Arrestin Is Required for Agonist-induced Trafficking of Voltage-dependent Calcium Channels

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Many metabotropic receptors in the nervous system act through signaling pathways that result in the inhibition of voltage-dependent calcium channels. Our previous findings showed that activation of seven-transmembrane receptor results in the internalization of calcium channels. This internalization takes place within a few seconds, raising the question of whether the endocytic machinery is in close proximity to the calcium channel to cause such rapid internalization. Here we show that voltage-dependent calcium channels are pre-associated with arrestin, a protein known to play a role in receptor trafficking. Upon GABA<sub>B</sub> receptor activation, receptors are recruited to the arrestin-channel complex and internalized. β-Arrestin 1 selectively binds to the SNARE-binding region of the calcium channel. Peptides containing the arrestin-binding site of the channel disrupt agonist-induced channel internalization. Taken together these data suggest a novel neuronal role for arrestin.

Inhibition of voltage-dependent calcium channels is a prominent target for G protein-mediated modulation (1, 2). Many metabotropic receptors in the nervous system act as synaptic transmission, muscle contraction, and regulation of calcium-dependent physiological processes. Here we demonstrate that β-arrestin 1 is associated with Ca<sub>2.2</sub> channels and that activation of 7TMRs results in the formation of an arrestin-receptor-channel complex. This interaction is required for internalization of calcium channels and plays a role in the modulation of calcium current.

EXPERIMENTAL PROCEDURES

Materials—Secondary antibodies were used in these experiments: donkey anti-rabbit (1:200, 10 μg/ml) (Alomone Laboratories, Jerusalem, Israel), anti-arrestin (1:500, BD Biosciences), rabbit anti-Ca<sub>2.2</sub> (1:500, BD Biosciences), and anti-GABA<sub>B</sub>-arrestin 1 antibodies, and recombinant β-arrestin 1 and β-arrestin 2 antibodies from the Lefkowitz laboratory. The following secondary antibodies were used in our studies: Oregon-3-conjugated goat anti-rabbit IgG (H+L) (1:200, 18 μg/ml), 5-iodo-2′-O-(4-fluorophenyl)-1′,3′-dicyanomethylene-3-phenylindole-dihydrochloride-conjugated goat anti-mouse IgG (H+L) (1:200, 10 μg/ml), and Cy5-goat anti-guinea pig IgG (H+L) (1:200, 7.5 μg/ml) (Jackson Laboratories). Antibodies from the Lefkowitz laboratory were used in experiments shown in Figs. 2a, 5d, and 6f. Calcium channels were detected by indirect immunofluorescence using an anti-pan-α<sub>1</sub> antibody that recognizes 1382–1400 of the rat α<sub>1</sub> subunit from skeletal muscle, a region conserved across all the α<sub>1</sub> subunits of high voltage-activated calcium channels; this has the advantage of not binding the SNARE-binding region.

Peptides—Sequences of the fluoresceinlabeled 894–929 and 920–944 peptides used in this study were based on Ca<sub>2.2</sub> α<sub>1</sub> sequence from chick dorsal root ganglion (DRG) neuron (CDB1, GenBank<sup>TM</sup> 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. The N terminus included the sequence of the penetratin domain of the Drosophila protein Antennapedia. Peptides were dissolved in 5 mM acetic acid at 1 mg/ml and diluted into HEPES-buffered saline (0.01 M HEPES, pH 7.4, and 0.15 M NaCl) for biochemical experiments.

The fluoresceinlabeled peptides that show no significant homology with other proteins was detected as tested by BLAST search. Control experiments were performed and no differences were detected between cells loaded with the peptide and unloaded cells. For each peptide used in our studies we performed time course and concentration-response experiments.

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The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. S1–S5.

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2 The abbreviations used are: TMR, transmembrane receptors; SNARE, soluble NSF attachment protein receptors; PIPES, 1,4-piperazineethanesulfonic acid; GABA<sub>B</sub>, γ-aminobutyric acid, type B; GABAB2R, γ-aminobutyric acid, type B receptor subunit 2; aa, amino acid(s).
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to determine optimal experimental conditions. Pilot studies were conducted in which fluoresceinated peptides were used to assess peptide entry into the cells.

