-type Ca\(^{2+}\) channel expressed in HEK cells crucially depends on the presence of G\(\beta\)\(\gamma\), which tonically inhibits the Ca\(^{2+}\) channel (15, 16). Thus, the finding of inter-relationships between syntaxin 1A and G\(\beta\)\(\gamma\) interactions with a Kv channel points to further analogy with the interaction of the N-type Ca\(^{2+}\) channel with these proteins, suggesting a general complex pattern of regulation of the activity of presynaptic voltage-gated channels, which may be related to the fine-tuning of presynaptic activity.

**Experimental Procedures**

**Constructs and Antibodies**—The primary antibodies used were polyclonal Kv.1.1-C terminus (Alamone Labs, Jerusalem, Israel), polyclonal Kv\(\beta\)-C terminus (a gift from Olaf Pongs, Hamburg), polyclonal syntaxin 1A (Alomone), monoclonal anti-HPC-1 (Sigma), Kv.1.1, Kv\(\beta\)1.1, G\(\beta\)\(\gamma\), \(\beta\)-adrenergic receptor kinase (\(\beta\)-ARK), and syntaxin 1A cDNAs and their mRNAs were described (11, 13). DNAs of Kv.1.1 fragments to create GST fusion proteins are described in Ref. 13. GST fusion constructs are described (4, 13). Enzymes were purchased from Roche Molecular Biochemicals, Promega (Madison, WI), or MBI Fermentas.
K⁺-channel Modulation by Syntaxin Requires Gβγ

(Vilnius, Lithuania). The degenerate phosphorothioate antisense oligodeoxynucleotide (AS-ODN) (including 5’ and 3’ end capping of 2- and 4-phosphorothioates, respectively, and a phosphorothioate at every third internal position to enhance resistance) was targeted against a nucleotide sequence 5’-GA/AG/AG/TCT- TCC/AG/AG/AG/ATG/CT/TNGA-3’ (AS-linker; Ref. 4, encoding amino acids ELED/EM/ED), corresponding to amino acids 163–170 in the linker separating helices H2B and H3 of human syntaxin 1A, the most highly conserved domain among syntaxins from different species.

The ODN is expected to hybridize to syntaxins from human, rodent, bovine, chick, aplysia, leech, and sea urchin homologs, as well as to rat syntaxin 3 and 4. The recombinant cytoplasmic part of syntaxin (corresponding to amino acids 4–264) was cleaved by thrombin from its corresponding GST fusion protein (4).

Preparation of Phodducin—C-terminal hexahistidine-tagged wild-type phodducin (phodducin-His₅), was expressed in Echerichia coli strain BL21(DEV)/E.coli as previously described (17). The induced cells were lysed in 50 mM sodium phosphate buffer, pH 7.4, by sonication. The lysate was centrifuged at 19,000 × g for 30 min and the histidine-tagged proteins were purified from the supernatant to >95% homogeneity by chromatography on nickel-nitrioltriacetic acid columns (Qiagen) followed by gel filtration on Superdex 200 column (Amersham Biosciences) in 50 mM Na phosphate buffer, pH 7.4.

The purified protein was aliquoted, frozen in liquid N₂, and stored at −80 °C.

Oocytes/Endocytosis Experiments—Oocytes of Xenopus laevis were prepared as described (18). Oocytes were injected (50 nl/oocyte) with 150–300 ng/µl K₁.1 and 1–3 µg/µl K₁.1 mRNA for biochemical experiments, and with 5–10 ng/µl K₁.1 and 15–100 ng/µl K₁.1 mRNAs for electrophysiological experiments. Gβγ, Gγ̄, and cβARK mRNAs (100 ng/µl each) were injected for both biochemical and electrophysiological experiments, 25 µg/µl syntaxin mRNA was injected for electrophysiological experiments and 25–100 ng/µl was injected for biochemical experiments. Dihydrocyclohexalasin B (DHCB; Sigma) treatment was done as described (11): oocytes were incubated in 40–60 µM of the drug for several hours prior to electrophysiological assay. Stock solution of DHCB was made in ethanol (kept at −20 °C); control solutions always included 40–60 µg ethanol. Two-electrode voltage clamp recordings were performed as described (19). To avoid possible errors introduced by series resistance, only current amplitudes up to 4 µA were recorded. Currents were elicited by stepping up the membrane potential from a holding potential of −80 to +50 mV for 350 ms. Net current was obtained by subtracting the scaled leak current elicited by a voltage step from −80 to −90 mV. Oocytes with a leak current of more than 3 nA/mV were discarded.

