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RGS12 Interacts with the SNARE-binding Region of the $Ca_v2.2$ Calcium Channel*[S]

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Activation of $GABA_B$ receptors in chick dorsal root ganglion (DRG) neurons inhibits the $Ca_v2.2$ calcium channel in both a voltage-dependent and voltage-independent manner. The voltage-independent inhibition requires activation of a tyrosine kinase that phosphorylates the α_1 subunit of the channel and thereby recruits RGS12, a member of the "regulator of G protein signaling" (RGS) proteins. Here we report that RGS12 binds to the SNARE-binding or "synprint" region (amino acids 726–985) in loop II-III of the calcium channel α1 subunit. A recombinant protein encompassing the N-terminal PTB domain of RGS12 binds to the synprint region in protein overlay and surface plasmon resonance binding assays; this interaction is dependent on tyrosine phosphorylation and yet is within a sequence that differs from the canonical NPXY motif targeted by other PTB domains. In electrophysiological experiments, microinjection of DRG neurons with synprint-derived peptides containing the tyrosine residue Tyr-804 altered the rate of desensitization of neurotransmitter-mediated inhibition of the $Ca_v2.2$ calcium channel, whereas peptides centered about a second tyrosine residue, Tyr-815, were without effect. RGS12 from a DRG neuron lysate was precipitated using synprint peptides containing phosphorylated Tyr-804. The high degree of conservation of Tyr-804 in the SNARE-binding region of $Ca_v2.1$ and $Ca_v2.2$ calcium channels suggests that this region, in addition to the binding of SNARE proteins, is also important for determining the time course of the modulation of calcium current via tyrosine phosphorylation.

Multiple G protein-mediated signaling pathways are known to modulate $Ca_v2.2$ (N-type) calcium channels (1, 2) via direct G protein-ion channel interactions, activation of second messenger cascades, and activation of tyrosine kinases (3, 4). This modulation of voltage-dependent calcium channels is a transient phenomenon. Upon prolonged exposure to a neurotransmitter, neurons become unresponsive or desensitized. Despite the common requirement for the activation of a G protein-coupled receptor kinase (GRK3) for desensitization of the neurotransmitter-mediated inhibition of calcium current (5), G_i- and G_o-mediated pathways exhibit different rates of desensitization (6) that may result from selective effects of the Gα-directed GTPase-accelerating activity borne by "regulator of G protein signaling" (RGS)[1] proteins (7, 8).

In dorsal root ganglion (DRG) neurons, the activation of γ-aminobutyric acid type B ($GABA_B$) receptors induces both voltage-dependent and voltage-independent inhibition of $Ca_v2.2$ channels (9). Voltage-independent inhibition requires the activation of a tyrosine kinase that phosphorylates the pore-forming α-subunit of the calcium channel (10). The tyrosine-phosphorylated form of the α-subunit becomes a target for the phosphotyrosine binding (PTB) domain of RGS12, a member of the RGS protein superfamily that specifically accelerates the rate of desensitization of this response (10).

To better understand the molecular basis for the RGS12-$Ca_v2.2$ channel interaction and its functional implications, we used recombinant proteins and peptides containing the cytoplasmic regions of the α_1 subunit of the Ca_v 2.2 channel to map the site of interaction with RGS12. Results from protein-protein interaction and electrophysiological experiments indicate that the SNARE-binding region of the channel interacts with RGS12. The SNARE-binding region of calcium channels has been shown to bind the target SNAREs syntaxin and SNAP-25 and the vesicular SNARE synaptotagmin (11). The binding of syntaxin to the channel is important for stabilization of the binding of G$\beta\gamma$ subunits (12). Our results suggest that a tyrosine-based motif within the synprint region (centered about Tyr-804) plays an important role in determining the rate of desensitization of GABA-mediated voltage-independent inhibition.

EXPERIMENTAL PROCEDURES

Materials—Recombinant active Src and the anti-phosphotyrosine 4G10 antibody (1:2000) were obtained from Upstate Biotechnology (Lake Placid, NY). The anti-RGS12 antiserum raised against the RGS box has been described previously by Schiff *et al.* (10). The ProFound pull-down biotinylated protein-protein interaction kit was obtained from Pierce. A pET30 vector (Novagen) was used for the expression of His_6-tagged channel loop constructs. Chemicals were purchased from Sigma. Anti-syntaxin (1:1000) was obtained from StressGen.

Peptides—Sequences of the peptides used in this study were based on the $Ca_v2.2$ α1 sequence from chick DRG neuron (CDB1; GenBank™

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[S] The on-line version of this article (available at http://www.jbc.org) contains a table of sequences for synthetic peptides containing Tyr-804 and/or Phe-815.

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[1] The abbreviations used are: RGS, regulator of G protein signaling; DRG, dorsal root ganglion; GABA, γ-aminobutyric acid; GST, glutathione *S*-transferase; PTB, phosphotyrosine binding; SNARE, soluble *N*-ethylmaleimide-sensitive factor attachment protein receptors; SPR, surface plasmon resonance.

accession number AAD51815). Peptides were synthesized by FastMoc chemistry at the Tufts University Core Facility (Boston, MA) and purified by high pressure liquid chromatography with >97% purity as determined by mass spectrometry. An amino-terminal biotin was included in every peptide. Peptides were first dissolved in 5 mM acetic acid at 1 mg/ml and then diluted into the internal solution for electrophysiological experiments or a HEPES-buffered saline solution for biochemical experiments.

Cell Culture—Embryonic chick sensory neurons were grown in culture as described previously (5). Dorsal root ganglia were dissected from 11–12-day-old chick embryos. Cells used for electrophysiology were plated at a density of ~50,000 cells per collagen-coated 35-mm tissue culture dish and studied for 1–3 days *in vitro*.

Electrophysiology—Whole-cell recordings were performed as described (5). For extracellular application, agents were diluted into standard extracellular saline and applied via a wide bore pipette. The calcium current was corrected for rundown by measuring calcium current as a function of time in control cells without a neurotransmitter. Cells used for experiments exhibited a rundown of the current of <1% per minute.

The external saline contained 133 mM NaCl, 1 mM $CaCl_2$, 0.8 mM $MgCl_2$, 10 mM tetraethylammonium chloride, 25 mM HEPES, 12.5 mM NaOH, 5 mM glucose, and 0.3 μM tetrodotoxin. The pipette internal solution contained 150 mM CsCl, 10 mM HEPES, 5 mM MgATP, and 5 mM bis-(*o*-aminophenoxy)ethane-tetraacetic acid. Pipettes resistances prior to forming high resistance seals ranged from 1–2 megaohms.