**Cell Culture**—Embryonic chick sensory neurons were grown in culture as previously described (6).

**Transmitter Application**—Agonist was prepared fresh in HBS Ca^{2+} external buffer (2.5 mM KCl) at 100 mM concentration (×1000) (±) baclofen (4-amino-3-(4-chlorophenyl)-butanoic acid) (Sigma). Stock solution was diluted in the appropriate HBS Ca^{2+} external buffer immediately prior to experiments. Cells were washed once with HBS Ca^{2+} external buffer (2.5 mM KCl) at room temperature followed by the addition of 2 ml of HBS Ca^{2+} external buffer (60 mM KCl), with or without a final concentration of 100 μM baclofen for 20 s or 5 min at room temperature.

**Immunohistochemistry**—Cultures grown on poly-L-lysine glass coverslips were fixed and permeabilized in methanol at −20°C for 15 min followed by three 5-min washes in PHEM buffer (60 mM PIPES, 25 mM HEPES, 10 mM EGTA, 2 mM MgCl_{2}, pH 6.9). Blocking was performed using 5% bovine serum albumin in PHEM buffer for 1-h at 4°C, and incubation with primary antibody in 1% bovine serum albumin and 1% normal goat sera in PHEM buffer was performed overnight at 4°C. After washes with PHEM buffer, coverslips were incubated with fluorophore-conjugated secondary antibodies in 1% bovine serum albumin PHEM buffer for 1.5 h at room temperature in the dark. Glass coverslips were washed three times (incubation each) in 1% bovine serum albumin PHEM buffer. Coverslips were mounted on glass slides with one drop of Vectashield anti-fade reagent (Vector Laboratories, Burlingame, CA).

**Confocal Imaging**—Confocal laser scanning microscopy was performed using a Zeiss Meta510 (Carl Zeiss, Inc., Thornwood, NY) equipped with a laser. Images of fixed cells were obtained with a pinhole setting of 1.0 using a UV × 63 1.4 NA oil objective lens at slow acquisition speed with ×4 frame averaging accumulation. The number of sections was calculated by MetaMorph software based on acquisition of sections at 240-nm intervals in the Z-plane. The confocal microscope settings were kept the same for all scans. All morphometric measurements were done using Metamorph image analysis software (Universal Imaging Corporation, West Chester, PA). Neurons were selected and carefully manually traced for maximum accuracy. The average intensity of fluorescence signal was measured in the traced regions and background staining (determined over neuron-free areas of the culture) was subtracted. Intensity measurements are expressed in arbitrary units of fluorescence per square area.

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 Metamorph. 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 integrated values were determined by measuring the fluorescence values as a function of area.

The plasma membrane was stained with 1 μM FM4-64X, a form of the FM4-64 dye that can be used in fixed cells (Invitrogen). Line scans of intensity profiles across the cells were generated with Metamorph (Universal Imaging). We measured the fluorescence intensity over a distance covering the membrane and the cytosol. Three line profiles, avoiding the nucleus, were performed to obtain an average profile of fluorescence intensity for each cell.

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 coefficient) between the two different signals was calculated using MetaMorph. 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. Imaging analysis was performed in a double-blind manner. Cells were scored by one person and analyzed in a randomized manner.

Statistical analyses were conducted using Student’s t-tests, or one-way analysis of variance as appropriate. Statistical differences were determined using one-way analysis of variance as appropriate. Statistical differences were considered significant.

Specificity of biotinylated ω-conotoxin GVIA (750 nM) and biotinylated ω-conotoxin GVIA-Quantum dot 655-streptavidin conjugate labeling of Ca_{2.2} channels was determined by preincubating cells with unlabeled ω-conotoxin GVIA (750 nM) and biotinylated ω-conotoxin GVIA-Quantum dot 655-streptavidin conjugate (120 nM) for 3 h at 30°C in a CO_{2} incubator prior to confocal imaging. Regions of interest were scanned at high acquisition speed at 2-s intervals in one X-Y focal plane with the appropriate stage head configured for the Bioptechs glass bottom culture dishes fitted with a perfusion pump.

