The C Terminus of the Ca Channel α_{1B} Subunit Mediates Selective Inhibition by G-Protein-Coupled Receptors

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Inhibition of calcium channels by G-protein-coupled receptors depends on the nature of the Gα subunit, although the G$\beta\gamma$ complex is thought to be responsible for channel inhibition. Ca currents in hypothalamic neurons and N-type calcium channels expressed in HEK-293 cells showed robust inhibition by G_i/G_o-coupled galanin receptors (GalR1), but not by Gq-coupled galanin receptors (GalR2). However, deletions in the C terminus of α_{1B-1} produced Ca channels that were inhibited after activation of both GalR1 and GalR2. Inhibition of protein kinase C (PKC) also revealed Ca current modulation by GalR2. Imaging studies using green fluorescent protein fusions of the C terminus of α_{1B} demonstrated that activation of the GalR2 receptor caused translocation of the C terminus of α_{1B-1} to the membrane and co-localization with Gαq and PKC. Similar translocation was not seen with a C-terminal truncated splice variant, α_{1B-2}. Immunoprecipitation experiments demonstrated that Gαq interacts directly with the C terminus of the α_{1B} subunit. These results are consistent with a model in which local activation of PKC by channel-associated Gαq blocks modulation of the channel by G$\beta\gamma$ released by Gq-coupled receptors.

Key words: Ca channels; G-proteins; Gαq; C terminus; galanin; G-protein receptors; PKC

Activation of G-protein-coupled receptors (GPCRs) is one of the major ways in which neurons respond to external signals. Activation of many GPCRs results in the inhibition of voltage-dependent Ca channels. The resulting reduction in Ca influx is a major mechanism by which neurons regulate the release of neurotransmitters (Miller, 1998). Activation of GPCRs produces several different types of Ca channel inhibition. The best studied of these processes is characterized by a slowing of Ca channel activation and is voltage-dependent, the inhibition being relieved by a depolarizing prepulse (Bean, 1989; Hille, 1994). It is thought that this type of inhibition is effected through the direct interaction of G-protein $\beta\gamma$ subunits with the pore-forming α_1 subunit of the Ca channel (Herlitze et al., 1996; Ikeda, 1996; De Waard et al., 1997). Interaction of $\beta\gamma$ subunits with the I/II loop and C terminus of the α_1 subunit has been demonstrated, although other regions of the channel appear to be involved, including the N terminus and domain I (Zhang et al., 1996; Zamponi et al., 1997; Page et al., 1998; Simen and Miller, 1998, 2000; Stephens et al., 1998). This mechanism of Ca channel inhibition has been described as "membrane-delimited," because it does not appear to involve freely diffusible intermediates. It is interesting to note, however, that the activation of GPCRs does not always produce voltage-dependent inhibition of Ca channels (Bernheim et al., 1991; Taussig et al., 1992; Shapiro and Hille, 1993; Shapiro et al., 1994; Liu et al., 1995; Margeta-Mitrovic et al., 1997).

According to the above discussion, one would expect that the productive activation of any GPCR would result in Ca channel inhibition by virtue of the fact that G$\beta\gamma$ subunits are released. However, Ca channel inhibition appears to depend on the nature of the Gα molecule with which a receptor is coupled. Activation of Gαi/Gαo-coupled (Dolphin and Scott, 1987; Ikeda and Schofield, 1989), Gαz-coupled (Jeong and Ikeda, 1998), and Gαs-coupled (Hille, 1994) receptors generally causes voltage-dependent inhibition of Ca channels, but the activation of receptors coupled to other Gα subunits (e.g., Gαq/11) usually does not (Shapiro and Hille, 1993; Hille, 1994; Shapiro et al., 1994). On the other hand, activation of Gαq/11-linked receptors often produces a slow, voltage-independent inhibition of Ca channels, the mechanism of which has not been determined. Recently, for example, it has been demonstrated that Gαq mediates voltage-independent inhibition of Ca channels produced by M1 muscarinic receptors (Haley et al., 2000), whereas activation of M2 receptors produces pertussis toxin-sensitive voltage-dependent inhibition (Toselli et al., 1995).

We have tried to determine why activation of some GPCRs fails to produce voltage-dependent Ca channel inhibition. Here we show that the differential susceptibility of N-type Ca channels to Gαi/Gαo- versus Gαq-coupled galanin receptors depends on structural elements in the C terminus of the Ca channel α_1 subunit and provide evidence that protein kinase C (PKC) may play an important role in mediating these effects.

MATERIALS AND METHODS

Acute isolation of hypothalamic neurons. Acutely isolated neurons from the hypothalamus were obtained from rat pups 10–16 d old. Rat pups were anesthetized and decapitated. The hypothalamus was rapidly removed and chilled to 4°C by submerging it in 4°C Ringer's solution (in

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mM: 126 NaCl, 26.2 $NaHCO_3$, 1.0 NaH_2PO_4, 3.0 KCl, 1.5 $MgSO_4$, 2.5 $CaCl_2$, and 10 glucose) while bubbling with 95% O_2 and 5% CO_2. The tissue was mounted in a vibratome (TPI), and 400 μm cuts were made though the hypothalamus containing the arcuate nucleus. The brain slices were transferred to a holding chamber containing Ringer's solution at 35°C for 1 hr. Tissues were then enzymatically treated with papain (15 U/ml; Roche Molecular Biochemicals, Indianapolis, IN) for 1 hr. Papain was then inactivated by treating the tissue with ovomucoid. Brain slices were returned to the holding chamber until needed.