Data Analysis—Data were filtered at 3 kHz, acquired at 10–20 kHz, and analyzed using PulseFit (HEKA) and IgorPro (WaveMetrics) on a Macintosh G3 computer. Strong depolarizing conditioning pulses (to 80 mV) that preceded test pulses (to 0 mV) reversed GABA-induced voltage-dependent inhibition without affecting voltage-independent inhibition. Such conditioning pulses had no effect on the control currents recorded in the absence of GABA. During neurotransmitter application, test pulse currents measured before and after the conditioning pulse were subtracted to yield the voltage-dependent component. Test pulses measured following the conditioning pulse were subtracted from control currents (measured in the absence of GABA) to yield the voltage-independent component.

In Vitro Phosphorylation—In vitro phosphorylation of recombinant channel loop proteins was performed using a recombinant active form of Src kinase (Upstate Biotechnology). 10–20 units of recombinant Src per assay were used. The kinase reaction buffer contained 100 mM Tris-HCl, pH 7.2, 125 mM $MgCl_2$, 25 mM $MnCl_2$, 2 mM EGTA, 0.25 mM sodium orthovanadate, 2 mM dithiothreitol, and 100 μM ATP. Reactions were incubated for 40 min at 30 °C and terminated by the addition of Laemmli sample buffer. Proteins were resolved in a 12% SDS acrylamide gel, and tyrosine phosphorylation was detected using immunoblotting with the anti-phosphotyrosine antibody 4G10 (1:1000).

For phosphorylation reactions using [γ-^{32}P]ATP (Amersham Biosciences), 10 μCi per reaction was used. Bands containing the recombinant proteins were excised from the gels and quantified in the scintillation counter using liquid scintillant. Reported values represent the results of three independent assays with each sample performed in duplicate.

Cellular Phosphorylation of the Tyr-804 Peptide—A biotinylated Tyr-804 peptide was synthesized with an N-terminal Antennapedia penetratin motif (Arg-Gln-Ile-Lys-Ile-Trp-Phe-Gln-Asn-Arg-Arg-Met-Lys-Trp-Lys-Lys) that allows the peptide to permeate the membrane. A stock solution of the peptide Antennapedia penetratin-Tyr-804 was incubated alone for 15 min at 37 °C prior to its addition to the DRG medium at a final concentration of 1.4 μg/ml. DRG neurons were treated in the presence of the peptide for 15 min in a humidified, 5% CO_2 incubator at 37 °C prior to exposure to 100 μM baclofen or saline (vehicle control) for 20 s. After agonist treatment, DRG neurons were lysed with ice-cold buffer (phosphate-buffered saline, pH 7.4, containing 250 μM sodium pervanadate, 1% (v/v) Nonidet P-40, 1 mM Pefabloc, 1 mM EDTA, 1 mM EGTA, 10 μg/ml pepstatin, 10 μg/ml leupeptin, 100 μg/ml soybean trypsin inhibitor, and 100 μg/ml calpain I and 100 μg/ml calpain II inhibitors). DRG neuron lysates (25 μl) were then applied onto a SAM^2 biotin capture membrane (Promega) to capture the biotinylated peptide, and tyrosine phosphorylation was detected using immunoblotting with the anti-phosphotyrosine antibody 4G10 (1:500; Upstate Biotechnology).

Immunoblot Quantitation—For quantitation of bands in immunoblots visualized using chemiluminescence, images were obtained using a Molecular Dynamics Personal Densitometer SI. ImageQuant 1.11 (for Macintosh) was used to analyze the data. Values were pooled for all of the experiments, and mean ± S.E. was plotted.

All experiments were repeated 2–5 times with similar results. Representative examples are shown for the biochemical experiments. For electrophysiological experiments, data derived using cells from three independent platings are shown.

Surface Plasmon Resonance (SPR) Biosensor Measurements—SPR binding assays were performed at 25 °C on a BIAcore 3000 (BIAcore Inc., Piscataway, NJ) at the University of North Carolina Department of Pharmacology Protein Core Facility. For the screening of relative binding specificity, N-terminally biotinylated, synthetic peptides were bound to a streptavidin-coated sensor surface (Sensor Chip SA, BIAcore, Uppsala Sweden) per the manufacturer's instructions at a density of 400 resonance units. Binding analyses were performed using HBS-EP buffer (0.01 M HEPES, pH 7.4, 0.15 M NaCl, 3 mM EDTA, and 0.005% surfactant P20) as the running buffer. The GST-rRGS12-(1–440) fusion protein, purified as described previously (20), was diluted in HBS-EP buffer and injected at a 10 μl/min flow rate over four flow cell surfaces simultaneously using the KINJECT command. Surface regeneration was performed by 10-μl injections of 0.05% SDS in HBS-EP buffer at a 20 μl/min flow rate. Background binding to a negative control peptide surface (control) was subtracted from all binding curves using BIAevaluation software version 3.0 (BIAcore Inc.) and plotted using GraphPad Prism version 4.0 (GraphPad Software Inc., San Diego, CA).

For quantitative binding analyses of the phosphotyrosine synprint motif, N-terminally biotinylated Tyr(P)-804/Tyr-815 peptide (biotin-Met-His-Asn-Phe-Arg-Asn-Ser-Cys-Glu-Ala-Leu-Tyr(P)-Asn-Glu-Leu-Asp-Pro-Glu-Glu-Arg-Val-Arg-Tyr-Ala-Thr-Thr-Leu) was bound to a streptavidin-coated sensor surface to a density of 0, 100, and 400 resonance units, respectively, on three independent flow cell surfaces. To eliminate any potential contribution of GST/GST dimerization to kinetic binding measurements, the open reading frame of the pGEX-RGS12-(1–440) construct was recloned by PCR into the hexahistidine-tagged prokaryotic expression vector pProEXHTb (Invitrogen), and the recombinant His_6-RGS12-(1–440) protein was purified from *Escherichia coli* by sequential application of a Ni^{2+}-nitrilotriacetic acid affinity matrix, anion exchange, and size exclusion chromatographic separations using previously described methods (13). Kinetic analyses of His_6-rRGS12-(1–440) binding to the Tyr(P)-804/Tyr-815 peptide were performed using a running buffer containing 0.05 M Tris-HCl, pH 7.5, 0.15 M NaCl, 1 mM dithiothreitol, and 5% (v/v) glycerol. Purified His_6-rRGS12-(1–440) protein was diluted in running buffer at concentrations ranging from 1 to 20 μM, and 50 μl of it was injected at a flow rate of 20 μl/min over four flow cell surfaces simultaneously using the KINJECT command. The sensorgrams were normalized to the signal achieved due to binding the control "blank" flow cell surface (0 resonance units), and the normalized data were globally fit to a 1:1 binding model (bulk refractive index effect = 0) using BIAevaluation software version 3.0. The resultant apparent dissociation constant (K_D) was derived from calculated k_{on} and k_{off} rates and is the result of four independent sets of titrations.