In Vivo Oocytes—Oocytes were subjected to immunoprecipitation as described (19). For co-precipitation of Gβγ only, oocyte lysate was incubated for 30 min at 37 °C with the anti-α antibody followed by 30 min incubation with 30–40 µl of protein A-Sepharose. Immunoprecipitates from 1% Triton X-100 homogenates of either oocytes or 20 plasma membranes (separated mechanically, as described in Ivanina et al. (29) were separated by SDS–PAGE and eletrophoretically transferred to membranes using the ECL detection system (Amersham Biosciences) or digitized scans of [35S]methionine/cysteine (Met/Cys)-labeled proteins were derived by PhosphorImager (Amersham Biosciences) and relative intensities were quantitated by ImageQuant (as described in Refs. 4 and 13).

In Vitro Binding of GST Fusion Proteins with Syntaxin 1A—The fusion proteins were synthesized and reacted with syntaxin as described (4, 13). Briefly, purified GST fusion proteins (150 pmol) immobilized on glutathione—Sepharose beads were incubated with either 1–10 µl of lysate containing ³²S-labeled Gβγ or syntaxin 1A (translated on the template of in vitro synthesized RNAs using a translation rabbit reticulocyte lysate kit (Promega) according to the manufacturer’s instructions) or 200 pmol of recombinant syntaxin peptide in 1 ml of phosphate-buffered saline with 0.1% Triton X-100 and 0.5 mg/ml bovine serum albumin, for 1 h at room temperature (except for Fig. 5D, see legend), eluted with 15 mM reduced glutathione in 30 µl of elution buffer (120 mM NaCl, 100 mM Tris—HCl, pH 8), and then subjected to SDS–PAGE.

“Pull-down” of Synaptosomal Proteins—This was done as described (4). GST fusion proteins (150 pmol) immobilized on glutathione—Sepharose beads were incubated with 150–200 µg of rat brain synaptosomes (P2 fraction; Ref. 20), stored in aliquots at −70 °C, and thawed once in HKA buffer (50 mM Hepes-KOH, pH 7.4, 140 mM K-acetate, 1 mM MgCl₂, and 0.1 mM EGTA), with 2% CHAPS or 4% Triton X-100 and a mixture of protease inhibitors (Roche Molecular Biochemicals) at 4 °C for 12 h. Samples were washed four times with HKA containing 0.1% Triton X-100, boiled for 10 min in SDS sample buffer, electrophoresed (12% polyacrylamide gel), and subjected to Western blot analysis using the ECL detection system (Amersham Biosciences). ECL signals were quantified with TINA software (Budapest, Hungary).

Statistical Analysis—Data are presented as mean ± S.E. Student’s t test was used to calculate the statistical significance of differences between two populations.