Neurons from the arcuate nucleus of the hypothalamus were isolated by micropunching the area just lateral to the third ventricle. Neurons were dissociated by gentle mechanical trituration using multiple pipettes of decreasing bore diameters. Cells were then plated onto glass coverslips precoated with poly-L-lysine. Cells were placed into a 35°C incubator and allowed to settle for a minimum of 30 min before electrophysiological recordings were made.

Receptor and α_1 subunit plasmid preparation. Rat galanin receptors 1 and 2 (GalR1 and GalR2) were cloned from a rat hypothalamic cDNA library (Clontech, Cambridge, UK) using PCR. Forward and reverse primers were designed from the reported sequences for GalR1 and GalR2 (GenBank accession numbers, U30290 and AF010318). The PCR products were isolated and subcloned into pGemT-Easy (Promega, Madison, WI). Multiple clones were sequenced with dRhodamine terminator cycle sequencing mix (PerkinElmer Life Sciences, Emeryville, CA) and an automated DNA sequencer (ABI 377; PerkinElmer Life Sciences). Error-free clones were selected and subcloned into a mammalian expression vector, pcDNA 3.1 (Invitrogen, San Diego, CA) or a modified pIRES-EYFP vector (Clontech).

Calcium channel subunit cDNAs encoding $\alpha_{1B\text{-}1}$, $\alpha_{1B\text{-}2}$, α_2/δ, and β_{1b} were kindly provided by SIBIA Neurosciences. cDNAs encoding the various wild-type G-protein α subunits (Gαi1, Gαo, and Gαq) and constitutively activated G-protein α subunits (Q-to-L mutations to eliminate GTPase activity: Gαi1Q240L, GαoQ250L, and GαqQ209L) were provided by Ronald Taussig (University of Michigan). cDNA for the κ-opioid receptor (κOR) was kindly provided by Dr. Graeme I. Bell (Howard Hughes Medical Institute, University of Chicago).

Modifications in the C terminus of $\alpha_{1B\text{-}1}$ were described previously (Simen and Miller, 2000). The Δ1875–2339 construct was created by deleting the nucleotides coding for amino acids (aa) 1875–2339 and adding a stop codon to the construct. The construct Δ2037–2087 is a deletion of aa 2037–2087. The Δ2037–2087 construct was created by replacing the nucleotides coding for residues 2037–2087 with a *Hin*dIII site, which codes for the amino acids Arg and Leu. Each of the C-terminal constructs was verified by DNA sequencing.

Transfections. Monolayers (<80% confluence) of HEK-293 or tsA-201 cells were replated on the day of transfection. Plasmids were transfected using Fugene 6 (Roche Molecular Biochemicals) per the manufacturer's instructions or polyethyleneimine as previously described (Simen and Miller, 1998). Twenty-four to 48 hr after transfection, cells were replated onto glass coverslips precoated with poly-L-lysine. Calcium currents were recorded 36–72 hr after transfection from CD8-positive or green fluorescence protein (GFP)-positive cells. CD8-transfected cells were labeled with a 1:1000 dilution of microspheres coated with an antibody against the CD8α antigen (Dynal, Oslo, Norway).

Electrophysiological recordings. Total Ba^{2+} currents were measured using the tight-seal whole-cell patch-clamp technique. The coverslips were mounted in a perfusion chamber and constantly perfused by a gravity feed system with a modified HEPES-balanced salt solution (in mM: 5 $BaCl_2$, 143 tetraethylammonium chloride, 1 $MgCl_2$, 10 Hepes, and 10 glucose, pH adjusted to 7.4 and osmolarity to 310 mOsm) to isolate the Ba^{2+} current. Patch pipettes of 2–6 MΩ resistance were filled with a solution containing 135 mM CsCl, 1 mM $MgCl_2$, 10 mM HEPES, 10 mM BAPTA, 14 mM phosphocreatine, 3.6 mM MgATP, 3.6 mM LiGTP, and 50 U/ml creatine phosphokinase, adjusted to pH 7.3 with CsOH and 290 mOsm. Data were digitized at 10 kHz and filtered at 2 or 5 kHz. Series resistance was compensated ≥70%, and currents were leak-corrected on-line using a P/5 protocol (Armstrong and Bezanilla, 1977).

Currents were measured and recorded with an Axopatch 200B (Axon Instruments) or EPC9 (Heka) amplifier using the Clampex program (pClamp 6 software suite; Axon Instruments) or the Pulse program (Heka). All experiments and solutions were used at room temperature. Each coverslip was used only once to prevent any possible effects of desensitization. However, no evidence of desensitization from multiple applications of galanin and its analogs was observed.

Unless otherwise noted, statistical analyses were performed using the Kruskal–Wallis variant of the ANOVA test followed by Dunn's *post hoc* test.