Binding of Endogenous RGS12 to Channel Peptides—Affinity columns were prepared by incubating 100 μg of N-terminally biotinylated synthetic peptides with streptavidin-Sepharose beads per the manufacturer's instructions (Pierce). 2 mg of DRG lysate was loaded onto the biotinylated peptide-avidin column and incubated for 4 h at 4 °C. Negative controls using beads without peptide and a lysate from HEK293T cells were run in parallel. Elution was performed per the manufacturer's instructions (Pierce). Eluate was mixed with Laemmli sample buffer and resolved by electrophoresis on a 7.5% SDS-polyacrylamide gel. Immunodetection of RGS12 was carried out as described previously using an antibody raised against the RGS box (1:1000) (10); syntaxin was detected using anti-syntaxin (1:1000, Stressgen)

RESULTS

RGS12 Binds to the Tyrosine-phosphorylated Synprint Region of Ca_v2.2 Channel—The PTB domain of RGS12 (amino acids 236–440) binds to the tyrosine-phosphorylated form of the Ca_v2.2 channel in a neurotransmitter-induced manner, and this interaction accelerates the desensitization of neurotransmitter-mediated channel inhibition (10). We have previously reported that the SNARE-binding (or synprint) region and the C terminus of the α_1 subunit of the Ca_v2.2 channel can be phosphorylated by Src kinase (14). To determine which region of the α_1 channel subunit binds to RGS12, we performed overlay assays using recombinant proteins encompassing cytoplasmic regions of the Ca_v2.2 α_1 subunit. In these overlay assays, a

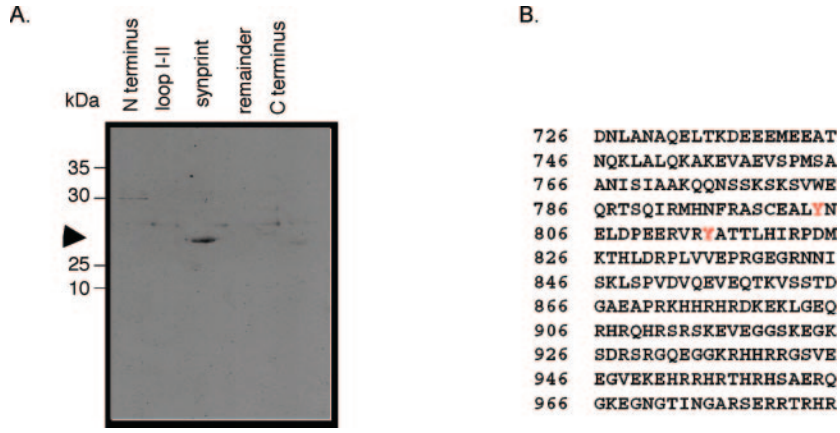

FIG. 1. **The N terminus of RGS12 binds to the synprint region.** *A*, a recombinant RGS12 N terminus binds to the Src-phosphorylated synprint region peptide in a protein overlay assay. Phosphorylated His_6-tagged recombinant proteins were resolved by SDS-PAGE and transferred to a nitrocellulose membrane. Membranes were incubated with 1 μg/ml recombinant GST fusion protein comprising the N-terminal 440 amino acids of RGS12. Data are representative of four experiments. An anti-GST antibody (1:2000) was used to detect binding. *B*, sequence of loop II-III of a chick $Ca_v2.2$ channel. Tyrosine residues Tyr-804 and Tyr-815 are indicated in *red*.

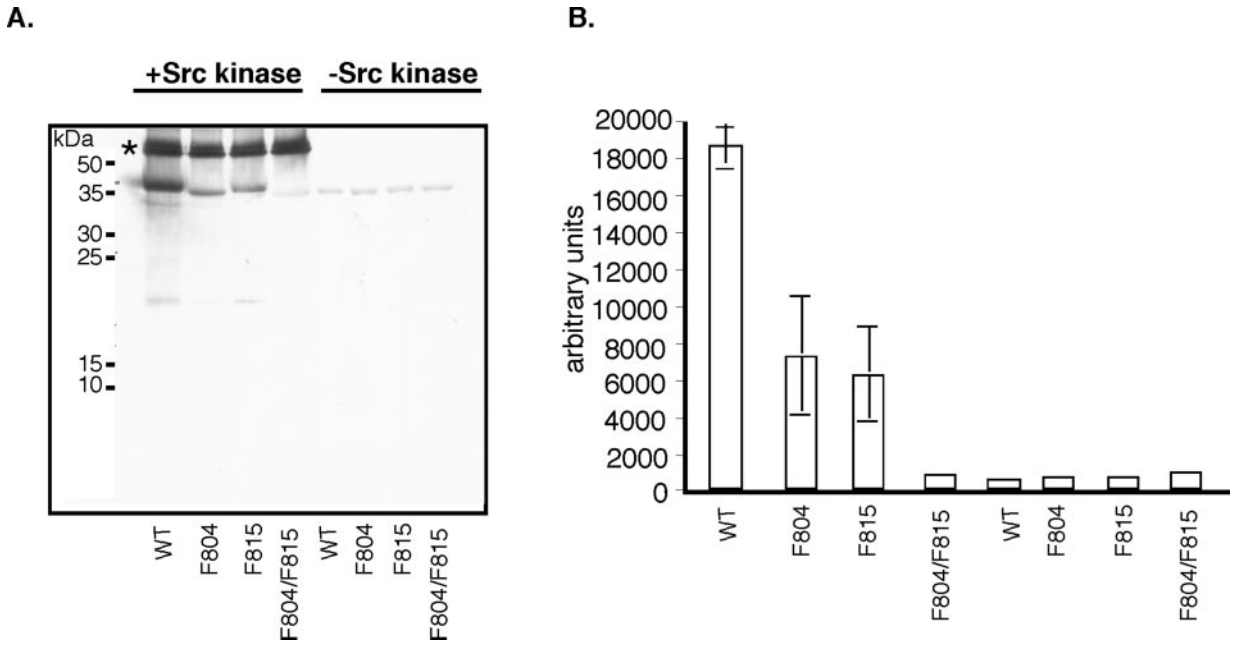

FIG. 2. ***In vitro* phosphorylation of phenylalanine-substituted mutant forms of the synprint region.** *A*, recombinant, GST-tagged channel loop proteins were expressed, phosphorylated by recombinant active Src, resolved by SDS-PAGE, and detected by immunoblotting with a 4G10 anti-phosphotyrosine antibody. *Asterisk* indicates immunoblot detection of input Src kinase. As negative controls, recombinant proteins were incubated in the kinase reaction mixture in the absence of kinase. Experiments were performed four independent times with similar results. *B*, histogram showing quantitation of density of bands. Values represent the mean of four independent experiments ± S.E. *WT*, wild-type; *F804*, Phe-804; *F815*, Phe-815.

recombinant GST fusion protein comprising the first 440 amino acids of rat RGS12 bound to an *in vitro* Src-phosphorylated channel loop encompassing the synprint region (Fig. 1*A*). This binding was dependent on phosphorylation, as no binding was seen without prior Src phosphorylation (data not shown). Despite the fact that the C terminus is heavily phosphorylated (14), no binding to GST-RGS12 was detected for this region of the channel.