For analysis, regions of interest were selected and carefully manually traced for maximum accuracy. The average intensity of fluorescence signal in the traced regions was measured using Physiology version 3.2 software (Zeiss) and background staining (determined over neuron-free areas of the culture) was subtracted. Intensity measurements are expressed in arbitrary units of fluorescence per square area.

**Antibody Microinjection**—Antibodies raised against β-arrestin 1 and β-arrestin 2 were microinjected into the cell body using an automated injector (Eppendorf 5246). Fluoresceinated dextran (Molecular Probes, Eugene, OR) was co-injected with...
the antibodies to allow subsequent identification using epifluorescence optics. Control experiments were performed by injecting vehicle, preimmune serum or rabbit IgG-containing internal solution. Confocal microscopy was performed 30 min after injection.

**Electrophysiology**—Whole cell recordings were performed as described in Ref. 6. For extracellular application, agents were diluted into standard extracellular saline and applied via a 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.

**Co-precipitation**—1 × 10⁶ DRG cells were used for each condition. DRG neurons were exposed to control solution or control solution containing 100 μM baclofen. 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 EDTA, 1 mM EGTA, 10 μg/ml pepstatin, 10 μg/ml leupeptin, and 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 (9).

**Pulldown**—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. Beads were mixed with 25 μl of Laemmli sample buffer and boiled for 5 min. After spinning down the sample the supernatant sample was resolved by 7.5% SDS-PAGE and the gel transferred to nitrocellulose. The antibodies to allow subsequent identification using epifluorescence optics.

**RESULTS**

**Pre-association of the Calcium Channel with Arrestin**—Imaging experiments in live embryonic chick DRG neurons have shown that, within seconds of receptor activation, calcium channels are cleared from the membrane and sequestered in clathrin-coated vesicles (8). The fast kinetics of internalization of calcium channels raises the question of whether components of the exocytic machinery exist in close proximity or pre-associated with the calcium channel. Whereas there is a high degree of co-localization between calcium channels and the endosomal markers Rab5 and clathrin heavy chain in cells exposed to agonist, there is a very low level of co-localization prior to receptor activation (8).

By analogy to 7TMR trafficking, we sought to determine whether arrestin associates with internalized calcium channels. Both β-arrestin 1 and 2 are expressed in DRG neurons as determined in immunoblotting experiments (Fig. 1a). We next determined whether calcium channels were spatially distributed in close proximity to arrestin. In the presence of saline, most of the calcium channels are localized in the plasma membrane as the channels co-localize with the plasma membrane marker FM4-64 (Fig. 1b). Optical slices from saline-treated neurons show that both calcium channels and arrestin co-localize as indicated by the yellow signal indicating overlap of green fluorescent signal (Cav₂.2 channels) and red fluorescent signal (arrestin). Both proteins are associated with the top slices; in the middle slices the fluorescence signal forms a ring around the periphery of the cell suggesting association with the membrane (Fig. 1, c and e, and supplementary materials Fig. S1). Upon exposure to agonist for 20 s, the fluorescence signal becomes more intense in the middle slices and a decrease is observed in the levels of membrane-associated arrestin and Cav₂.2 channel (Fig. 1d and e, and supplementary materials Fig. S2). The Pearson correlation coefficient between calcium channels and arrestin is 0.73 ± 0.09 in saline-treated cells and 0.8 ± 0.06 in baclofen-treated cells (n = 25). Together these results suggest that arrestin and Cav₂.2 channels are preassociated in the cell surface and are internalized upon 7TMR activation.