Measurement of $[Ca]_i$ with fura-2. After isolating neurons as described above, cells were loaded with fura-2 methyl-ester (Molecular Probes, Eugene, OR; 3 μM fura-2 for 20 min at room temperature). Cells were then washed with a HEPES-balanced salt solution (in mM: 140 NaCl, 10 HEPES, 2 $CaCl_2$, 2 $MgCl_2$, 5 KCl, and 10 glucose, pH 7.4 and adjusted to ~310 mOsm.) for 20 min to allow for deesterification of Fura-2. Changes in free internal calcium concentration ($[Ca]_i$) were monitored using digital video microfluorimetry. An intensified CCD camera (Hamamatsu, Hamamatsu City, Japan) coupled to a Nikon (Mellville, NY) Diaphot microscope and Metafluor software (Universal Imaging Corp., West Chester, PA) was used to gather intensity values. Cells were excited at 340 and 380 nm using a 150 W Xe arc and computer-controlled filter wheel. Ratio intensities were calibrated via an eight point curve derived from imaging droplets of 50 μM fura-2 in calibrated free calcium buffers (Molecular Probes). Ratio intensities and calculated calcium concentrations from marked areas of interest were logged to a computer. Drugs were bath-applied using a gravity feed system at room temperature.

Fusion constructs. Fragments of the C terminus were expressed as fusion proteins with GFP. The GFP-C1 construct consisted of GFP fused to aa 1768–2339 of the human $\alpha_{1B\text{-}1}$ Ca channel. The GFP-C2 construct consisted of GFP fused to aa 1768–2237 of the $\alpha_{1B\text{-}2}$ Ca channel. The GFP-CC1 construct consisted of GFP fused to aa 1871–2339 of $\alpha_{1B\text{-}1}$. The GFP-CC2 construct consisted of GFP fused to aa 1871–2237 of $\alpha_{1B\text{-}2}$. The GFP-CCC1 construct consisted of aa 2164–2339 of $\alpha_{1B\text{-}1}$ fused to GFP. The GFP-N construct consisted of aa 1768–2109 of $\alpha_{1B\text{-}1}$ fused to GFP. The GFP-NN construct consisted of aa 1768–2009 of $\alpha_{1B\text{-}1}$ fused to GFP. The GFP-NNN construct consisted of aa 1768–1875 $\alpha_{1B\text{-}1}$ fused to GFP. Each of these constructs was constructed by ligating the appropriate fragment into the *Xho*I and *Xba*I sites of the pEYCP-C1 vector (Clontech).

Immunoprecipitation experiments. tsA-201 cells were transfected with GFP-C1, GFP-C2, GFP-CC1, GFP-CC2, or GFP-CCC1 in combination with rat Gαq, rat Gαq*, rat GalR2, or rat μOR. Two to 3 d after transfection, cells were washed once and dissociated for 10 min in 2 ml of PBS. Cells were then centrifuged at 800 rpm for 8 min at 4°C in flat-sided 10 ml tubes. The cells were then resuspended in 500 μl of labeling medium devoid of methionine and cysteine (Life Technologies, Gaithersburg, MD) and incubated for 20 min at 37°C. One hundred fifty microcuries of ProMix (300 μCi/ml; Amersham Pharmacia Biotech, Arlington Heights, IL) was then added, and cells were incubated at 37°C for 3 hr. The cells were then centrifuged at 800 rpm for 8 min at 4°C and resuspended in 200 μl of lysis buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 20 mM iodacetamide, 5 mM KCl, 5 mM $MgCl_2$, 1% IGEPAL CA-630, and 20 U/ml aprotinin), in addition to a protease inhibitor mixture (in μg/ml: 10 *N*-P-tosyl-L-arginine methyl ester, 10 tosyl-L-phenylalanine-chloromethyl ketone, 10 soybean trypsin inhibitor, 1 leupeptin, and 1 pepstatin A, final concentrations). The cells were lysed for 25 min on ice and centrifuged at 10,000 × *g* for 10 min at 4°C. Incorporation of radioactivity in total protein was determined as TCA-precipitable counts in duplicates of 1 μl of lysate and used to normalize input of lysates in immunoprecipitation experiments.

Samples were precleared by adding 60 μl of recombinant protein A coupled to Sepharose CL6 beads (Repligen) to the lysates. The samples were taken up to a total volume of 700 μl with TNNB (50 mM Tris, pH 8.0, 250 mM NaCl, 0.5% IGEPAL CA-630, 0.5 mM PMSF, 0.02% NaN_3, 0.1% BSA, and protease inhibitor mixture). The reactions were mixed on a rotator for 1 hr at 4°C and were then centrifuged, and the supernatants were mixed overnight at 4°C with fresh protein A-Sepharose and the appropriate antibody. Two microliters of rabbit anti-Gαq antiserum (Calbiochem, La Jolla, CA), 5 μl of rabbit anti-GFP (Molecular Probes), or 5 μl of rabbit anti-β subunit antibody (Calbiochem) were used for immunoprecipitation. The protein A beads were washed three times with 1 ml of TNNB at 4°C and then three times with 1 ml of TNNB without BSA at 4°C. The proteins were then eluted with 80 μl of 1× reducing sample buffer by brief mixing and boiling for 3 min. Forty microliters of eluate were then loaded on 9 or 13% SDS-PAGE gels. The gels were run at 30 mA for ~4 hr, dried onto Whatman (Maidstone, UK) 3M paper for 1 hr at 80°C under vacuum, exposed to a low-energy PhosphorImager screen overnight (Molecular Dynamics, Sunnyvale, CA), and analyzed in a Storm 860 PhosphorImager (Molecular Dynamics).