RESULTS

Functional Interaction of Gβγ and Syntaxin 1A with the K₁.1/K₁.1 Channel Are Similar—Co-expression of α with β in oocytes injected with the corresponding mRNAs results in the formation of a heteromultimeric αβ channel that conveys a rapidly inactivating current with a fast inactivating component (I₁) and a sustained, noninactivating component (Iᵢ) (see Fig. 1A). The extent of inactivation (the inactivating fraction) is defined as I₁/Iᵢ (Iᵢ = peak current). Inactivation of the αβ current, but not the inactivation rate constant, depends on the level of β, which provides the “ball” for inactivation. The extent of inactivation increases up to saturation at I₁/Iᵢ = 0.5–0.8 as the ratio of β-mRNA to α-mRNA injected into oocytes is increased to about 50:1 (depending on the batches of mRNAs and oocytes) (11). As previously shown by our group (4, 13) (Fig. 1A, A and B), co-expression of either GβγGγ (Gβγ) or syntaxin 1A (syntaxin) with the αβ channel (expressed from nonsaturating ratios of β-mRNA to α-mRNA, usually 5–10:1) increases the extent of fast inactivation. Conversely, scavenging of endogenous Gβγ by co-expression of the myristoylated C-terminal fragment of the cβARKI (13) reduces the extent of inactivation. Knock-down of endogenous syntaxin by an AS–ODN (termed AS-linker; Ref. 4), has a similar effect (Fig. 1A and Ref. 4). Further similarity between the functional effects of Gβγ and syntaxin was observed by examining the action of DHCB, a microfilament-disrupting agent previously shown to increase the extent of inactivation (11). As shown in Fig. 1C, the effect of treatment of oocytes with DHCB and the effect of syntaxin were additive when channels were expressed with nonsaturating β-mRNA to α-mRNA ratios (low β to α), whereas upon saturation with β (high β to α), which increased the inactivation, both effects were occluded. These results were similar to those obtained previously for the Gβγ effect (Ref. 13; see also Fig. 1C, inset).

Functional Interaction of Syntaxin with the αβ Channel Requires Gβγ, and Functional Interaction of Gβγ with the αβ Channel Requires Syntaxin—The similarity between the functional interactions of Gβγ and syntaxin with the channel led us to examine the possibility of coupling between the effects of Gβγ and syntaxin. Fig. 1D shows electrophysiological analysis of channels co-expressed with Gβγ, with syntaxin, or with both. The effects of Gβγ and syntaxin on the extent of inactivation are nonadditive (black bars), implying a possible convergence of signaling pathways. Moreover, injection of 30 pg of the antisyntaxin AS linker into oocytes co-expressing the channel with Gβγ abolished the effect of Gβγ on inactivation. These results indicate that endogenous syntaxin is required for generation of the Gβγ effect. We then looked for symmetry by examining whether Gβγ is required for generation of the syntaxin effect. We analyzed the effect of co-expressed syntaxin in the absence or presence of co-expressed cβARK. cβARK abolished the effect of syntaxin on inactivation, indicating that endogenous Gβγ is needed for generation of the syntaxin effect (Fig. 1D).

Interaction of Both Syntaxin and Gβγ with the Functional αβ Channel at the Plasma Membrane Is Required for the Increase in the Extent of Inactivation—Furthermore, we addressed the question whether both Gβγ and syntaxin have to be present at the plasma membrane to jointly regulate the inactivation. We first considered the interaction between the channel and syntaxin. A previous study showed that the effect of co-expressed syntaxin requires the physical interaction of syntaxin with the
functional channel (4). This was achieved by taking advantage of the fact that the N718–963 ("synprint") peptide, a Ca\(^{2+}\)-channel domain (found to block co-immunoprecipitation of native N-type Ca\(^{2+}\) channels with syntaxin (21)) competed efficiently, in an in vitro binding assay, with the binding of syntaxin to the \(\beta\) subunit of the channel. Thus, by microinjection of this peptide into oocytes co-expressing syntaxin and the \(\alpha\beta\) channel, we were able to acutely reverse the effect of syntaxin on the channel. Because the synprint peptide was injected 20–60 min prior to the electrophysiological assay it could not affect significantly \(\alpha\beta\) synthesis or assembly in this time frame so that it must affect properties of plasma membrane channels. As a control we used the shorter N718–859 peptide, which did not compete for syntaxin binding and did not rescue the channel from the effect of syntaxin (Ref. 4; see also Fig. 2, inset). These results indicated that interaction of the channel with exogenous syntaxin, resulting in increased inactivation, occurs at the plasma membrane. In this study we further examined whether interaction of the channel with endogenous syntaxin, which is needed for increasing the extent of inactivation in oocytes co-expressing \(\gamma\delta\), also occurs at the plasma membrane. To this end we tried to rescue the functional channel from the effect of \(\gamma\delta\) by injecting the synprint peptide into oocytes co-expressing \(\gamma\delta\) with the channel. We found that the increased inactivation by \(\gamma\delta\) was indeed acutely reversed by this peptide and not by the control peptide (Fig. 2, left panel). The effect of the synprint peptide in oocytes expressing \(\alpha\beta\) alone was statistically insignificant, however, when compared with the effect of the control peptide that enhanced inactivation, there was a clear reduction in inactivation, as was shown before (Ref. 4, and Fig. 2, right panel).
pressing the channel alone increased the extent of inactivation. Overexpression of at least one of these agents, syntaxin or Gβγ, enhances the extent of inactivation. Overexpression of endogenous syntaxin (like exogenously expressed syntaxin) interacts with the functional channel at the plasma membrane to support the Gβγ effect.