Arrestin co-precipitates with Cav₂.2 channel protein from chick DRG neurons exposed to saline or baclofen (Fig. 2a) giving further indication that arrestin is pre-associated with the Cav₂.2 channel upon agonist-independent internalization. A rabbit IgG containing anti-β-arrestin antibody to pre-association of Cav₂.2 channels and arrestin (9, 25). Arrestin co-precipitates with the bovine thyrochonic chick DRG neuron Cav₂.2 channel/Arrestin Interaction

**Ca₄₂.₂ Channel-Arrestin-Receptor Complexes Are Formed during Calcium Channel Internalization**—Because arrestin is known to interact with phosphorylated 7TMRs (10, 11), we determined whether arrestin and the Cav₂.2 channel are part of a complex that contains the receptor. Indirect immunofluorescence using confocal laser microscopy was used to visualize the GABAB₁ subunit of the GABA₁ receptor (blue signal), Cav₂.2 channel (green signal), and arrestin (red signal). X-Y optical slices were taken from the top to bottom of DRG neurons treated with saline or baclofen. Very low co-localization was detected between the receptor and arrestin or Cav₂.2 channel in saline-treated neurons (Fig. 3a and supplementary materials Fig. S3). The Cav₂.2 channel-arrestin correlation coefficient is 0.70; the other values for co-localization are below 0.10 (n = 20, Fig. 3e).

Upon a 20-s exposure to baclofen, DRG neurons exhibited an increase in the degree of co-localization between Cav₂.2 channels and arrestin, and receptors as indicated by the white fluorescence signal (Fig. 3, b and e, and supplementary materials Fig. S4). Most of the co-localization takes place in the middle slices suggesting that the signal is cytoplasmic and that these proteins are internalized together. The co-localization of the Cav₂.2
**FIGURE 1.** Ca$_{2.2}$ channels co-localize with β-arrestin. 

- **a.** Immunodetection of β-arrestin in DRG neurons. 100 μg of embryonic chick DRG lysate was resolved by 4–20% acrylamide mini-gel SDS-PAGE and proteins were transferred to membrane and probed for β-arrestin 1 and β-arrestin 2 (1:1000). Data are representative of four independent experiments.

- **b.** Immunodetection of calcium channels at the plasma membrane. The plasma membrane was stained with 1 μM FM4-64X (red signal, second panel) and calcium channels were detected by indirect immunofluorescence using anti-pan-Cav1 antibody followed by Oregon Green-conjugated anti-rabbit IgG (green signal, third panel). Right panel shows a merged image (yellow signal). X-Y optical slices were acquired at 0.2-μm intervals from the top to bottom of each cell. A middle optical slice is shown. Scale bar represents 10 μm. Data are representative of three independent experiments.

- **c** and **d.** Calcium channels and β-arrestin were detected by indirect immunofluorescence using anti-pan-Cav1 antibody followed by Cy3 anti-mouse IgG, respectively. A series of merged images (Ca$_{2.2}$ channel/arrestin) of X-Y optical slices acquired at 0.2-μm intervals from the top to bottom of each cell is shown. Cells were treated with saline (c) or 100 μM baclofen (d) for 20 s. Scale bar represents 10 μm. Data are representative of seven independent experiments.

- **e.** Histogram showing integrated fluorescence values for the merged signal for the calcium channel and arrestin associated in the membrane (M) and cytoplasm (C). Error bars represent mean ± S.E. and analysis between independent matched groups (matched by saline alone or agonist) was significant at: *, p < 0.001 and **, p < 0.0001.
channel-arrestin-receptor is transient, as the analysis of neurons exposed to agonist for 1 or 5 min revealed a very low degree of co-localization (Fig. 3, c, d, and e). Our results show that co-localization of the receptor with arrestin and the calcium channel is highest at 20 s, a time point in which inhibition of calcium channels by 7TMRs is still at its maximum (12). The degree of co-localization between the proteins is lower at 1 and 5 min, which parallels the desensitization of transmitter-mediated inhibition of calcium current.

Activation of GABA_{\alpha} receptors increases the amount of receptor that co-precipitates with Ca_{\alpha2.2} channel (Fig. 4). When the presence of the GABABR2 was probed by immunoblotting of calcium channel precipitates a band in the 130-kDa region was observed in agreement to published reports (13). Exposure of neurons to agonist resulted in a 4-fold increase in the amount of receptor detected in the immunoprecipitates (Fig. 4). When membranes were probed for arrestin, no change in the amount of arrestin associated with the channel was observed, which is in agreement with the results shown in Fig. 2a. The biochemical and imaging data suggest that receptor-arrestin-channel complexes are formed upon 7TMR activation and that these complexes are internalized.