Confocal imaging of staining patterns. Cells were fixed for 20 min with 4% paraformaldehyde 48–72 hr after transfection and mounted in 60% glycerol, 5% *n*-propyl gallate, and PBS, buffered to pH 7.8 with Tris.

Some cells were permeabilized with Triton X-100 and treated with anti-Gα primary antibody (Calbiochem) or anti-hemagglutinin (HA) primary antibody (Molecular Probes) for 1–12 hr. Staining of Gαq and HA epitope-tagged PKC-δ was revealed by a Cy5-conjugated secondary antibody (Jackson ImmunoResearch, West Grove, PA) and Texas Red-conjugated secondary antibody (Covance), respectively. Slides were scanned on an Olympus Optical (Tokyo, Japan) Fluoview LSM confocal system typically using a 60×, numerical aperture 1.4 objective and excitation at 488 and 647 nm for GFP and Cy5, respectively. Emissions at 510–550 and 700–775 nm were collected on separate detectors. Optical sections were taken at 0.3 nm vertical steps throughout the entire cell volume. Staining controls (processed without primary antibody or non-transfected cells) were scanned under identical machine settings to verify that the fluorescence was specific. Cells with clumped GFP contents were excluded from analyses. Volume reconstructions were created in Metamorph version 4.5 (Universal Imaging). Fluorescence intensity maps were plotted for linear transects drawn through the cytosol at the equatorial plane.

RESULTS

Selective modulation of N-type channels by galanin receptors

An example of the selectivity of Ca channel regulation by GPCRs is afforded by comparison of the effects of activating two galanin receptors, GalR1 and GalR2. GalR1 exhibits low affinity for the GalR2 specific agonist [D-Trp^2] galanin (Smith et al., 1997, 1998; Wang et al., 1997a,b, 1998). GalR1 and GalR2, unlike GalR3, have been shown to be highly expressed in the hypothalamus (Smith et al., 1997, 1998; Wang et al., 1997a,b, 1998). We therefore examined the effects of galanin and it analogues on Ba^{2+} currents in acutely isolated neurons from the arcuate nucleus of the hypothalamus. [D-Trp^2] galanin was used to distinguish between the effects of the two receptors.

Figure 1*a* demonstrates that the application of multiple galanin receptor agonists, with the notable exception of [D-Trp^2] galanin, inhibited the Ba^{2+} current in acutely isolated hypothalamic neurons ($n = 13$). This pharmacological profile suggests that activation of GalR1 receptors, but not GalR2 receptors, in these neurons is linked to inhibition of the Ba^{2+} current. The inhibition was voltage-dependent, being substantially relieved by a strong depolarizing prepulse to +80 mV (Fig. 1*b, i*). Although application of [D-Trp^2] galanin did not produce any inhibition of the Ba^{2+} current (e.g., Fig. 1*a*), robust $[Ca]_i$ mobilization ($n = 8$; data not shown) was observed, as expected from the activation of a Gαq-coupled receptor. Thus, activation of GalR1 but not GalR2 receptors in hypothalamic neurons produces voltage-dependent inhibition of the Ba^{2+} current. However, activation of GalR2 receptors mobilizes $[Ca]_i$, consistent with previous expression studies (Smith et al., 1997, 1998).

Similar results were obtained with cloned rat galanin receptors (GalR1 and GalR2) expressed in HEK-293 cells together with the N-type Ca channel subunits $\alpha_{1B\text{-}1}$, α_2/δ, and β_{1B}. As with the hypothalamic neurons, GalR1-expressing HEK cells showed a large voltage-dependent inhibition of the Ba^{2+} current on application of galanin or galanin agonists (Fig. 1*c*). Galanin and its analogs ([1–16] galanin, M15, M32, M40, and C7) blocked the Ba^{2+} current by 73.00 ± 2.70% ($n = 8$), 61 ± 1.70% ($n = 6$), 63 ± 1.90% ($n = 5$), 65 ± 3.70% ($n = 7$), 62 ± 2.90% ($n = 5$), and 81 ± 5.90% ($n = 5$), respectively. Application of [D-Trp^2] galanin had no effect on the Ba^{2+} current in GalR1-expressing cells (Fig. 1*c*). When GalR2 receptors were expressed in HEK cells together with N-type Ca channels, neither application of galanin ($n = 9$; data not shown) nor [D-Trp^2] galanin ($n = 7$; Fig. 1*d*) produced inhibition of the Ba^{2+} current, even though activation of G_i/G_o-coupled κORs expressed in the same cells by the κOR selective

