G Protein-induced Trafficking of Voltage-dependent Calcium Channels*

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Calcium channels are well known targets for inhibition by G protein-coupled receptors, and multiple forms of inhibition have been described. Here we report a novel mechanism for G protein-mediated modulation of neuronal voltage-dependent calcium channels that involves the destabilization and subsequent removal of calcium channels from the plasma membrane. Imaging experiments in living sensory neurons show that, within seconds of receptor activation, calcium channels are cleared from the membrane and sequestered in clathrin-coated vesicles. Disruption of the L1-CAM-ankyrin B complex with the calcium channel mimics transmitter-induced trafficking of the channels, reduces calcium influx, and decreases exocytosis. Our results suggest that G protein-induced removal of plasma membrane calcium channels is a consequence of disrupting channel-cytoskeleton interactions and might represent a novel mechanism of presynaptic inhibition.

EXPERIMENTAL PROCEDURES

Materials—The following primary antibodies were used in these studies: rabbit anti-pan α1 (1:200, 0.5 μg/ml) (Alomone Labs, Jerusalem, Israel), mouse anti-chick β2 subunit (1:500) (BD Transduction Laboratories), mouse anti-Rab5 (1:2000) (BD Transduction Laboratories), mouse anti-Rab3 (1:2000) (BD Transduction Laboratories), mouse anti-chick N-glycosylated natrium channel (1:1000) (The Jackson Laboratory, Bar Harbor, ME). The fluoresceinated peptide contains the scrambled sequence from the 795–814 peptide and a fluorescein peptide containing the scrambled sequence of N terminus 1–25 used in this study were based on the Ca2,2 α1 sequence from chick dorsal root ganglion (DRG)3 neuron (CDB1, GenBankTM AAD51815). Peptides were synthesized by FastMoc chemistry at the Tufts University Core Facility (Boston, MA) and purified by high performance liquid chromatography with >97% purity as determined by mass spectrometry. An N-terminal biotin was included in every peptide; also, the N terminus was tested.

Activity- and receptor-dependent trafficking of ionotropic receptors has been widely studied in the post-synaptic density (8, 9). Such studies have not been extended to proteins in the presynaptic active zones. In this study we have found that activation of G protein-coupled receptors induces destabilization and subsequent removal of calcium channels from the plasma membrane. Transmitter-induced trafficking of calcium channels is a consequence of disrupting the interaction of the channel with L1-CAM and ankyrin B and might represent a novel mechanism of presynaptic inhibition.

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The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. 1 and 2.

The abbreviations used are: DRG, dorsal root ganglion; GABA, γ-aminobutyric acid; PIPES, 1,4-piperazinediethanesulfonic acid; PBS, phosphate-buffered saline; AP, Antennapedia penetratin; SNARE, soluble N-ethylmaleimide factor attachment protein receptor, H, heavy chain; L, light chain.

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showed that the peptide from loop II-III did not disrupt Gβγ-mediated pathways.

**Cell Culture**—Embryonic chick sensory neurons were grown in culture as previously described (Diversé-Pierluissi et al. 10).

**Electrophysiology**—Whole-cell recordings were performed as described in Diversé-Pierluissi et al. (10). For extracellular application, agents were diluted into standard extracellular saline and applied via wide-bore pipette. For the experiments presented in this report, calcium current has been corrected for rundown by measuring calcium current as a function of time in control cells without transmitter. Cells used for experiments exhibited a rundown of the current of less than 1%/min.

The external saline contained 133 mM NaCl, 1 mM CaCl2, 0.8 mM MgCl2, 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 (BAPTA). Pipettes resistances before forming high resistance seals ranged from 1 to 2 megohms.

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

**Living Neuron Experiments**—Culture dishes were preincubated with 250 nM tetramethylrhodamine-labeled κ-conotoxin GVIA-tetramethylrhodamine conjugate (Calbiochem) to remove unbound fluorophore conjugate. For live neuron experiments, 50 μM Trollox (final concentration) (Calbiochem), a water-soluble, cell-permeable derivative of vitamin E with antioxidant properties, was added to 1 mM Ca2+ external buffer to inhibit photobleaching. Images were taken every 2 s from the top surface of the cells. Specificity of κ-conotoxin GVIA-tetramethylrhodamine conjugate labeling of Ca2.2.2 channels was determined by preincubating cells with unlabeled κ-conotoxin GVIA (750 nM) and κ-conotoxin GVIA-tetramethylrhodamine conjugate (250 nM) for 3 h at 30 °C in a CO2 incubator before confocal imaging.