Mapping of Arrestin-Ca_{\alpha2.2} Channel Interaction—The Ca_{\alpha2.2} channel/arrestin interaction was further supported by
results from experiments in which His<sub>6</sub>-tagged recombinant proteins containing sequences from the cytoplasmic regions of the α<sub>1</sub> subunit of Ca<sub>2.2</sub> channels were used in arrestin pull-down assays from rat brain lysate. Recombinant protein containing the SNARE-binding or synprint region from this loop II–III region (amino acids (aa) 726–984) bound to arrestin (Fig. 5a), whereas no binding was detected in samples incubated with the remainder of loop II–III or the C terminus. Truncation of the synprint domain protein showed that aa 894–944 are required for binding to arrestin (Fig. 5b).
Recombinant β-arrestin 1 binds to recombinant protein containing the sequence of the synprint region of the Cav2.2 channel suggesting a direct interaction between the two proteins (Fig. 5c). This interaction is selective for β-arrestin 1, as no binding to the synprint region of the calcium channels was detected when recombinant β-arrestin 2 was used in the experiments (Fig. 5d).

β-Arrestin 1 Is Required for Agonist-mediated Internalization of Calcium Channels — We tested whether arrestin plays a role in agonist-induced internalization of voltage-dependent calcium channels. Calcium channels at the cell surface were labeled using biotinylated ω-conotoxin GVI A bound to streptavidin-conjugated quantum dots. Chick DRG neurons express only one type of calcium channel, Cav2.2 channel (N type) (14), which is located both at the terminals and the soma. Channels at both locations are coupled to the exocytic machinery (15, 16). Cav2.2 channels exhibited a punctate distribution in the top plane of the membrane (Fig. 6, a and b). Preincubation of DRG neurons with unlabeled toxin prevents the binding of Qdot 655-streptavidin-biotinylated ω-conotoxin GVI A demonstrating that the probe binds selectively (supplementary materials Fig. S5). Incubation of DRG neurons with the Qdot 655-conjugated streptavidin alone did not result in significant labeling of the surface of DRG neurons (supplementary materials Fig. S5). These results are not qualitatively different from those that we have previously obtained using rhodamine-conjugated ω-conotoxin GVI A (8).

In live cell imaging experiments, exposure of DRG neurons to baclofen, a GABAB receptor agonist (100 μM) and well-established modulator of these channels, produced a decrease in fluorescence signal in the top surface of the cell within 2 s (Ref. 8, FIGURE 4. Association of calcium channels with the GABA<sub>B</sub> receptor. Calcium channels were precipitated (IP) from DRG neurons treated with saline or baclofen for 20 s using anti-pan-α<sub>1</sub> antibody and immunoblotting (IB) for GABA<sub>B</sub> receptor (GABABR2). The membrane was stripped and probed for arrestin. Histogram shows quantitation of the GABABR2 band from 5 independent experiments. n=5, mean ± S.E. and analysis between independent matched groups (matched by saline alone or agonist) was significant at: *, p < 0.05. FIGURE 5. Arrestin binds to the SNARE-binding region of the calcium channel. a, rat brain lysate was incubated with His<sub>6</sub>-tagged recombinant proteins containing sequences from the cytoplasmic regions of the α<sub>1</sub> subunit of the Cav2.2 channel. Precipitation was performed for arrestin. Data are representative of four independent experiments. b, interaction of arrestin with loop II–III of calcium channel. Rat brain lysate was incubated with His<sub>6</sub>-tagged recombinant proteins with sequences spanning regions of loop II–III. Precipitation was performed using nickel beads. Immunoblotting was performed for arrestin. Data are representative of four independent experiments. c, direct interaction of arrestin with aa 894–944 of the calcium channel. Recombinant β-arrestin 1 was incubated with His<sub>6</sub>-tagged recombinant proteins containing aa 728–795 or 894–944 of the calcium channel. Precipitation was performed using nickel beads. Immunoblotting was performed for arrestin. d, precipitation was performed using recombinant β-arrestin 1 or β-arrestin 2.
Ca,2.2 Channel/Arrestin Interaction