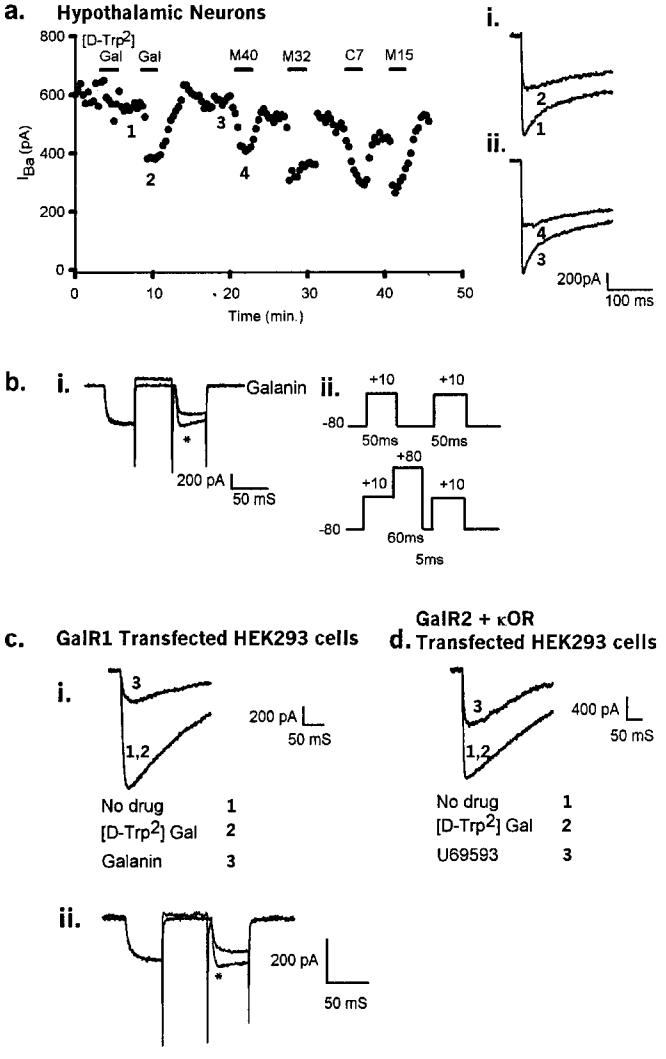

Figure 1. Effects of galanin analogs on acutely dissociated hypothalamic neurons and on HEK293 cells transiently transfected with GalR1 or GalR2 together with N-type Ca channel subunits ($\alpha_{1B\text{-}1}$, α_2/δ, and β_{1b}). *a*, Typical experiment on a hypothalamic neuron in which the Ba^{2+} current was inhibited by repeated application of different galanin analogs. All peptides were used at 100 nM. Peak Ba^{2+} currents expressed in picoamperes are plotted versus time expressed in minutes. The Ba^{2+} current was elicited every 20 sec from a holding potential of −80 mV to a test potential of +10 mV for 200 msec. *a*, *i, ii*, Corresponding currents at the times specified in the current plot. *b, i*, Representative hypothalamic neuron demonstrating prepulse facilitation of the Ba^{2+} current during an application of galanin. The plot is an overlay of two current traces during two separate voltage protocols, where the second trace (*) contains a voltage step to a positive potential of +80 mV interspersed between two test pulses. *b, ii*, Voltage protocols used to generate the currents in *b, i. c, i*, Typical experiment with an HEK293 cell expressing GalR1 and N-type Ca channels. The Ba^{2+} current was inhibited by application of galanin but not [D-Trp^2] galanin. Galanin and [D-Trp^2] galanin were used at 100 nM. The Ba^{2+} current was elicited using the same protocol in *a*. *c, ii*, Representative GalR1-expressing HEK cell demonstrating prepulse facilitation of the Ba^{2+} current during an application of galanin. The plot is an overlay of two current traces during two separate voltage protocols in which the second trace (*) contains a voltage step to a positive potential of +80 mV interspersed between two test pulses. The voltage protocols used are identical to the one illustrated in *b*, *ii. d*, Typical experiment with a HEK293 cell expressing GalR2, κOR, and N-type Ca channels. The peak Ba^{2+} current was not inhibited by application of galanin (100 nM) but was inhibited by the application of the κOR agonist U69593 (1 μM). The Ba^{2+} current was elicited using the same protocol in *a*.

agonist U69593 was clearly effective (Fig. 1*d*). Overnight pretreatment with pertussis toxin (PTX) completely blocked inhibition of N-type currents by galanin ($n = 6$; data not shown), suggesting that GalR1 preferentially couples to Gαi, Gαo, or both.

Mobilization of $[Ca]_i$ was examined in HEK cells transfected with either GalR1 or GalR2. In cells expressing GalR1, application of galanin ($n = 15$) or [D-Trp^2] galanin ($n = 15$) did not increase $[Ca]_i$ (data not shown). However, application of carbachol to activate endogenous muscarinic receptors produced $[Ca]_i$ mobilization, and this effect could be blocked by treating the cells with thapsigargin (data not shown). In contrast, HEK cells transfected with GalR2 showed large increases in $[Ca]_i$ after application of [D-Trp^2] galanin and other galanin analogs (data not shown). The mobilization of $[Ca]_i$ after GalR2 activation was blocked by pretreating the cells with thapsigargin (data not shown; $n = 12$), suggesting that the source of Ca was from thapsigargin-sensitive internal stores. Overnight incubation with PTX did not block the ability of either galanin ($n = 16$) or [D-Trp^2] galanin ($n = 8$; data not shown) to increase $[Ca]_i$ in GalR2-expressing cells, suggesting that GalR2 preferentially couples to a PTX-insensitive G-protein such as Gαq or Gαi1.