Next, we considered the interaction between the channel and Gβγ. In a previous study (13) we could not determine whether the effect of Gβγ requires interaction of Gβγ with functional channels at the plasma membrane, or if the interaction with the channel is limited to the biosynthesis and assembly phase at the endoplasmic reticulum. In an attempt to answer this question in this study we used phosducin, a cytoplasmic protein characterized by its ability to specifically bind Gβγ subunits with high affinity and to efficiently scavenge Gβγ (22). We were able to show that microinjection of 1 μM (final concentration) recombinant phosducin (17) into oocytes co-expressing Gβγ could acutely reverse the effect of Gβγ on the inactivation, whereas heat-denatured (2 h incubation at 65 °C) phosducin could not (Fig. 3, left panel). This indicated that exogenous Gβγ exerts its effect by interacting with the channel at the plasma membrane. We further showed that phosducin, but not the denatured phosducin, also reverses the increase in extent of inactivation in oocytes co-expressing syntaxin (Fig. 3, right panel), indicating that endogenous Gβγ also exerts its effect by interacting with the channel at the plasma membrane. Injection of either intact or denatured phosducin into oocytes expressing the channel alone increased the extent of inactivation. This effect, probably irrelevant to the ability of the channels to bind Gβγ, was manifest only with oocytes devoid of overexpressed Gβγ and could mask the effect relevant to the ability of phosducin to scavenge the endogenous Gβγ. Overall, these data are consistent with a mechanism in which the concomitant interaction of Gβγ and syntaxin with the functional channel enhances the extent of inactivation. Overexpression of at least one of these agents, syntaxin or Gβγ, is necessary to produce a maximal effect.