**Retraction Notice:**

*Retracted May 13, 2011*

**Figure Legend:**

- **Figure a:** Top slice images showing Baclofen and saline treatments.
  - Top slice images at different time points (0s, 10s, 20s, 30s, 40s, 50s).
  - Middle slice images for Baclofen treatment.

- **Figure b:** Top slice saline treatment images.
  - Top slice images at different time points (0s, 10s, 20s, 30s, 40s, 50s).
  - Middle slice images for saline treatment.

- **Figure c:** Images of 0s time point for Baclofen and saline treatments.

- **Figure d:** Bar graph showing integrated area (intensity/pixel) for saline and baclofen treatments.
  - Saline: C
  - Baclofen: M

- **Figure e:** Graph showing normalized fluorescence over time for different treatments.
  - Baclofen
  - Cav 894-929 peptide
  - Cav 920-944 peptide
  - Control

- **Figure f:** Graph showing normalized fluorescence over time for baclofen and anti-beta arrestin treatments.
  - Baclofen
  - Anti-beta arrestin 2
  - Control

- **Figure g:** Graph showing percentage of voltage-dependent inhibition.
  - Anti-mouse IgG
  - Anti-arrestin
  - * indicates significance
FIGURE 6. β-Arrestin 1/calcium channel interaction is required for channel trafficking. a and b, time-lapse confocal images of live DRG neurons. Images from the top and middle planes of the cell were acquired at 2-s intervals (10-s interval images are shown here for clarity); calcium channels were visualized with Quantum dot 655-conjugated streptavidin bound to biotinylated ω-conotoxin GVIA. Neurons were exposed to 100 μM baclofen, a GABAB receptor agonist (a) or saline (b). Negative time values represent time points prior to addition of agonist. Images are representative of 20 independent experiments; each experiment consisting of a minimum of 4 cells. c, confocal images from the top surface of DRG shown in panel a after a second application of baclofen. d, histogram plot of agonist-induced changes in fluorescence in the top and middle optical slices. X-Y optical slices were taken before (t = −10) and during the application of 100 μM baclofen (t = +10). The integrated density of each optical slice was measured and the total surface and cytoplasmic intensity per pixel was calculated. Membrane (M) and cytoplasmic (C) staining was assessed by integrated morphometric analysis using Metamorph (Universal Imaging) of peripheral staining relative to total cells staining. Data values are the mean from 20 cells and error bars represent mean ± S.E. Data analysis between independent matched groups (matched by saline alone or agonist) was significant at *p < 0.001. e, cells were incubated with fluoresceinated peptides containing aa 894–929 or 920–944. Agonist was added when indicated by the bar and fluorescence in the top slice was measured as a function of time. Values plotted represent the mean from 10 cells. f, β-arrestin-1 selectively mediates agonist-induced sequestration of calcium channels. Effect of antibodies raised against β-arrestin 1 and β-arrestin 2 on baclofen-induced calcium channel sequestration. Antibodies (1:1000) were introduced into the cell bodies by microinjection. The antibodies were injected in the presence of fluoresceinated-dextran to allow subsequent visualization using epifluorescence. Experiments were performed 1 h after injection. Fluorescence as a function of time was measured for the region of interest using Physiology software version 3.2. Each time point represents the mean of 5 cells. g, effect of antibodies raised against β-arrestin 1 on agonist-induced voltage-independent inhibition. Antibodies (1:1000) were introduced into the cell bodies by microinjection. The antibodies were injected in the presence of fluoresceinated-dextran to allow subsequent visualization using epifluorescence. Experiments were performed 1 h after injection. Analysis between independent matched groups (matched by saline alone or agonist) was significant at *p < 0.005.