The synprint region of the chick $Ca_v2.2$ α_1 subunit contains two tyrosine residues, Tyr-804 and Tyr-815 (Fig. 1*B*). To test which tyrosine residue was phosphorylated, synprint region proteins with tyrosine-to-phenylalanine (Tyr → Phe) mutations were expressed and purified as His_6-tagged fusion proteins and phosphorylated by Src *in vitro* (Fig. 2*A*) (conversion to phenylalanine removes the hydroxyl group on the phenyl ring of the residue side chain required for phosphorylation). Mutation of tyrosine 804 caused a 55% decrease compared with a positive control containing both tyrosine residues (wild-type), whereas mutation of the second tyrosine residue (Tyr-815) decreased phosphorylation by 45% (Fig. 2*B*). *In vitro* phosphorylation assays using [γ-^{32}P]ATP confirmed these observations. In the wild-type recombinant protein the stoichiometry of phosphorylation was 1.9 mol of phosphate/mol peptide, with a 57 ± 9 and 43 ± 10% decrease in phosphate incorporation in Phe-804 and Phe-815 peptides, respectively (n = 3). These results suggest that both Tyr-804 and Tyr-815 can be phosphorylated. Neither tyrosine residue falls within the canonical Asn-Pro-Xaa-Tyr(P) motif commonly recognized by PTB domains (15, 16).

Synprint Peptides Slow the Desensitization Rate of GABA-mediated $Ca_v2.2$ Channel Inhibition—We have shown previously that the endogenous RGS12 protein, and not other RGS family members, controls the timing of desensitization of the GABA-mediated, voltage-independent inhibition of calcium current in embryonic DRG neurons (10). Thus, synthetic peptides containing Tyr-804 and/or Tyr-815 (supplemental table I, available in the on-line version of this paper) were tested in electrophysiological assays to determine their ability to interfere with the rate of desensitization of GABA-mediated inhibition of calcium current. Embryonic chick DRG neurons express only one type of voltage-dependent calcium channel, the $Ca_v2.2$ (N-type, ω-conotoxin GVIA-sensitive) channel (17). Calcium current as a function of time was monitored using whole-cell