Selectivity of Ca channel inhibition depends on structural elements in the C terminus of the α_1 subunit

Although activation of GalR2 receptors produced no inhibition of wild-type $\alpha_{1B\text{-}1}$ Ca channels, we found that certain modifications to the C terminus of the channel α_1 subunit rendered it susceptible to inhibition. We compared wild-type $\alpha_{1B\text{-}1}$ with two C-terminal mutations of $\alpha_{1B\text{-}1}$. The first C-terminal change we examined was a truncation of $\alpha_{1B\text{-}1}$ (Δ1875–2339). This C-terminal truncation includes a region previously implicated in interactions with Gαi (Furukawa et al., 1998a,b). The second modified channel we expressed was Δ2037–2087, containing a deletion encompassing a region in $\alpha_{1B\text{-}1}$ homologous to a putative G$\beta\gamma$ binding site previously described in the C terminus of α_{1E} (Qin et al., 1997). We have previously shown that these C-terminal alterations have little to no effect on the ability of a G_i/G_o-coupled receptor (κOR) to modulate the channel (Simen and Miller, 2000). Therefore, we used κOR in these studies as a positive control.

As expected, activation of κOR with U69593 (1 μM) produced voltage-dependent inhibition of Ba^{2+} currents in cells expressing wild-type $\alpha_{1B\text{-}1}$, GalR2, and κOR (51 ± 3.4% inhibition; $n = 6$), whereas [D-Trp^2] galanin (100 nM) had no significant effect (2.2 ± 0.5% inhibition; $n = 6$; Fig. 1*d*). κOR activation by U69593 also inhibited the Ba^{2+} current in cells expressing Δ1875–2339 or Δ2037–2087 in a voltage-dependent manner, similar to its effects on wild-type Ca channels (Fig. 2*a–c*). However, in contrast to wild-type $\alpha_{1B\text{-}1}$, activating GalR2 with galanin (10–100 nM) elicited a robust inhibition of the Ba^{2+} current in Δ1875–2339-expressing cells (Fig. 2*a*) and Δ2037–2087-expressing cells (Fig. 2*b*). The magnitude of the inhibition of Δ1875–2339 and Δ2037–2087 seen after GalR2 activation was consistently smaller than that observed with κOR activation (Fig. 2*a,b*). U69593 blocked the Ba^{2+} current by 52 ± 5% ($n = 6$) and 55 ± 2% ($n = 6$) in Δ1875–2339- and Δ2037–2087-expressing cells, respectively, whereas galanin blocked the Ba^{2+} current by 30 ± 3.2% ($n = 9$) and 27 ± 2% ($n = 6$) in Δ1875–2339- and Δ2037–2087-expressing cells, respectively. When larger truncations in the C terminus of $\alpha_{1B\text{-}1}$ were made (construct Δ1768–2339), no functional channel expression was obtained (data not shown).

We also characterized the voltage dependence of the GalR2

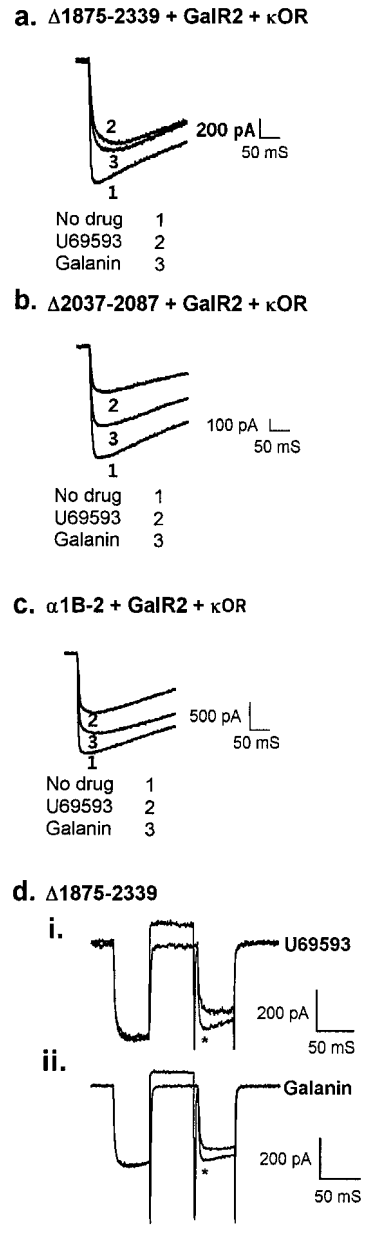

Figure 2. Effects of galanin analogs on HEK293 cells transiently transfected with GalR2 and κOR together with Δ1875–2339, Δ2037–2087, or $\alpha_{1B\text{-}2}$. In all three instances, the Ba^{2+} current was inhibited by the application of galanin (100 nM) and U69593 (1 μM). The Ba^{2+} current was elicited using same protocol as in Figure 1*a*. *a–c*, Representative currents from HEK cells expressing Δ1875–2339, Δ2037–2087, or $\alpha_{1B\text{-}2}$, respectively, showing robust inhibition by U69593 as well as galanin. *d, i, ii*, Representative traces from GalR2-, κOR-, and Δ1875–2339-expressing HEK293 cells, demonstrating prepulse facilitation of the Ba^{2+} current during application of U69593 and galanin, respectively. The plot is an overlay of two current traces during two separate voltage protocols, where the second trace (*) contains a voltage step to a positive potential of +80 mV interspersed between two test pulses. The voltage protocols used are identical to those illustrated in Figure 1*b, ii*.