Physical Interaction of Syntaxin with the αβ Channel Is Similar to That of Gβγ—The similarity and coupling between the functional effects of syntaxin and Gβγ with the αβ channel led us to compare their physical interactions with the channel. A previous study by our group (4) demonstrated that syntaxin 1A associates with the αβ protein complex in brain synaptosomes and in a ~1:1 stoichiometry with the αβ channel in plasma membranes of oocytes. We specifically characterized the interaction of β with syntaxin and showed that immobilized β—GST fusion protein could pull down syntaxin from synaptosomal lysates and bind recombinant syntaxin. Gβγ binds in vitro not only to β but also to the N terminus of α (13), therefore, we examined the interaction of syntaxin with α. Toward this end three approaches were adopted. First, we performed a pull-down assay, using synaptosomal lysates and immobilized GST fusion proteins corresponding to the major intracellular parts of α. The following fusion proteins were constructed (Fig. 4A): αC, the full-length C terminus of α; αN, the full-length N terminus of α; αT1“A” and αT1“B”, regions of the N terminus of α that participate in tetramerization of Kvα subunits (23–25); the latter also participates in Kvβ subunit binding. Two types of detergents were used, 2% CHAPS or 4% Triton X-100, to control for artifacts because of possible aggregations of proteins. The results of the two experimental conditions were similar. The pulled down syntaxin was visualized using Western blot analysis. Fig. 4B shows one experiment (two left panels) and a summary of values obtained in five experiments (right panel) demonstrating that αN and αT1B but not αC or GST itself pulled down syntaxin. The second approach was an in vitro binding assay using the same immobilized GST fusion proteins and either the recombinant cytoplasmic part of syntaxin (see “Experimental Procedures”) or 35S-labeled full-length syntaxin synthesized in reticulocyte lysate. The results of both settings were similar. Syntaxin interacted with the full-length N terminus of α and no interaction was detected with the full-length C terminus (Fig. 4C, middle panel). More specific analysis of this interaction showed that syntaxin bound to both the T1A and T1B domains, more strongly (by about 1.5-fold) to T1A (Fig. 4C, left and right panels). It thus appears that syntaxin, similarly to Gβγ, interacts in vitro not only with β (4) but also with the N terminus of α. To further compare the binding of Gβγ with that of syntaxin we examined the interaction of Gβγ with the N-terminal domains. The binding pattern was somewhat different from that of syntaxin, as it was hard to detect any preferential binding of Gβγ to one of the domains (Fig. 4C, left and right panels).

Next, we applied a third, in vivo, approach to establish the interaction of the α subunit with syntaxin. In a previous study (4), co-immunoprecipitation experiments with Xenopus oocyte lysates (1% Triton X-100), using anti-α antibody, showed that syntaxin co-precipitates with the αβ channel from lysates of oocytes expressing syntaxin with α and β subunits. In this study, binding of syntaxin to α itself was substantiated by successful co-precipitation of syntaxin with α from lysates of oocytes co-expressing syntaxin with α alone (Fig. 4D). To compare this interaction of syntaxin with that of Gβγ we first had to determine the conditions for obtaining specific co-immunoprecipitation of Gβγ with the channel in oocytes, which we were previously unable to do (13). By incubating oocyte lysate for a short period at 37 °C with the anti-α antibody (see “Experimental Procedures”), we were able to demonstrate Gβγ-immunoreactive bands that were precipitated together with α (or with αβ; not shown) from oocytes co-expressing Gβγ with the channel, but not from oocytes expressing Gβγ alone (Fig. 4E). These results show that Gβ can co-precipitate with the α subunit. Taken together, the in vitro and in vivo findings point to a similarity between the physical interactions of Gβγ and of syntaxin with α.
**FIG. 4.** Syntaxin 1A interacts physically with α. A, schematic presentation of α showing fragments generated as GST fusion proteins. B, pull down of syntaxin 1A from brain synaptosomes by the GST fusion proteins (shown in α). GST fusion proteins immobilized on GSH–agarose beads (each at 150 pmol) were incubated with 2% CHAPS synaptosomal lysate (200 μg) for 12 h at 4 °C. Precipitated proteins were separated by SDS–PAGE (12% polyacrylamide) and immunoblotted (IB) with either anti-syntaxin 1A antibody (IB Syx, upper left panel) or anti-GST antibodies (IB GST, lower left panel). 45 μg of synaptosomes were loaded on the Lysate lane. Normalized relative ECL signal intensities of bound syntaxin (derived from IB Syx) for each of the GST fusion proteins normalized to its relative amount (derived from IB GST) were averaged from five experiments (in one of which we used 4% Triton X-100 instead of CHAPS lysate) (right panel). Numbers on the right refer to the mobility of prestained molecular weight standards. C, in vitro interaction of syntaxin 1A or Gβ1/2 with GST fusion proteins of α fragments. In vitro synthesized 35S-labeled syntaxin 1A or Gβ1/2 were incubated with 200 pmol of the indicated GST fusion proteins immobilized on GSH–agarose beads in a 1-ml 0.1% Triton X-100 reaction volume for 1 h. The glutathione-eluted proteins were analyzed by SDS–PAGE. Upper left and upper middle panels are digitized PhosphorImager scans and the lower left and lower middle panels are scans of Coomassie Blue staining. Left panel shows results from one of three similar experiments. Numbers on the left refer to the mobility of prestained molecular weight standards. D, co-immunoprecipitation (IP) of syntaxin with α from Xenopus oocytes. SDS–PAGE analysis of [35S]Met/Cys-labeled α and syntaxin 1A proteins co-precipitated by anti-α antibody (IPα) from homogenates of plasma membranes of oocytes that were injected with α or syntaxin 1A mRNAs only (α or Syx), or co-injected with syntaxin 1A and α (α + Syx). Arrows indicate the relevant proteins. The results shown are from one of three independent experiments. E, co-immunoprecipitation of α from Xenopus oocytes. Proteins were precipitated by anti-α antibody from homogenates of whole oocytes that were injected with Gβγ mRNA alone (Gβγ) or together with α (α + Gβγ) mRNAs. Protein samples were analyzed on a 5–15% gradient gel. The results shown are from one of three independent experiments. Numbers on the right in D and E refer to the mobility of prestained molecular weight standards.
Binding of Gβγ did enhance the binding of syntaxin by 2.89 ± 0.49-fold (p < 0.004; n = 9; range 1.56–5.7). The enhancing effect of Gβγ was observed when the ratio of the reacting proteins was adjusted so that the initial Gβγ binding was severalfold larger than that of syntaxin (which was set at the limit of the experimental resolution) (see Fig. 6A, left panel for one representative experiment and right panel for a summary of nine experiments). Importantly, under the same conditions we could not detect any enhancement by Gβδ of syntaxin binding to the partial fragment of the N terminus, αT1A (which binds syntaxin; rather, Gβγ reduced the syntaxin binding (Fig. 6A, left panel). In fact, such an apparent reduction in the binding of a protein to a GST-fused peptide was observed also in the presence of another protein that does not bind the peptide (13) and thus is probably an experimental artifact. The enhanced binding of syntaxin to αT was Gβγ dose-dependent and was saturable (Fig. 6B). The enhancement was also dependent on the amount of syntaxin, as only above a certain concentration the enhancement could be detected (Fig. 6C). Analysis of the time dependence of Gβγ and syntaxin bindings revealed that the enhancement of syntaxin binding in the presence of Gβγ followed the binding of Gβγ (Fig. 6D), again indicating that syntaxin binding is dependent on Gβγ binding.