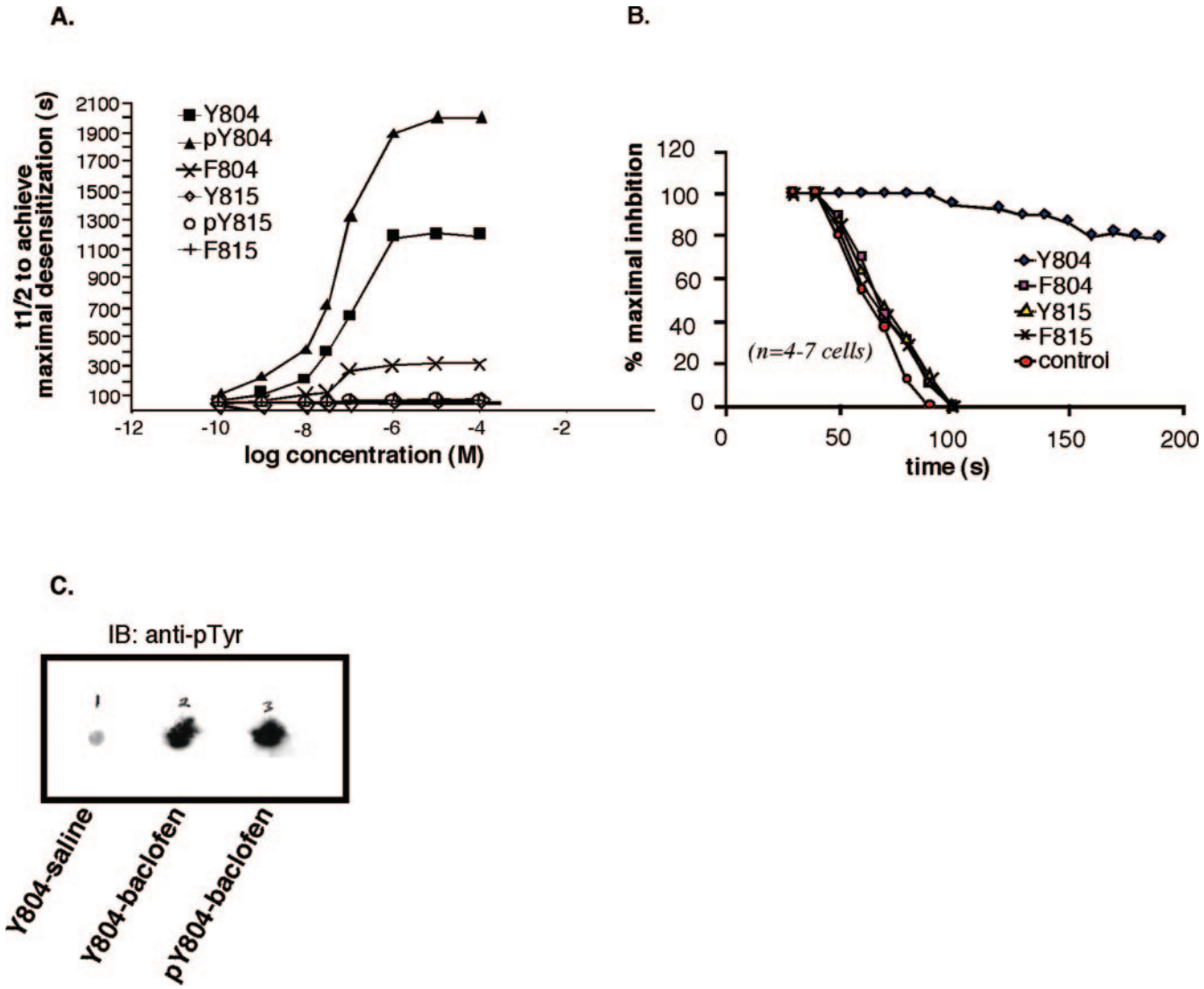

FIG. 3. **Effect of synprint region peptides on the rate of desensitization of GABA-mediated, voltage-independent inhibition of a $Ca_v2.2$ channel in chick DRG neurons.** *A*, concentration dependence of the synprint peptide effect. Inward calcium current was evoked by stepping from −80 to 0 mV for 50 ms. A protocol with +80 mV, a 20-ms prepulse, and a 5-ms interval prior to the test pulse was used to measure the voltage-independent component of the inhibition. Peptides were introduced by passive diffusion through the recording pipette at the concentrations indicated. Peak calcium current as a function of time was measured and plotted. The half-time to achieve maximal desensitization was calculated as a function of peptide concentration. *B*, percentage of inhibition as a function of time. Each peptide listed was introduced at 1 μM into the DRG neurons, and the percentage of GABA-induced voltage-independent inhibition was measured as a function of time. *C*, tyrosine phosphorylation of the Tyr-804-containing peptide inside DRG neurons. Neurons were incubated with the peptide and exposed to saline or baclofen (to activate $GABA_B$ receptors) for 20 s. As a positive control, cells were incubated with Tyr(P)-804 peptide. Immunodetection was performed with an anti-phosphotyrosine antibody (4G10; 1:500). Data are representative of four experiments. *IB*, immunoblot; *Y804*, Tyr-804; *pY804*, Tyr(P)-804; *Y815*, Tyr-815; *pY815*, Tyr(P)-815; *F804*, Phe-804; *F815*, Phe-815.

electrophysiology to determine any agonist-independent effect that the peptides might have on the basal calcium current. Peptide concentrations from 1 nM to1 mM were introduced into the cytosolic environment through the recording pipette. DRG neurons were exposed to 100 μM GABA (in the presence of 100 μM bicuculline to block $GABA_A$ receptors), and both the magnitude and the time course of the GABA-mediated voltage-independent inhibition were determined. Under these experimental conditions, the peptides did not cause a significant change in the magnitude of voltage-independent inhibition; GABA-induced voltage-independent inhibition was 34 ± 8% in control cells compared with 31 ± 9% in the presence of peptide.

At 1 μM, the 16-amino acid Tyr(P)-804-containing peptide (supplemental table I) slowed the rate of desensitization by a factor of 48, whereas the Tyr-804-containing peptide slowed the rate of desensitization by a factor of 23 (peptides Tyr(P)-804 and Tyr-804 respectively; Fig. 3*A*). The mutation of Tyr-804 to phenylalanine severely curtailed this effect on the desensitization rate (peptide Phe-804 (*F804*); Fig. 3). Peptides containing solely the second tyrosine motif (Tyr-815) had no significant effect on the desensitization rate. Effects of Tyr-804-containing peptides were observed at concentrations down to the nanomolar range (1 nM; Fig. 3*A*). In contrast, even at high concentrations the Tyr-815-containing peptides were without effect.

Although our previous data suggested that RGS12 interacts with the calcium channel (10) in a phosphotyrosine-dependent manner, both the Tyr(P)-804- and Tyr-804-containing peptides altered the rate of desensitization of GABA-induced voltage-independent inhibition of calcium current. We therefore tested whether the Tyr-804-containing peptide could be phosphorylated within the cellular environment upon the activation of $GABA_B$ receptors in DRG neurons. To answer this question, a biotinylated and cell-permeant form of the Tyr-804 peptide was introduced into DRG neurons, and then cells were exposed to