In living neuron studies, images were scanned using a Zeiss LSM 510 META microscope in an inverted configuration with a pinhole setting of 1.0 using a UV Planapochromat 63× 1.4 NA oil objective at 2-s intervals in one x-y focal plane with the appropriate stage adapter configured for the Delta T controlled culture dish system fitted with a micro-perfusion pump and with the temperature control set at 25 °C (Biotechins Inc., Butler, PA). To obtain optical slices at high speed, regions of interest were used. Fluorescence values as a function of time were measured using Zeiss Physiology Version 3.2 software.

**Transmitter Application**—Transmitters were prepared fresh in Heps-buffered saline Ca2+ external buffer (2.5 mM KCl) at 100 mM concentrations (1000×) and γ-aminobutyric acid (GABA) (Sigma) with and without baclofen (4-amino-3-[4-chlorophenyl] butanoic acid (Sigma)). Transmitter was diluted in the appropriate Heps-buffered saline Ca2+ external buffer immediately before experiments. Cells were washed once with Heps-buffered saline Ca2+ external buffer (2.5 mM KCl) at room temperature followed by the addition of 2 ml of Heps-buffered saline Ca2+ external buffer (60 mM KCl) with or without a final concentration of 100 μM transmitter for 20 s or 5 min at room temperature.

**AP-YF Peptide Treatment**—Peptide Scr (scrambled) and peptide AP-YF were incubated 15 min at 37 °C before addition to DRG medium for a final concentration of 1.4 μg/ml. Cells were treated in the presence of peptide for 30 min in a CO2 incubator at 37 °C before saline or transmitter treatment.

**Immunohistochemistry**— Cultures grown on poly-l-lysine glass coverslips were fixed and permeabilized in methanol at −20 °C for 15 min followed by 3× 5-min washes in PHEM buffer (60 mM PIPES, 25 mM HEPES, 10 mM EGTA, 2 mM MgCl2, pH 6.9). Cells were alternatively fixed with 4% paraformaldehyde in PHEM buffer, pH 6.9, for 20 min, washed 3× 3 min with PHEM buffer, and permeabilized with 0.1% Triton X-100, PHEM buffer for 5 min. Blocking was performed using 5% bovine serum albumin in PHEM buffer for 1 h at 4 °C. Cells were incubated overnight with primary antibody in 1% bovine serum albumin and 1% normal goat serum in PHEM buffer, pH 6.9. After washing with PHEM buffer, coverslips were incubated with fluorophore-conjugated secondary antibodies (1:200; Sigma) in PHEM buffer for 1.5 h at 20 °C. Coverslips were washed four times (5 min each) in PHEM buffer and mounted on glass slides with one drop of Vectashield anti-fade reagent (Vector Laboratories) and anti-fade sealed.

Confocal laser-scanning microscopy was performed at Mount Sinai School of Medicine Microscopy Shared Resource Facility using a Leica TCS-SP (UV) microscope in an inverted configuration. Images of fixed cells were obtained with a pinhole setting of 1.4 NA oil objective lens and an optical zoom between 1.4 and 2× at slow acquisition speed with 4× frame-averaging accumulation. The number of sections was calculated by the Leica TCS software based on acquisition of sections at 240-nm intervals in the z plane. Confocal z stack images were saved as individual z slices as well as average intensity two-dimensional projections.

**Distribution Plots and Analysis**—Images for distribution analysis were acquired using an Olympus BX60 microscope configured for fluorescence imaging and fitted with a cooled color CCD camera and an Optronix DEI-750D CE digital output frame grabber. Images were acquired using a 40× objective where imaging parameters were kept constant and were obtained at comparable focal planes. Additional images were taken with a 100× oil immersion objective for later analysis. Images were taken in successive non-overlapping steps, and 20–30 images were typically acquired for each time point. Images were imported into Adobe Photoshop and normalized with the auto levels adjustment, and cells were scored for the number of puncta present on a grid.

The integrated density of each optical slice was measured, and the total surface and cytoplasmic intensity per pixel were calculated. Membrane and cytoplasmic staining were assessed by integrated density morphometric analysis using ImageJ (National Institutes of Health). We used regions of interest, and for every optical slice the whole area was defined as total fluorescence and the interior of the cell as the cytosolic fluorescence. The membrane fluorescence was defined as the difference of total cytosolic. The average membrane area was 1.5 ± 0.7 μm (n =
The integrated values were obtained by obtaining the fluorescence values as a function of area.