Ca2.2 Channel/Arrestin Interaction

We have shown that, in both embryonic chick DRG neurons and rat brain, β-arrestin 1 is bound to Ca2.2 channels in the absence of receptor activation. This is in contrast to previous reports of arrestin-binding proteins that showed a requirement for receptor activation for the nucleation of signaling complexes. Unlike the interaction of arrestin with receptors (17) and NHE5 (18), the interaction of calcium channels with arrestin does not require phosphorylation, as channel phosphorylation is undetectable prior to activation of GABAB receptors (9). Furthermore, the arrestin-binding site in the calcium channels does not contain consensus sites for phosphorylation.

The arrestin-binding site in Ca2.2 channels is located within loop II–III in the SNARE-binding region, a region important for the regulation of both channel activity and secretion (19). Binding of syntaxin to this channel region plays a role in voltage-dependent inhibition by stabilizing the binding of G protein β-γ subunits to the channel (20). Synaptotagmin also binds to the channel in this region upon depolarization, in a calcium-dependent manner (21). Deletion of the SNARE-binding region (rat 726–984) from loop II–III results in mistargeting of Ca2.1 channels (22); future studies should address whether this is due to long-term disruption of arrestin/calcium channel interaction. It should be noted that arrestin-binding, aa 894–929, is highly conserved (98% homology) among voltage-dependent calcium channels, suggesting that arrestin/channel interaction will be found in different regions of the channel.

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calcium influx into the neurons rather than termination or desensitization of the response.

Recent studies from Zamponi and colleagues (23) have shown that Ca$_{2.2}$ channels are internalized with ORL (nociceptin) receptors upon a prolonged 30-min exposure of tsA-201 cells and primary rat DRG neurons to ligand. Whereas this internalization of calcium channels is a response to chronic exposure to an agonist, our observations on the spatiotemporal distribution of calcium channels have focused on early events after receptor activation (<1 min) (8). Agonist-induced desensitization of calcium current inhibition and return of the calcium channels to the surface occur in the time scale of 1 min (8, 12). In contrast to the nociceptin receptor studies (23), our biochemical and imaging experiments do not show a significant degree of GABA$_B$ receptor/Ca$_{2.2}$ channel association prior to receptor activation.

In contrast to the cell surface abundance of NHE5 that can be altered by β-arrestin 1 or β-arrestin 2 (18), agonist-induced channel internalization is selectively regulated by β-arrestin 1. The selectivity of these two forms of arrestin is largely unexplored in intact primary cells or tissue. Both β-arrestin 1 and β-arrestin 2 play a role in receptor endocytosis and activation of the mitogen-activated protein kinase pathway (24). Whereas GABA$_B$ receptors increase mitogen-activated protein kinase activity, mitogen-activated protein kinases do not play a role in the modulation of calcium current (25).

Various reports suggest that the selectivity in β-arrestin arises at the level of the receptor, whereas β-arrestin 1 plays a role in β-arrestin-mediated activation of phosphodiesterase 4, β-arrestin 2 regulates opioid-mediated presynaptic inhibition by regulating synaptic release probability (34). Recently it has been shown that β-arrestin 2 associates with Akt and phosphatase PP2A to mediate dopaminergic responses (33).

β-Arrestin 1 is the predominant form of arrestin found in the small diameter DRG neurons (35). As these neurons are involved in nociception, one would expect alterations in the regulation of painful stimuli. In conclusion, our studies have shown that β-arrestin 1 associates with Ca$_{2.2}$ channels and it is required for agonist-mediated internalization of calcium channels. Accurate calcium signaling requires the proper spatiotemporal organization of voltage-gated calcium channels and signaling molecules. Arrestin could work both as a scaffold for calcium channels to facilitate internalization and as a switch for recovery from presynaptic inhibition as it plays a role in the recycling of calcium channels. We cannot exclude the possibility that other components of the active zone are being sequestered along with the calcium channels, raising the possibility that this is part of a more extensive remodeling of the active zone. Given the abundance of arrestin in the nervous system and its spatial correlation with components of the release machinery, this new function for arrestin could have important implications for the modulation of synaptic function.

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