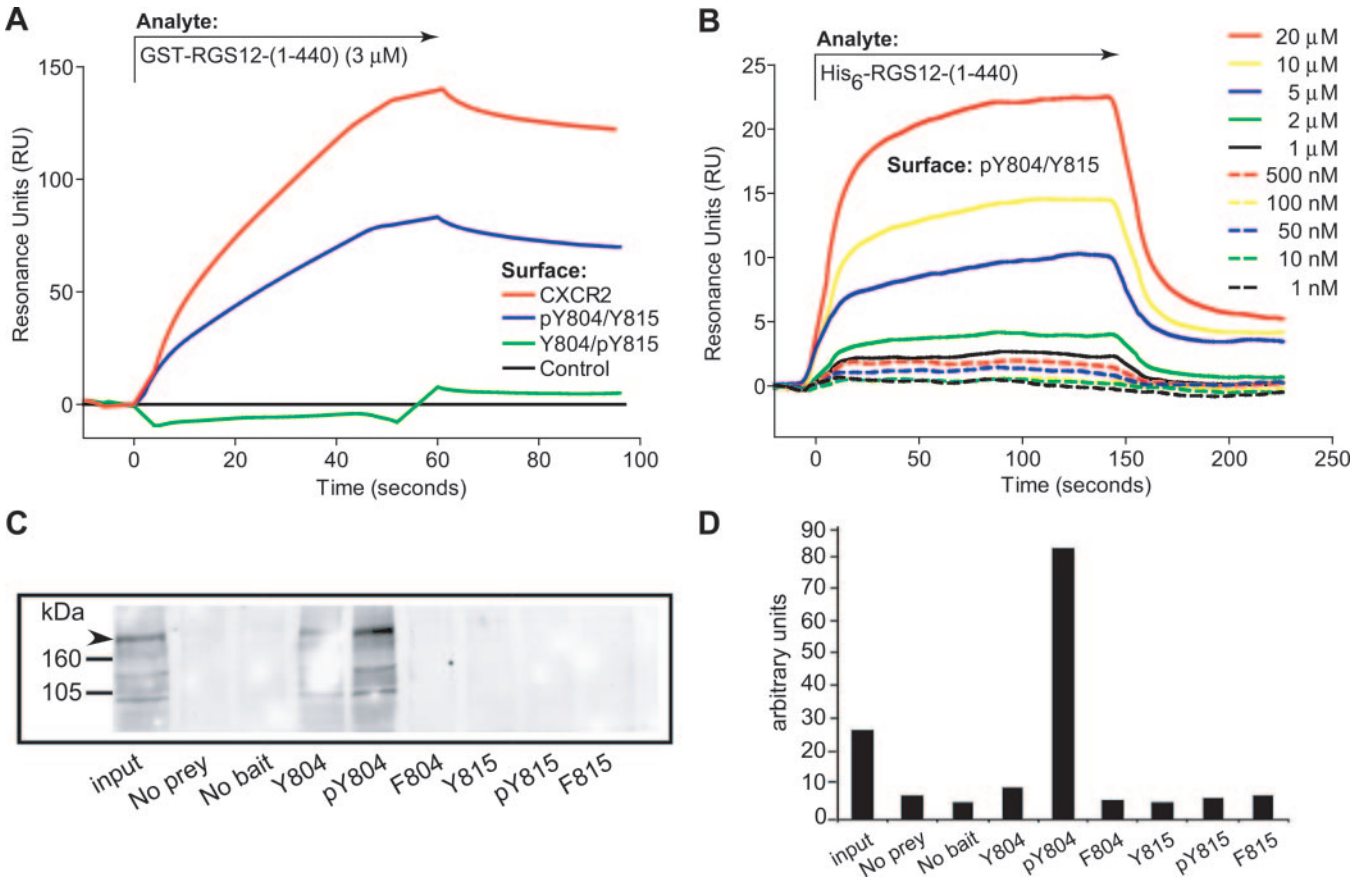

FIG. 4. **Binding of RGS12 to the tyrosine 804 motif in the SNARE-binding (synprint) site of the Ca$_v$2.2 channel.** *A*, specific phosphorylation-dependent binding of GST-RGS12 N terminus (amino acids 1–440) fusion protein to synprint motif peptides from the domain II-III linker of Ca$_v$2.2 as assessed by SPR. Simultaneous SPR measurement of binding to biotinylated peptides from the chick synprint site (Met-His-Asn-Phe-Arg-Asn-Ser-Cys-Glu-Ala-Leu-Tyr(P)-Asn-Glu-Leu-Asp-Pro-Glu-Glu-Arg-Val-Arg-Tyr-Ala-Thr-Thr-Leu (Tyr(P)-804/Tyr-815) or Met-His-Asn-Phe-Arg-Asn-Ser-Cys-Glu-Ala-Leu-Tyr-Asn-Glu-Leu-Asp-Pro-Glu-Glu-Arg-Val-Arg-Tyr(P)-Ala-Thr-Thr-Leu (Tyr-804/Tyr(P)-815) and to the C-terminal peptide from human interleukin-8 receptor B (CXCR2) after injection (time 0 s, flow rate 10 μl/min) of 10 μl of 3 μM GST-RGS12-(1–440) fusion protein at 25 °C. All SPR binding curves were subtracted from the response measured on an irrelevant peptide negative control surface (*Control*). *B*, sample sensorgrams from a titration of a His$_6$-RGS12-(1–440) protein injected over a Tyr(P)-804/Tyr-815 peptide surface. Biotinylated Tyr(P)-804/Tyr-815 peptide was bound to the SPR biosensor surface, and the indicated concentrations of the His$_6$-RGS12-(1–440) recombinant protein (1 nM to 20 μM) were serially injected as described under "Experimental Procedures." The apparent dissociation constant for the interaction between the His$_6$-RGS12-(1–440) protein and the Tyr(P)-804/Tyr-815 peptide was 13.4 ± 0.8 μM, (mean ± S.E. of four independent sets of titrations). *C*, binding of endogenous RGS12 from chick DRG lysates to channel peptides. Biotinylated peptides from the chick synprint site were bound to streptavidin-Sepharose beads and used to affinity-purify chick DRG lysates. Immunodetection of eluates was performed using anti-RGS12 antibody (1:1000). *D*, values plotted in histogram showing quantitation of band density from immunoblots as in part C represent the mean value from three independent experiments. *F804*, Phe-804; *Y804*, Tyr-804; *pY804*, Tyr(P)-804; *Y815*, Tyr-815.

saline or an agonist prior to lysis. Cell lysates containing the biotinylated peptide were spotted onto a streptavidin membrane and detected by immunoblotting with an anti-phosphotyrosine antibody. The Tyr-804 peptide introduced into cells exposed to baclofen exhibited a much higher degree of phosphorylation (Fig. 3*C*). This finding suggests that the Tyr-804 peptide becomes phosphorylated in the cellular environment of the DRG neurons.

In time course electrophysiological experiments, cells containing peptide Tyr-804 did not exhibit significant desensitization after exposure to GABA for 200 s (Fig. 3*B*), whereas cells containing other peptides or control internal solution exhibited complete desensitization by 100 s. Results obtained in experiments with the 27 amino acid peptides spanning Tyr-804 (supplemental table I in the on-line version of this article) were the same as the results obtained with the 16 amino acid peptides spanning Tyr-804 (data not shown). These results suggest that, whereas both Tyr-804 and Tyr-815 can be phosphorylated *in vitro* (Fig. 2), only those peptides containing the Tyr-804-centered motif interfere with the rate of desensitization.

Mapping the Determinants of the RGS12/Synprint Motif Interaction—SPR biosensor measurements were carried out to test which phosphotyrosine residue-containing motif had the potential to mediate association with RGS12 *in vitro*. Initially, the ability of the recombinant GST-RGS12-(1–440) fusion protein to bind biosensor surfaces pre-adsorbed with Tyr(P)-804/Tyr-815 and Tyr-804/Tyr(P)-815 peptides was tested. (Fig. 4*A*). As a positive control, a 20-amino acid biotinylated peptide comprising the C terminus of the interleukin-8 receptor CXCR2, which we have previously shown interacts with the N-terminal RGS12 PDZ domain (18), was used. The recombinant RGS12 N terminus was observed to bind both the control CXCR2 peptide and the Tyr(P)-804/Tyr-815 channel-loop peptide surfaces (Fig. 4*A*), whereas any interaction with the Tyr-804/Tyr(P)-815 peptide was below the detection limit of the SPR biosensor.

Fusion of the RGS12 N terminus with the 26-kDa GST moiety allows for a higher signal-to-noise ratio in SPR measurements, because the biosensor response is directly proportional to the molecular mass of the analyte binding to the biosensor (18). However, as mass transport and GST/GST dimerization artifacts can complicate kinetic determinations (19), independent SPR experiments were performed using low density peptide surfaces and varying concentrations of the