For co-localization of two different proteins of interest, pictures, usually green in one case and red in the other, were merged, and co-localized puncta, which appear yellow, were counted for each cell. For the measurement of the degree of co-localization, the correlation coefficient (Pearson’s coefficients) between the red signal (Cav2.2 channel) and the green signal (Rab5 or clathrin heavy chain) were calculated using the Wright University Co-localization Plug-in for ImageJ (National Institutes of Health). For each experiment random groups of cells were scored for individual puncta and overlapping puncta of two proteins of interest in matched pairs per cell with a minimum of 25 cells scored per experiment and conditions for manual counting and 10 cells per experiment and conditions for automated counting using IP Labs (Scanalytics) image software at the Mount Sinai School of Medicine Microscopy Shared Resource Facility.

IP Labs image software analysis was performed on tiff format confocal images only, utilizing voxel-size parameters obtained from the stored Leica TCS NT data file. The cut-off intensity level was set at 50, and thresholds were maintained across the entire analysis. Each experimental condition was performed in duplicate, and the raw data were pooled. S.E. were calculated for frequency distribution plots using the binomial S.E. (\( \sqrt{(p \cdot q)/n} \)). Determinations of significance (\( p \) values) between the independent matched frequency distributions of saline alone controls and transmitter-treated groups were performed utilizing the \( \chi^2 \) test, with a \( p \) value \( \leq 0.05 \) judged as significant, using publicly available
online software (Interactive Chi-Square) from Ohio State University, Department of Psychology.

**Co-precipitation**—$1 \times 10^6$ DRG cells were used for each condition. DRG neurons were exposed to control solutions containing 100 μM bicuculline or 100 μM GABA in the presence of 100 μM bicuculline. 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, 100 μg/ml calpain I, and 100 μg/ml calpain II inhibitors. The α₁ subunit of the Cav2.2 channel was immunoprecipitated as previously described (11).

**Pull Down**—2 mg of rat brain lysate was incubated with 100 μg of His₆-tagged recombinant protein bound to nickel beads for 4 h at 4°C. The beads were spun down and washed three times. Beads were mixed with 25 μl of Laemmli sample buffer and boiled for 5 min. After spinning down the beads, the supernatant sample was resolved by 7.5% SDS-acrylamide gel. Immunodetection of L1-CAM and ankyrin B was carried out using anti-L1-CAM (1:1000, 8D9 Developmental Studies Hybridoma Bank) and anti-ankyrin B (1:1000, BD Transduction Laboratories).

**Secretion Assay**—Secretion of substance P was analyzed using the single-cell immunoblot method adapted from Huang and Neher (12). Briefly, polyvinylidene difluoride transfer membranes (Immobilon P brand from Millipore) were cut to 22 × 22 mm and placed in 6-well plates. Membranes were pre-wetted with methanol for 20 s, rinsed with distilled water, and allowed to equilibrate in test solution for >1 h. In parallel, DRG cells plated were incubated with media containing AP-YF.
or AP-Scr (1.4 μg/ml) for 1 h. This medium was removed and replaced with 70 μl of test solution. The membranes were then placed on top of the cells and allowed to incubate at 37 °C in a humidified CO2 incubator for 30 min. The membranes were then carefully removed from the cells and allowed to dry completely and fixed with powdered paraformaldehyde at 80 °C for 1 h. Fixed membranes were rinsed in phosphate-buffered saline (PBS; 1 mM KH2PO4, 10 mM Na2HPO4, 137 mM NaCl, 2.7 mM KCl) containing 1% Triton X-100 (PBS-T) to remove excess paraformaldehyde and blocked with PBS-T containing 3% fetal bovine serum for 30 min at room temperature. The membranes were incubated with anti-substance P antibody (Santa Cruz Biotechnologies) at 1:200 dilution in PBS-T with 1% fetal bovine serum overnight in 37 °C incubator. The next day the membranes were developed with a peroxidase/diaminobenzidine reaction using the ABC staining kit from Santa Cruz Biotechnology. Images were taken using a CCD camera and analyzed using Photoshop and National Institutes of Health image software. Data were plotted by assigning an upper and lower limit of gray values and plotting the percent of cells at a range within those limits. By plotting graphs as percent of cells in a dynamic range, multiple experimental runs (n = 4) were pooled.