As it was demonstrated that Gβγ binds physiologically to syntaxin (15), the obvious interpretation of the synergistic effect of Gβγ on syntaxin binding would be that syntaxin is simply recruited to the channel by binding physically to Gβγ. However, the fact that in several experiments, while enhancing syntaxin binding, Gβγ binding decreased substantially (e.g. Fig. 6C), together with the finding that the binding patterns of Gβγ and syntaxin to the N terminus were not identical (Fig. 4C), leads us to favor a mechanism in which binding of Gβγ allosterically increases the binding affinity of the N terminus for syntaxin. It is possible that both mechanisms, recruitment of syntaxin via the binary complex syntaxin-Gβγ and allosteric interaction, contribute in concert to the enhancement of syntaxin binding. These experiments support the idea that Gβγ and syntaxin can interact concomitantly with the channel to produce the observed effect on inactivation.

**Discussion**

We reported the existence of a physical interaction in brain synaptosomes between an αβ channel complex and syntaxin 1A (syntaxin), occurring at least partially within the context of a larger macromolecular complex that also contains synaptotagmin and SNAP-25. The interaction was sensitive to the physiological state of the synaptosomes, being relieved upon stimulation of neurotransmitter release (4). We further demonstrated, in Xenopus oocytes, that the direct interaction of the αβ channel with syntaxin affects the fast inactivation of the channel. In this study we focus on another protein, Gβγ, which may exist in a complex with both the channel and syntaxin in synaptosomes, and plays a role in mediating the functional interaction of syntaxin with the channel, leading to enhanced inactivation. A somewhat similar interplay between Gβγ and syntaxin 1A was described for the voltage-dependent inhibition of presynaptic N-type Ca2+ channels (14, 15). Thus, the emerging picture is one in which the activities of presynaptic voltage-dependent ion channels are regulated by proteins that participate in presynaptic neurotransmitter release, possibly with the function of fine-tuning presynaptic activity.