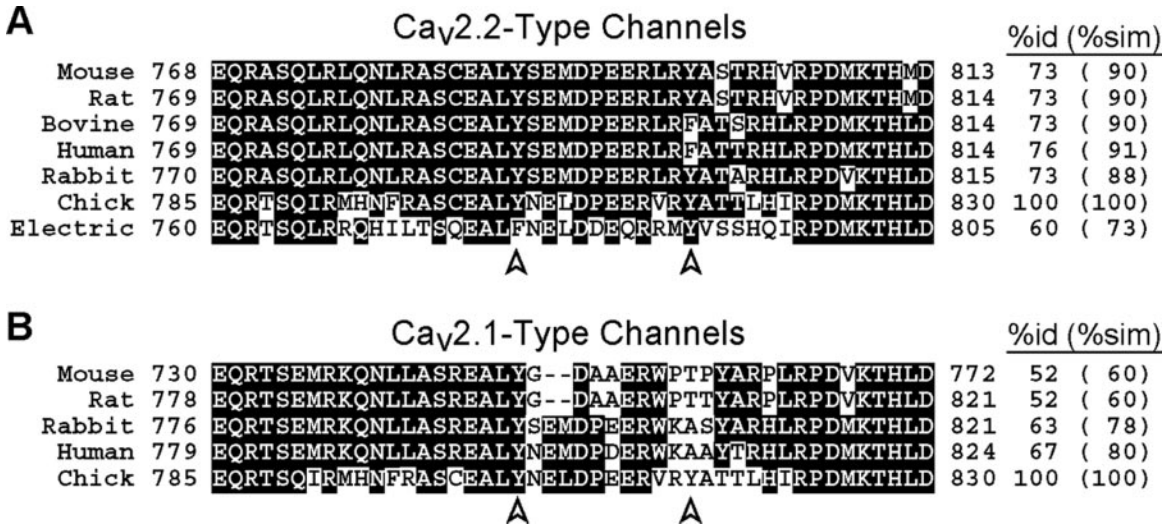

FIG. 5. **Sequence alignment of the RGS12-binding region of the calcium channel.** *A*, sequence alignment of the RGS12-binding region of Ca_v2.2 channels. Sequence alignment was performed using ClustalX. The positions of Tyr-804 and Tyr-815 have been highlighted with *arrowheads*. GenBank™ accession numbers are as follows: mouse (*Mus musculus*), NP_031605; rat (*Rattus norvegicus*), NP_671482; rabbit, Q05152; bovine (*Bos taurus*), NP_777057; and human (*Homo sapiens*), NP_000709. *B*, sequence comparison between the RGS12-binding regions of Ca_v2.2 channel (*bottom sequence*, chick Ca_v2.2) with the same region in Ca_v2.1 channels from different species. Accession numbers are as follows: mouse, AAC52940; rat, A41098; rabbit, P27884; human, AAB61613.

hexahistidine-tagged RGS12 N terminus protein to obtain an apparent dissociation constant (K_D) of 13.4 ± 0.8 μM for the interaction with the Tyr(P)-804/Tyr-815 peptide (see representative dose-response curves in Fig. 4*B*).

We also tested the ability of Tyr-804- and Tyr-815-containing peptides to interact with full-length, endogenous RGS12 by micro-affinity chromatography of chick DRG neuron lysates using these biotinylated peptides bound to streptavidin resin. Although RGS12 binding was detected in eluates from peptide columns containing Tyr-804- and Tyr(P)-804-containing peptide columns, no RGS12 was detected in eluates from peptide columns solely containing Tyr-815- or Tyr(P)-815-centered motifs (Fig. 4*C*).

Collectively, our results suggest that a polypeptide span surrounding Tyr-804 within the Ca_v2.2 channel synprint region is the binding site for RGS12. An alignment of this Ca_v2.2 channel region across different species indicates that this Tyr-804 tyrosine residue has been conserved in multiple species (Fig. 5). The surrounding residues are also conserved. To test whether residues surrounding Tyr-804 contribute to the interaction with RGS12, peptides were designed with single residue substitutions and tested for their ability to bind endogenous RGS12 by streptavidin micro-affinity chromatography of chick DRG neuron lysates. Most of the amino acid substitutions N- and C-terminal to the Tyr-804 position decreased RGS12 binding to the background level seen for the Phe-804 negative control (Fig. 6, *a* and *b*); three of the four positions at which alanine substitutions did not abrogate RGS12 binding (Phe-796, Asn-805, and Leu-807; Fig. 6*a*) are not conserved between chick and mammalian Ca_v2.2 sequences (Fig. 5).

Because the RGS12 binding site is part of the SNARE-binding region of the calcium channel, we tested whether syntaxin could bind to the synprint region peptides. By micro-affinity chromatography, syntaxin was detected in eluates from Tyr-804-containing peptide columns but not detected in eluates from Tyr-815 or Tyr(P)-815 peptide columns (Fig. 6*b* and data not shown). Unlike RGS12, the binding of syntaxin to Tyr-804-containing peptides was not dependent on the phosphorylation status of the tyrosine residue (Tyr-804 (*Y804*) *versus* Tyr(P)-804 (*pY804*); Fig. 6*c*).

To test whether syntaxin and RGS12 compete for the same binding region, Tyr-804 and Tyr(P)-804 peptide columns were pre-incubated with the GST-RGS12-(1–440) fusion protein prior to loading of the cell lysates. Tyr(P)-804 peptide columns bound with recombinant RGS12 showed a decrease in syntaxin binding (Fig. 6*d*). RGS12 was confirmed to be present in eluates from Tyr(P)-804 peptide columns upon anti-GST immunoblotting (Fig. 6*d*). These results raise the possibility that syntaxin and RGS12 may compete for the same binding site *in vivo*.

DISCUSSION

RGS12 has the potential to serve as a signal transduction "nexus" that modulates multiple signaling pathway components by virtue of its multi-domain architecture including N-terminal PDZ and PTB domains, a central RGS-box with $G\alpha_{i/o}$-specific GTPase-accelerating activity, C-terminal Ras-binding domains, and a GoLoco motif (13, 18, 20, 21). Our previous studies of the involvement of RGS12 in modulating presynaptic $GABA_B$-receptor signaling in chick DRG neurons suggest that RGS12, in binding to the calcium channel, modulates channel activity directly in addition to its ability to accelerate Gα-subunit GTPase activity (10). Here we have mapped the region of the calcium channel that interacts with RGS12 to the SNARE-binding or synprint region (amino acids 726–985). Introduction of a peptide that contains Tyr-804 and flanking residues is sufficient to alter the rate of desensitization of the GABA-mediated inhibition of calcium current.

Several proteins are known to bind to the synprint region, an intracytosolic portion of the channel that has been extensively studied as important for the regulation of both channel activity and secretion (22). Binding of syntaxin to this channel region plays a role in voltage-dependent inhibition (23) and is thought to stabilize the binding of G protein $\beta\gamma$ subunits to the channel (24). Synaptotagmin also binds to the channel in this region upon depolarization in a calcium-dependent manner (25). We have shown here that RGS12 and syntaxin are both capable of binding the Tyr-804-based peptide motif; however, the RGS12 interaction appears to be phosphorylation-dependent (see below), whereas the syntaxin interaction occurred irrespective of phosphorylation status. The binding of RGS12 to the phosphorylated synprint region prevents syntaxin binding. We are currently investigating whether the binding of RGS12 to the calcium channel synprint region can displace syntaxin protein from this site and thus reverse the inhibition of the calcium channel.