**Binding of Endogenous Ankyrin B to 795–814 Channel Peptides**—Affinity columns were prepared by incubating 100 μg of N-terminally biotinylated, synthetic 795–814 or scrambled peptides with streptavidin-Sepharose beads as 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. Elution was performed as per the manufacturer’s instructions (Pierce). Eluates were mixed with Laemmli sample buffer and resolved by 7.5% SDS-polyacrylamide gel electrophoresis. Immunodetection of ankyrin B was carried out with anti-ankyrin B antibody (1:1000, BD Transduction Laboratories).

**RESULTS**

**Activation of G Protein-coupled Receptors Induces Removal of Cav2.2 Calcium Channels**—Chick DRG neurons express only one type of calcium channel, CaV2.2 channels (N type) (13). In these neurons, Cav2.2 channels are located both at the terminals and the soma and are coupled to the exocytic machinery (12, 14). To determine whether activation of G protein-coupled receptors alters the distribution of the Cav2.2 channels (N type), we used rhodamine-conjugated ω-conotoxin GVIA to visualize the channels in the surface of live DRG neurons. We found a punctate distribution of channels in the top plane of the membrane which was rapidly (within 2 s) reduced by the application of baclofen, a GABAA receptor agonist (100 μM) and a well-established modulator of these channels (Fig. 1a). The decrease in fluorescence was transient as channel puncta reappeared at the cell surface with some delay (within several minutes) under the continued presence of the transmitter (Fig. 1, a and b). The flow of saline had no effect on channel distribution (Fig. 1a). Experiments in which x-y optical slices were taken before and during agonist application showed a loss of fluorescence signal in the top surface that was accompanied by an increase in fluorescence in the middle optical slice (Fig. 1c). When the neurons are in saline, most of the fluorescence signal is in the top surface or membrane-associated (Fig. 1d). Upon exposure to the agonist, most of the fluorescence is found in the middle optical slices (Fig. 1d). In experiments in which the transmitter was applied for a 20-s interval followed by a washout with saline, the fluorescence signal reappeared to the cell surface within 10 s (see Fig. 5d, control).

Preincubation of DRG neurons with unlabeled toxin prevents the binding of rhodamine-conjugated ω-conotoxin GVIA, demonstrating that the probe binds selectively (Supplemental Fig. 1a). Because ω-conotoxin GVIA blocks the channel pores, these results suggest that the trafficking of calcium channels does not require calcium influx through the CaV2.2 channel.

The time course for removal and reappearance of the channels in the membrane parallels the time course of transmitter-mediated inhibition of calcium current and desensitization of the response, respectively, suggesting that channel trafficking might provide a mechanism to modulate calcium channel activity.

The reduction in surface label was associated with an increase in cytoplasmic label, as shown by imaging of optical slices from top to bottom at 240-nm intervals from fixed DRG neurons (Fig. 2). Calcium channels were detected using an anti-pan α1 antibody that recognizes...
Inhibition of voltage-dependent calcium channels has two biophysical components, voltage-dependent and voltage-independent inhibition (15). The contribution of each component varies according to the cell type and subtype of voltage-dependent calcium channel. Whole-cell patch clamp experiments were performed to correlate the magnitude of agonist-induced internalization of calcium channels with the inhibitory components of the current. In parallel electrophysiological experiments the magnitude of calcium current inhibition by baclofen was 70 ± 10%; 50 ± 8% was voltage-independent and 20 ± 9% was voltage-dependent (Supplemental Fig. 1d). In the imaging experiments we observed a 50% loss of fluorescent signal from the top surface that correlates with the magnitude of the voltage-independent inhibition.

The punctate distribution of fluorescence in the cytosol suggests that Cav2.2 channels are likely to be sequestered into vesicles. To test this idea we determined whether Cav2.2 channels co-distributed with the vesicle-associated proteins Rab5 (an early endosome marker) and clathrin (clathrin-coated vesicle marker). Under saline or control conditions, a low level of co-localization was observed between the calcium channels. Because DRG neurons express only Cav2.2 calcium channels (13), we used a cell-permeant peptide containing the epitope sequence blocks the signal (Supplemental Fig. 1b), demonstrating specificity. Together these results suggest that upon application of agonist, Cav2.2 channels are removed from the plasma membrane and sequestered into clathrin-coated vesicles.

Gi/o Mediates Removal of Cav2.2 Channels—Transmitter-induced inhibition of voltage-dependent calcium channels is mediated through the activation of pertussis toxin-sensitive, heterotrimeric Gi/o proteins (16). To determine whether activation of Gi/o is required for agonist-induced removal of calcium channels from the membrane, DRG neurons were pretreated with 100 ng/ml pertussis toxin for 16 h before exposure to transmitter or saline. Pertussis toxin treatment blocked transmitter-induced removal of Cav2.2 channels (Fig. 4, a and b).