**Modulations by Syntaxin and Gβγ Are Linked**—Different lines of evidence point to a possible coupling between Gβγ-induced and syntaxin-induced modulations of the fast inactivation of the αβ channel in Xenopus oocytes. First, the functional effects of both agents seem similar and are nonadditive
Thus, overexpression of each of them resulted in enhanced inactivation and, conversely, knock down of endogenous syntaxin or sequestration of endogenous free $G\beta\gamma$ resulted in decreased inactivation. Also, the effects exerted both by $G\beta\gamma$ and syntaxin were occluded in the same way by disruption of microfilaments and by saturation with $\beta$ subunits of the channel (Fig. 1C), treatments both known to enhance inactivation. Second, the physical interactions of $G\beta\gamma$ or syntaxin with the channel are similar. Each agent was co-immunoprecipitated from oocyte lysates, both with $\alpha\beta$ or with
α alone and interacted in vitro with both β and the N terminus of α (Fig. 4, C–E) (4, 13).

To prove a link between the two modulations by Gβγ and syntaxin we first showed that the effect of overexpressed syntaxin is absolutely dependent on the level of free endogenous Gβγ (which can be scavenged by cGARK). The converse was also true, i.e. the effect of overexpressed Gβγ was absolutely dependent on the level of endogenous syntaxin (which can be knocked down by syntaxin antisense ODN). These findings indicated that interaction of the channel with both Gβγ and syntaxin is a requirement for the enhanced inactivation. However, this approach did not distinguish between a functional channel that is already in the plasma membrane and a channel that is still in the process of biosynthesis. Therefore, we verified that the enhanced inactivation in oocytes co-expressing Gβγ is abolished by acute administration of the synprint peptide, previously shown (4) to impair the effect of syntaxin (Fig. 2). Symmetrically (Fig. 3), the enhanced inactivation in oocytes co-expressing syntaxin was abolished by the acute administration of phosducin, which also abolished the enhanced inactivation in oocytes co-expressing Gβγ, by binding to and thereby sequestering Gβγ (22, 17). These results strongly suggest that both synprint and phosducin reverse the changes in inactivation by acutely disrupting physical interactions within the ternary complex comprised of the Gβγ–syntaxin–functional channel, implying that the enhanced inactivation is the result of protein–protein interactions at the cell surface. To substantiate this conclusion, we demonstrated that the N terminus of α can concomitantly interact with syntaxin and Gβγ in vitro, to form a ternary complex (Fig. 6). Also, we provided evidence supporting the notion that the channel can simultaneously interact with syntaxin and Gβγ in oocytes (Figs. 5).

Mechanism of Syntaxin/Gβγ Modulation—In the case of syntaxin-mediated Gβγ inhibition of N-type Ca2+ channels, syntaxin optimized the G-protein modulation of the Ca2+ channel, rather than acting as an essential participant. It was therefore suggested that syntaxin mediates a co-localization of Gβγ and the Ca2+ channel by virtue of its ability to bind Gβγ (15). Our electrophysiological results concerning the modulation of the inactivation of a Kv channel show an absolute requirement for both syntaxin and Gβγ. This, together with the in vitro binding results that demonstrate enhanced syntaxin binding to the channel by Gβγ, are consistent with a mechanism in which the concomitant physical interaction of the channel with both syntaxin and Gβγ is mandatory to enhance inactivation.