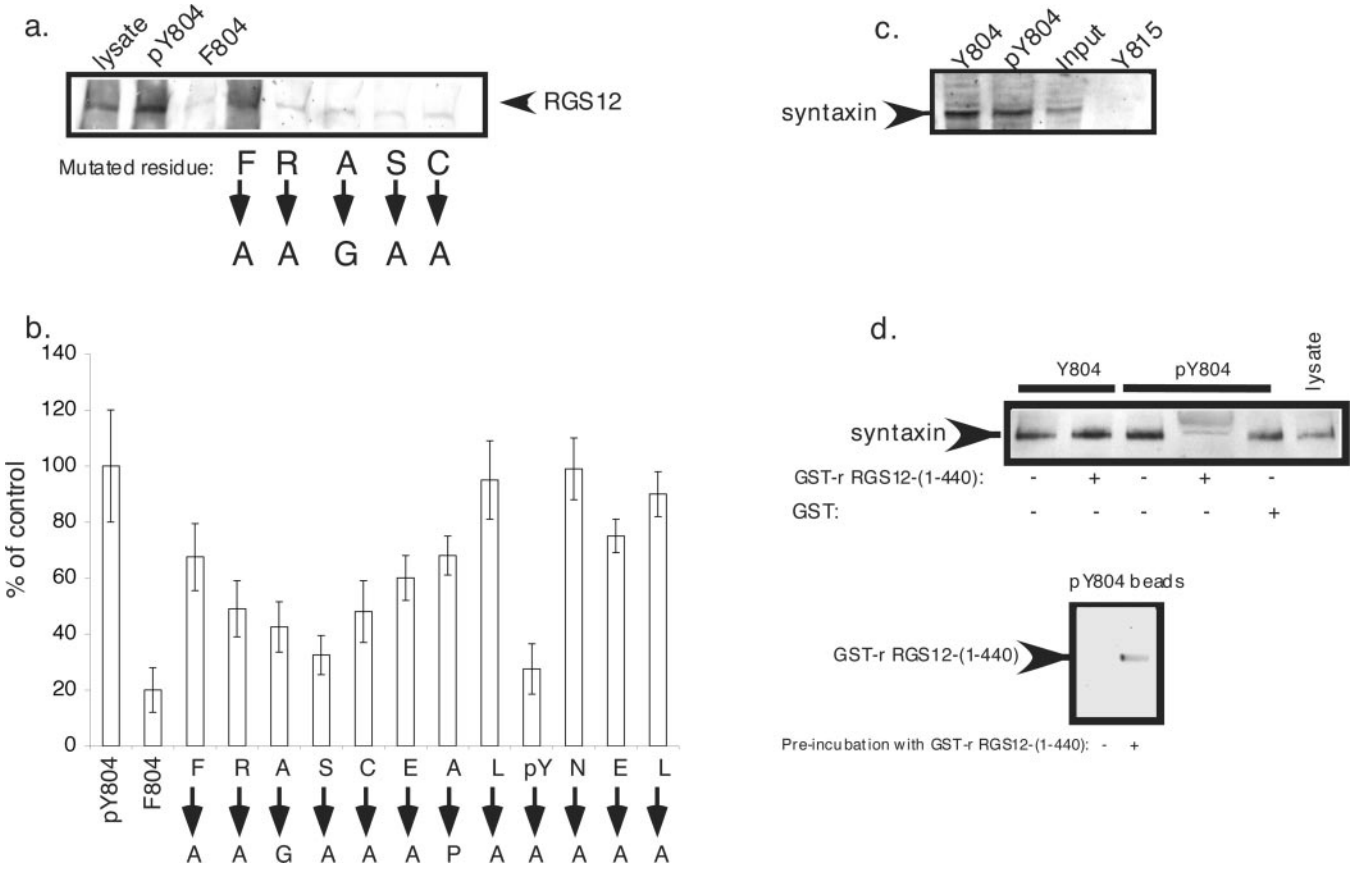

FIG. 6. *a*, binding of endogenous RGS12 from chick DRG lysates to Tyr-804-containing synprint peptides (amino acids 796 to 807) with single amino acid substitutions (as indicated by *arrows*). Experiment was performed as described for Fig. 4*C*. The *lane* labeled *lysate* at the *extreme left* represents total chick DRG lysates. Data are representative of six independent experiments. *b*, histogram shows quantitation of endogenous RGS12 bound to mutant peptides. Density of the RGS12 band was normalized to the density of the RGS12 band from samples bound to wild-type Tyr(P)-804 peptide and expressed as a percentage. *Error bars* represent mean ± S.E. Data are representative of six independent experiments. *c*, binding of syntaxin to the synprint region peptides. Binding of endogenous syntaxin from chick brain lysates to channel peptides was performed by micro-affinity chromatography as described for the RGS12 binding experiments. Immunodetection was performed using an anti-syntaxin antibody (1:1000). Data are representative of five independent experiments. *d*, competition of RGS12 and syntaxin for binding to synprint region peptides. Binding of endogenous syntaxin from chick brain lysates to channel peptides was performed by micro-affinity chromatography as described for RGS12 binding experiments. Peptide columns were pre-incubated with control or GST-RGS12-(1–440)-containing solutions prior to loading the cell lysates. Immunodetection was performed using anti-syntaxin antibody (1:1000) to detect syntaxin or anti-GST (1:3000; Sigma) to detect the GST-RGS12 recombinant (*GST-r*) protein. Data are representative of four independent experiments.

The PTB domain was first identified in Shc (26, 27), a signaling adaptor protein that mediates phosphotyrosine-dependent interactions between growth factor receptor tyrosine kinases and their downstream effectors (28). PTB domains were first assumed to be strictly targeted to phosphotyrosine residues, as the prototypic PTB domains of Shc and IRS-1 were found to recognize phosphotyrosine sites in the context of a conserved sequence motif: Asn-Pro-Xaa-Tyr(P) (29, 30, 31). However, as other PTB domains have been identified and their binding sites characterized, it is clear that a PTB domain can also have non-phosphorylated and non-Asn-Pro-Xaa-Tyr motif-based polypeptide targets (15, 16). Here we have determined that the RGS12 N terminus binds in a phosphotyrosine-dependent manner to the synprint region polypeptide of the sequence Arg-Ala-Ser-Cys-Glu-Ala-Leu-Tyr(P)-Asn-Glu. This sequence, centered about Tyr-804, represents a novel target for PTB domain interactions as it does not possess an Asn-Pro-Xaa-pY motif and, moreover, mutagenesis of this sequence suggests that at least one residue C-terminal of the phosphotyrosine (namely Glu-806) is required for the RGS12 interaction. The interaction between recombinant RGS12-N-terminal fusion proteins and the Tyr-804-centered synprint motif was strictly dependent on tyrosine phosphorylation; however, both electrophysiological measurements and cell lysate chromatography suggested some activity also for the non-phosphorylated Tyr-804-containing peptide. The simplest explanation for this apparent dichotomy is phosphorylation of the synthetic Tyr-804 peptide (*e.g.* Fig. 3*C*) by endogenous tyrosine kinase activity upon its addition to the DRG neurons (and lysates thereof), which is under study.

Future experiments are necessary to determine whether the tyrosine residue involved in the interaction between RGS12 and the Ca_v2.2 channel also plays a role in the onset of channel modulation by a neurotransmitter. Point mutations of residues Tyr-804 and Tyr-815 to phenylalanine in the context of the full-length Ca_v2.2 channel will help to distinguish which residue(s) are important for the onset of neurotransmitter-mediated modulation and whether the phosphorylation of Tyr-804 causes inhibition of calcium influx prior to its interaction with RGS12.

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