GABA activates separate signaling pathways to inhibit Cav2.2 channels in chick DRG neurons; one that is voltage-independent and involves tyrosine kinase (17) and a second one that mediates voltage-dependent inhibition by direct binding to the channel (2, 3). To test whether Gβγ subunits mediate the trafficking of calcium channels, we used a cell-permeant peptide containing the Gβγ binding region of the β-adrenergic receptor kinase. The N terminus of the peptide contains a biotin group and an Antennapedia penetratin (AP) domain that allows the peptide to permeate the membrane. We have previously used this peptide to prevent Gβγ-mediated inhibition of calcium current in chick DRG neurons (10). Cells were pretreated in medium containing 1 μg/ml peptide for 1 h before the experiment. Control experiments were performed in which the biotinylated peptides were detected with fluorophore-conjugated streptavidin to determine whether the peptide was present in the cell cytoplasm (Supplemental Fig. 2A). Pretreatment with the peptide failed to alter calcium channel trafficking induced by the
GABAB receptor agonist baclofen (Fig. 5, a and b), suggesting that G protein βγ subunits are not mediating this process.

We then determined whether transmitter-induced trafficking of calcium channels is mediated by the signaling pathways associated with voltage-independent inhibition of Ca,2,2 channels induced by kinase activation. Because GABA,

mediated voltage-mediated inhibition of calcium current requires the activation of a tyrosine kinase (17), we tested the effect of tyrosine kinase inhibitors on GABA-induced traf-
ficking of calcium channels. To test this, we pretreated the cells with 100 μM genistein, an inhibitor of tyrosine kinases. x-y optical slices of confocal images from the middle section of the cells show that in cells pretreated with genistein, baclofen failed to induce movement of the channels into the cytoplasm (Fig. 5b). The channels are seen in the edge of the image associated with the plasma membrane in a pattern similar to that of saline-treated neurons (Fig. 5b). In control cells baclofen treatment induced movement of channels into the cytoplasm (Fig. 5b). Daidzein, an inactive analog of genistein, did not alter baclofen-induced channel trafficking in the presence of orthovanadate the magnitude of the response was larger, as measured by the loss of fluorescence from the top surface (Fig. 5d). In addition, recovery after agonist washout was slower compared with control cells. We have previously reported similar effects of orthovanadate on baclofen-mediated inhibition of calcium current (17).

We had previously reported that injection of active Src kinase mimicked GABA<sub>A</sub> receptor voltage-independent inhibition, whereas injection of antibodies raised against Src kinase blocked this inhibitory component (25). Active Src kinase induced calcium channel internalization in the absence of receptor activation (Fig. 5e), further suggesting that tyrosine kinase activation is required for internalization. To test the role of Src kinase on channel internalization, we injected antibodies raised against members of the non-receptor tyrosine kinase family. Injection of antibodies selective for p60Src prevented baclofen-induced calcium channel internalization (Fig. 5f). Antibodies raised against PY2 and Yes were without effect.

These results together with the lack of baclofen-induced peptide containing the Gβγ-binding site of Src kinase, the requirement for G protein activation to obtain these results of the electrophysiological recordings (Supplemental Fig. 1), further suggest that the transmission G protein-coupled receptor activates Src kinase and plays a role in the voltage-independent inhibition of calcium current.

Disruption of L1-CAM-Ankyrin Interaction Induces Removal of Ca<sub>2.2</sub> Channels from the Plasma Membrane—The fluorescent puncta are likely to represent clusters of many calcium channels. Such clusters may require the interaction of the channels with cytoskeletal components. The receptor-induced removal of calcium channels from the membrane raises the possibility that activation of heterotrimeric G proteins causes disruption of cytoskeletal elements that might anchor the channels in the membrane. Although the mechanisms of synaptic targeting of calcium channels have been widely studied (18), the molecular mechanisms involved in the retention of Ca<sub>2.2</sub> channels at the plasma membrane are largely unknown. The best-characterized example of selective retention of a voltage-dependent ion channel is that of the anchoring of Na<sub>channels through indirect interactions with L1 family member neurofascin in neurons (19). Based on what is known about the retention and membrane organization of Na<sub>channels, we tested whether L1-CAM-ankyrin interaction plays a role in the retention of calcium channels in the membrane. We employed a cell-permeant peptide that disrupts L1-CAM-ankyrin B interaction (AP-YF peptide, Ref. 20). This peptide has two domains; they are an Antennapedia penetratin domain that allows the peptide to permeate the membrane and the 12-amino acid ankyrin interaction domain of L1-CAM with the terminal tyrosine residue replaced with a phenylalanine (21). Pretreatment of DRG neurons with AP-YF peptide (1.4 μg/ml, 30 min) induces removal of Ca<sub>2.2</sub> channels in a manner similar to that mediated by G proteins (Fig. 6, a and b). The peptide produces a change in the subcellular distribution of calcium channels from the membrane to the cytosol similar to that induced by baclofen (Fig. 6b). Under these experimental conditions calcium channels are found in the cytoplasm of the neurons in the absence of receptor activation. A scrambled peptide (Scr) in which the L1-CAM domain sequence is reversed had no effect (Fig. 6a).