inhibition of Δ1875–2339 and Δ2037–2087 currents using a prepulse protocol. The inhibition of the Ba^{2+} current by galanin was partially relieved by a prepulse to +80 mV (Fig. 2*d*). The ratio of postpulse to prepulse currents (P2:P1) was similar for U69593 and galanin in Δ1875–2339-expressing cells, 1.6 ± 0.10 ($n = 5$) and 1.5 ± 0.10 ($n = 4$), respectively. For Δ2037–2087 expressing cells,

somewhat lower P2:P1 ratios were observed for U69593 and galanin, 1.4 ± 0.09 ($n = 5$) and 1.3 ± 0.06 ($n = 6$), respectively. Thus, it appears that modifications to the $\alpha_{1B\text{-}1}$ C terminus render it susceptible to inhibition in a voltage-dependent manner by activation of GalR2 receptors, in a manner that is typically observed with G-protein G$\beta\gamma$ subunits. These data strongly suggest that G$\beta\gamma$ subunits released by GalR2 activation are capable of inhibiting $\alpha_{1B\text{-}1}$ but that the C terminus is somehow involved in blocking these effects.

Interestingly, a C-terminal splice variant of the α_{1B} subunit of the Ca channel ($\alpha_{1B\text{-}2}$) was previously described (Williams et al., 1992). $\alpha_{1B\text{-}2}$ differs from $\alpha_{1B\text{-}1}$ in that $\alpha_{1B\text{-}2}$ is shorter (2237 vs 2339 nucleotides) than $\alpha_{1B\text{-}1}$ and differs in its last 74 amino acids when compared with $\alpha_{1B\text{-}1}$. However, there has been little functional description of the properties of $\alpha_{1B\text{-}2}$. We expressed $\alpha_{1B\text{-}2}$ and examined its modulation by GalR1, GalR2, and κOR. Expression of $\alpha_{1B\text{-}2}$, α_2/δ, β1b, κOR, and GalR2 produced a Ba^{2+} current that was inhibited by κOR. Application of U69593 blocked the Ba^{2+} current (51 ± 7%; $n = 7$) to an extent similar to that seen with $\alpha_{1B\text{-}1}$ (Fig. 2*c*). As with $\alpha_{1B\text{-}1}$, inhibition of $\alpha_{1B\text{-}2}$ was voltage-dependent (P2:P1, 1.5 ± 0.1; $n = 4$). However, in contrast to $\alpha_{1B\text{-}1}$, $\alpha_{1B\text{-}2}$ was also inhibited by the activation of GalR2. GalR2 inhibited $\alpha_{1B\text{-}2}$ Ca currents by 27 ± 0.9% ($n = 6$; Fig. 2*c*). The modulation of $\alpha_{1B\text{-}2}$ by GalR2 was voltage-dependent, because a strong depolarizing prepulse partially relieved the observed inhibition. The P2:P1 ratio for galanin was 1.5 ± 0.1 in $\alpha_{1B\text{-}2}$-expressing cells ($n = 4$). Overall, the prepulse ratios (P2:P1) for Δ1875–2339 and $\alpha_{1B\text{-}2}$ are similar to the ratios we previously reported for κOR and $\alpha_{1B\text{-}1}$ (1.7 ± 0.13; Simen and Miller, 1998). The ratios for Δ2037–2087 are somewhat lower, suggesting a lower degree of voltage dependence of inhibition for this particular construct, which involved the smallest alteration to the C terminus that we tested.

We explored the role of Gα subunits in these effects by overexpressing various wild-type and mutant Gαq subunits. When we overexpressed wild-type Gαi, Gαo, and Gαq, activation of GalR1 inhibited $\alpha_{1B\text{-}1}$ Ca currents to an extent similar to that in the control situation (Fig. 3*a, i, b*). Galanin inhibited the Ba^{2+} current in the presence of overexpressed wild-type Gαi by 68 ± 4.9% ($n = 4$), inhibited the Ba^{2+} current by 71 ± 3.7% ($n = 6$) with Gαo, and inhibited the Ba^{2+} current by 56 ± 5.9% ($n = 10$) with Gαq (Fig. 3*b*). Overexpression of constitutively active Gαi (Gαi*) or Gαo (Gαo*) reduced the inhibition produced by GalR1 when compared with overexpression of wild-type Gαi or Gαo but to a lesser extent than Gαq*. When we overexpressed Gαi*, galanin inhibited the Ba^{2+} current by 33 ± 2.3% ($n = 8$), and in the presence of Gαo*, galanin inhibited the Ba^{2+} current by 35 ± 4.1% ($n = 6$; Fig. 3*b*). In contrast, overexpression of constitutively active Gαq (Gαq*) potently blocked the ability of GalR1 to inhibit the Ba^{2+} current (Fig. 3*a, ii, b*). Activation of GalR1 inhibited wild-type Ca currents by 6.4 ± 2.5% ($n = 12$) when Gαq* was overexpressed (Fig. 3*b*). Overall, overexpression of Gαi or Gαo yielded similar inhibition than overexpression of Gαq ($p > 0.05$, Bonferroni corrected *t* test). However, overexpression of Gαi* and Gαo* allowed for significantly more inhibition than Gαq* ($p < 0.001$; Bonferroni corrected *t* test). Therefore, overexpression of all three constitutively active Gα species reduced inhibition to some extent, but Gαq* was significantly more effective than Gαi* or Gαo* in blocking inhibition.