How do the binding of Gβγ and syntaxin enhance inactivation? Various modulators of the αβ channel (protein kinases, phosphatases, cytoskeleton disrupters, PSD-95-like proteins, syntaxin, Gβγ) alter the extent of inactivation, but not its rate (4, 11–13). Therefore, we suggested that the interaction with each of these agents shifts the equilibrium between two gating modes of the αβ channel, the one inactivating and the other noninactivating, toward the inactivating mode (4, 11, 13, 26). We demonstrated such a shift in the case of phosphorylation-induced enhancement of αβ inactivation on the single-channel level (26). The determined stoichiometry of Kvα1 and Kvβ1 is consistent with the αβ model, where n = 0–4, depending on the relative concentrations of α and β (as opposed to that with Kvβ2 which is αβ only (27)). The inactivating ball particle in these channels can be provided by the N terminus of the β subunit. The Gβγ modulation (13) that is occluded by microfilament disruption and saturation of α with β, suggested to us that in order for the αβ channel to exist in the inactivating mode, one β subunit per tetramer of α subunits is sufficient, as long as the ball is detached from the microfilaments. However, with only one ball per tetramer, chances are that the ball is attached to the microfilaments and that the channel therefore resides mostly in the noninactivating mode. Increasing the β/α ratio increases the probability that at least one ball per channel will be detached from the microfilaments to implement inactivation and shift the channel to the inactivating mode. In support of our theory, compelling evidence that the N terminus of β strongly associates with the microfilaments was presented (28). Furthermore, on the basis of our biochemical analysis of the Gβγ modulation (15) showing enhanced assembly between α and β in the presence of co-expressed Gβγ, we assigned a role for Gβγ, early in biosynthesis, in enhancing the assembly of α tetramers with an increased number of β subunits, thereby shifting the channel to the inactivating mode.

In view of the results of the present study that show the inter-relationship between Gβγ and syntaxin interactions with the channel, this model should now be modified to account for the following facts: (i) concomitant interaction of Gβγ and syn-
taxin with the channel at the plasma membrane is necessary for the functional effect (this study); (ii) concomitant binding of Gβγ and syntaxin to the channel is suggested from co-precipitation and in vitro binding results (this study); (iii) physical association of syntaxin with the channel occurs mainly in plasma membranes of oocytes (as evident from co-immunoprecipitation experiments (4)); (iv) no reliable indication of an enhanced assembly of β with α in oocytes overexpressing syntaxin, in contrast to oocytes overexpressing Gβγ, could be obtained (4). Thus, in the modified model the binding of Gβγ at the endoplasmic reticulum to enhance assembly may be necessary but is insufficient to shift the channel from the nonactivating (NI) to the inactivating mode (I); the subsequent binding of syntaxin at the plasma membrane to the Gβγ-bound channel is essential for the enhanced inactivation to occur. In oocytes overexpressing syntaxin alone, the endogenous Gβγ is probably sufficient to ensure the enhanced inactivation driven by syntaxin. Furthermore, in co-immunoprecipitation experiments, we observed a tendency for overexpressed cβARK, which reduces endogenous Gβγ levels (13), to reduce the basal ratio of β assembled with α by 0.77 ± 0.155 (n = 5) in oocytes injected with a nonsaturating mRNA ratio of β/α. This effect was, however, statistically insignificant (p > 0.14), possibly because the changes in basal assembly of the reduction in endogenous Gβγ levels were close to the resolution limit (data not shown). In any scenario, whether or not it includes Gβγ-induced assembly of β with α (because of the interaction of Gβγ with both the α and β subunits (13)), the data presented here strongly suggest that, to enhance inactivation, both Gβγ and syntaxin are required to be bound to the functional channel. We further suggest, in view of the finding that Gβγ enhances syntaxin binding to the N terminus of α in vitro, that Gβγ, which binds to the channel already in the early stage of biosynthesis (13), facilitates allosterically the binding of syntaxin to the channel at the plasma membrane (Fig. 7). However, we cannot exclude the possibility that syntaxin is recruited to the channel via formation of the binary complex syntaxin-Gβγ (15).

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