Under saline conditions there is a significant degree of co-localization between the calcium channels and ankyrin B and L1-CAM (Fig. 6c). After treatment with neurotransmitter, Ca<sub>2.2</sub> channels move to the cytoplasm, whereas L1-CAM and ankyrin are not endocytosed with the channels (Fig. 6c) and stay at the plasma membrane. These results suggest that L1-CAM-ankyrin interaction function to retain Ca<sub>2.2</sub> channels at the plasma membrane.

Consequences of Disruption of L1-CAM-Ankyrin Interaction on Calcium Channel Activity—We tested the effect of disrupting L1-CAM-ankyrin B-containing complexes on whole-cell calcium currents in embryonic chick DRG neurons. Because these neurons only express Ca<sub>2.2</sub> channels, the voltage-dependent calcium current measured under our experimental conditions are carried through these channels. AP-YF or Scr peptide was introduced into the cytoplasmic environment by passive diffusion through the recording pipette, and calcium current was measured as a function of time. AP-YF peptide (1.4 ng/ml) inhibited calcium current by 83% after 2 min of recording (Fig. 7, a and b), whereas Scr peptide had no significant effect.

AP-YF peptide should result in altered calcium-dependent processes such as exocytosis. To examine this, we measured secretion of substance P from chick DRG neurons (12). After the removal of Ca<sub>2.2</sub> channels and their associated function, the peptide induces a decrease in the density of substance P from DRG neurons, whereas cells in low K<sup>+</sup> secrete low levels of substance P (Fig. 7c). Preincubation of neurons with AP-YF peptide blocks high K<sup>+</sup>-induced secretion, decreasing secretion to levels observed in low K<sup>+</sup> (Fig. 7c). Baclofen has an additional effect on AP-YF peptide-induced inhibition of secretion, with 75% of DRG neurons secreting low levels of substance P. The scrambled peptide did not affect secretion significantly. These results demonstrate that under experimental conditions in which we have observed removal of calcium channels, AP-YF peptide causes a robust inhibition of high K<sup>+</sup>-induced secretion of substance P. The results from the secretion assays show that the disruption of the L1-ankyrin B interaction results in a decrease in the K<sup>+</sup>-induced secretion to the levels observed under non-depolarizing conditions. This is consistent with a loss of calcium channels in the plasma membrane; even if the neurons are depolarized, calcium influx is reduced.

Activation of G Protein-coupled Receptors Disrupts the Interaction of Calcium Channels with the L1-CAM-Ankyrin Complex—Because AP-YF peptide mimics G protein-mediated removal of calcium channels from the membrane, we tested whether calcium channels directly associate with L1-CAM and ankyrin B and if so, whether activation of G protein-coupled receptors alters the association. L1-CAM and ankyrin B co-precipitate with Ca<sub>2.2</sub> channel protein from chick DRG neurons treated with saline; when we probed for L1-CAM the main bands were observed in the 200–220-kDa region, in agreement to published reports. When membranes were probed for ankyrin B, bands were observed at around 220 and 150 kDa, in agreement with the predicted size for ankyrin B. Pretreatment with AP-YF but not Scr abolished the
interaction (Fig. 8, a and b). Activation of GABA<sub>B</sub> receptors (Fig. 8, panels a and b; Supplemental Fig. 2) reduced the amount of L1-CAM and ankyrin B that co-precipitated with the calcium channel. These results suggest that activation of G protein-coupled receptors results in disruption of a complex containing L1-CAM, ankyrin B, and Cav2.2. Furthermore, interaction with L1-CAM seems to be necessary for ankyrin B binding to the calcium channel.

**L1-CAM-Ankyrin Complex Interacts with the SNARE Binding or “Synprint” Region of the Calcium Channel**—We further tested for Cav2.2-L1-CAM-ankyrin B interactions by using His<sub>6</sub>-tagged recombinant proteins containing sequences from the C terminus or loop II-III of Cav2.2 channels in pull-down assays for L1-CAM and ankyrin B from rat brain lysate. The loop II-III represents the SNARE binding or synprint region of the channel and has been postulated to play a role in the targeting of calcium channels (22). His<sub>6</sub>-tagged recombinant protein from this region (amino acids 726–984) precipitated both ankyrin B and L1-CAM (Fig. 8, c and d), whereas no binding was detected in samples incubated with the remainder of loop II-III or the C terminus (Fig. 8c). Mutagenesis of the synprint domain protein showed that residues amino acids 795–814 are required for binding to L1-CAM-ankyrin B (Fig. 8, c and d). Preincubation of the rat brain lysate with AP-YF peptide (1.4 μg/ml) abolished binding of the recombinant synprint peptide with
FIGURE 7. a, AP-YF, scrambled peptide (Scr) (1.4 ng/ml), or control internal solution was included in the recording pipette. Inward Ca\(^{2+}\) current was evoked by stepping from –80 to 0 mV for 50 ms. Calcium current as a function of time was measured and plotted. Error bars represent S.E. Values from five cells were used for each experimental condition. b, electrophysiological traces from representative cells are shown. The bottom trace shows Ca\(^{2+}\) current measured at 30 s after achieving whole-cell configuration, and the top trace shows Ca\(^{2+}\) current after equilibration for 2 min with peptide-containing internal solution. c, secretion of substance P was measured from individual DRG neurons using anti-substance P antibodies (1:200, Santa Cruz) and chemiluminescence. Data were analyzed using National Institutes of Health Image. The plot shows the frequency of cells showing a given density value for secreted substance P. Data are representative of four independent experiments.

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Our experiments have revealed a new form of G protein-mediated modulation of Cav2.2 channels that involves their removal from the plasma membrane and trafficking. G protein-coupled receptor-mediated removal of calcium channels parallels the onset of transmitter-mediated inhibition of calcium current. Removal of calcium channels from the membrane can be observed within a few seconds during the time in which we have observed maximal inhibition of calcium current. At 5 min, when the channels can be observed at the cell surface, transmitter-mediated inhibition of calcium current has desensitized (10). Two lines of evidence suggest that agonist-induced internalization of calcium channels might play a role in the voltage-independent inhibitory component: 1) in parallel imaging and electrophysiological experiments performed in sister cultures the magnitude of internalization correlates with the magnitude of the voltage-independent inhibitory component; 2) trafficking of calcium channels is mediated by signal pathways associated with voltage-independent inhibition of calcium channels.

The activation of G protein-coupled receptors destabilizes the channels from the plasma membrane and might disrupt interactions that mediate the retention of Ca$_{\text{v}}$ channel in the membrane. Voltage-dependent sodium channels have been shown to interact with the L1-family member neurofascin and ankyrin G at the axon initial segment and nodes of Ranvier (26). However, this interaction is stable in nature and has not been demonstrated to be altered by receptor activation. Our data suggest that L1-CAM-ankyrin B interaction is required for retention of the calcium channel at the plasma membrane as well but that this interaction is dynamic and regulated (Fig. 9c). The basis for the dynamic nature of the L1-CAM-ankyrin B-calcium channel interaction seems to reside on the modulation of calcium channels by G protein-coupled receptors. The disruption of L1-CAM-ankyrin B interaction by the AP-YF peptide mimics the effects of activation of G protein-coupled receptors. One potential mechanism by which G protein-coupled receptors could disrupt this interaction is by phosphorylation of one of the components of the complex. Previous data from our laboratory have shown that, upon activation of GABA$_{\text{A}}$ receptor, the $\alpha_1$ subunit of the channel becomes tyrosine-phosphorylated; one of the phosphorylation sites is residue Tyr-804 (Fig. 9b). The phosphorylation of the residue Tyr-804 (Fig. 9b).
to be a substrate for both serine-threonine (28) and tyrosine kinases (29). Phosphorylation of L1 family members at the highly conserved FIGQY sequence disrupts their binding to ankyrin (21). We also cannot exclude the involvement of other molecules that could bridge the interaction between L1-CAM-ankyrin B with the calcium channel.

Published reports implicate two different regions of Cav2.2 channels in synaptic targeting. Results from experiments using heterologous expression or overexpression of tagged-channels implicate two different regions of Cav2.2 channels in synaptic targeting. First, deletion of the Cav2.2 channel C terminus PDZ-interacting domain that interacts with Mint-1, a munc-18-interacting protein, prevents targeting of the channel to synaptic terminals (18). A second potential molecular loci for the targeting of voltage-dependent calcium channels was revealed in experiments in which the SNARE binding or synprint region (rat 726–984) from loop II-III was deleted from the \( \alpha_1 \) subunit of Cav2.1 calcium channels. In the absence of this region Cav2.1 calcium channels were inserted in the membrane of the somata (22). The results presented here suggest that interaction of L1-CAM-ankyrin B with the SNARE binding or synprint region of the Cav2.2 channel is needed for retention of these channels in the plasma membrane. Disruption of L1-CAM-ankyrin B

FIGURE 9. a, binding of endogenous ankyrin from chick DRG lysates to the 795–814 peptide. Biotinylated 795–814 or scrambled peptides were bound to streptavidin-Sepharose beads and used to affinity-purify chick DRG lysates. Immunodetection of eluates was performed using anti-ankyrin B (1:500) antibodies. WB, Western blot. Data are representative of three independent experiments. b, interaction of ankyrin B with region 795–814 of calcium channel disrupted by tyrosine phosphorylation. Chick DRG lysate was incubated with a biotinylated peptide containing the 795–814 region of the calcium channel (795–814) or 795–814 region containing phosphorylated tyrosine residue 804 (p795–814). Data are representative of three independent experiments. c, calcium channels exist in association with L1CAM and ankyrin B (left). G protein-coupled receptor (GPCR)-mediated activation of kinases results in phosphorylation of the channel which in turn results in disruption of channel-L1CAM-ankyrin interactions (middle). The disruption of these interactions destabilizes the channel in the membrane, and channels are endocytosed (right).
interaction abolishes association of ankyrin with the calcium channel. One potential interpretation of this observation is that the interaction with L1-CAM stabilizes or facilitates the interaction of ankyrin B with the calcium channel.

Most of the information on the subcellular distribution of voltage-dependent calcium channels comes from developmental studies on the formation of the presynaptic active zones. These studies have shown that voltage-dependent calcium channels are localized to the Bassoon-containing active zone precursor vesicles (30). Our results suggest that transmitters can induce trafficking of calcium channels. This transmitter-induced trafficking is a different process from the compensatory endocytosis described in presynaptic terminals. The G protein-coupled receptors used in our studies inhibit calcium channels and exocytosis, whereas compensatory endocytosis takes place after opposite events, calcium influx and exocytosis, to retrieve the contents of the synaptic vesicles (31). Furthermore, we have observed internalization of syntaxin, whereas this is not the case in compensatory endocytosis (32).

Lateral diffusion in the plane of the membrane has been suggested to play a role in the regulation of receptor function at the cell surface of the postsynaptic density (33). For instance, activity-dependent lateral diffusion of AMPA receptors has been postulated to regulate receptor activity in the postsynaptic density (34). The experiments presented here do not exclude the possibility that movement of calcium channels in the plasma membrane might be sufficient to interfere with calcium channel activity prior to endocytosis.

Removal of calcium channels from the plasma membrane induced by AP-YF results not only in a decrease in calcium current but in the inhibition of calcium-dependent processes such as secretory activity. P. We cannot discard the possibility that L1-CAM receptor action could play a role in anchoring elements of the exocytic machinery. However, because the loss of secretion results in the loss of the synaptic vesicles, the simplest interpretation of this result is that removal of the calcium channels from the plasma membrane results in a change in the number of channels available in the plasma membrane.

Because calcium channels are known to have a change in the number of calcium channels in the plasma membrane could have important physiological consequences. Although these changes in the number of functional calcium channels will have consequences in short-term modulation of synaptic activity, future studies will focus on the long-term physiological consequences of this reorganization. The mechanism described in this paper could be physiologically relevant to CNS neurons such as the synaptic depression induced by the dendritic release of GABACh from bitufted neurons, which activate presynaptic GABAA receptors in pyramidal neurons (35).

Numerous studies have shown activity-dependent reorganization of the postsynaptic density. In the presynaptic active zone, accurate calcium signaling requires the proper spatiotemporal organization of voltage-gated calcium channels and signaling molecules. We cannot predict the possibility that other components of the active zone are sequestered along with the calcium channels, raising the possibility that this is part of a more extensive remodeling of the active zone. Our results demonstrate the dynamic nature of the organization of the active zone and its regulation by the activation of G protein-coupled receptors, a process that has important implications for the modulation of synaptic function.

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