We tested the hypothesis that the actions of Gαq were mediated through the C terminus of the Ca channel by overexpressing Gαq* with either Δ1875–2339 or $\alpha_{1B\text{-}2}$ Ca channels to see whether it would inhibit GalR1 modulation as it did with $\alpha_{1B\text{-}1}$. In contrast to its effects on $\alpha_{1B\text{-}1}$, overexpression of Gαq* was unable to block the GalR1 mediated inhibition of either Δ1875–2339 or $\alpha_{1B\text{-}2}$. Galanin inhibited the Ba^{2+} current in Δ1875–2339- and Gαq*-expressing cells by 28 ± 1.9% ($n = 6$) and by 34 ± 4.8% ($n = 6$) in $\alpha_{1B\text{-}2}$- and Gαq*-expressing cells. This should be compared with the inability of GalR1 to inhibit $\alpha_{1B\text{-}1}$ in cells overexpressing Gαq* (Fig. 3*b*). These results strongly suggest that

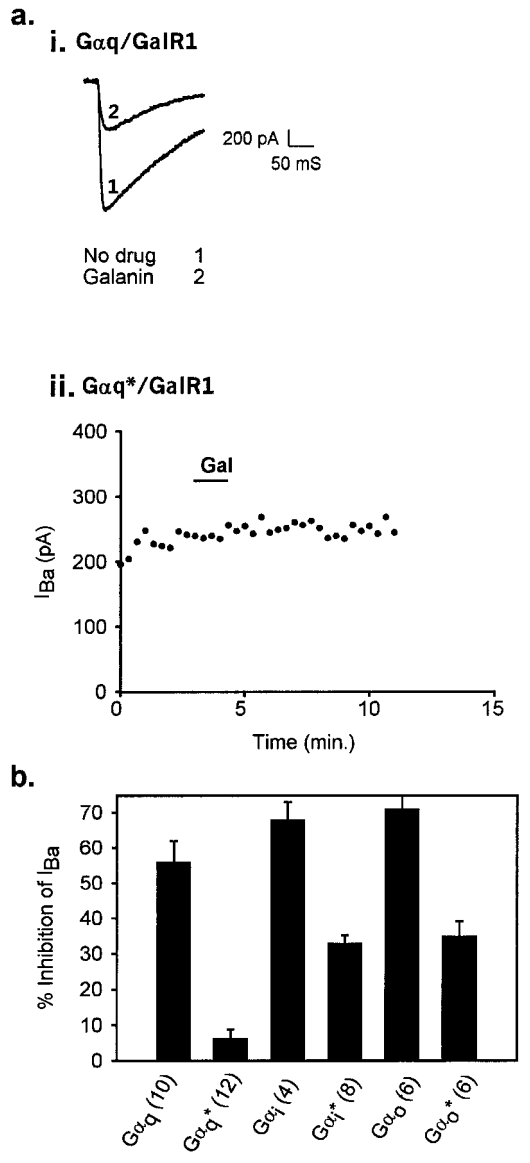

Figure 3. Effects of expression of different wild-type and constitutively active G-protein α subunits (denoted as Gα*) on the ability of GalR1 to inhibit the Ba^{2+} current. *a*, Typical experiment illustrating an HEK293 cell expressing GalR1 and $\alpha_{1B\text{-}1}$ with either Gαq or Gαq*. *a, i*, Currents from a typical cell expressing wild-type Gαq, in which the Ba^{2+} current was inhibited by application of galanin (100 nM). *a, ii*, Typical cell expressing Gαq* in which the application of galanin no longer inhibited the Ba^{2+} current. The Ba^{2+} current was elicited with the same protocol as in Figure 1*a*. *b*, Summary graph of the inhibitory effects of galanin on the Ba^{2+} current in HEK293 cells transfected with Gαi, Gαo, Gαq, Gαi*, Gαo*, and Gαq*. Data are expressed as percent inhibition of the peak Ba^{2+} current after the application of galanin (100 nM). Data are plotted as mean ± SEM. The *number* of responding cells in each group is in *parentheses*.

the inhibitory actions of Gαq are mediated in some manner by the C terminus of the channel.

GalR2 activation causes translocation of GFP-tagged C-terminal fragments of α_{1B}

The above results suggest that activation of GalR2 is ineffective in inhibiting $\alpha_{1B\text{-}1}$ Ca channels by a mechanism involving Gαq and the C terminus of the channel. However our results clearly suggest that even though $\alpha_{1B\text{-}1}$ Ca channel inhibition is not observed after GalR2 activation, activation of the receptor might influence the state of the channel in a Gαq-dependent manner. To demonstrate that activation of GalR2 does influence the $\alpha_{1B\text{-}1}$ Ca channel, the cellular localization of various C-terminal fragments of $\alpha_{1B\text{-}1}$ fused to GFP was determined by confocal fluorescence imaging before and after the activation of GalR2. The C terminus of $\alpha_{1B\text{-}1}$ fused to GFP (GFP-C1; aa 1768–2339) was found to be distributed throughout the cytoplasm in untreated tsA-201 cells but translocated to the plasma membrane and co-localized with immunohistochemically localized Gαq after stimulation of GalR2 (Fig. 4*a,b*). In contrast, the C terminus of $\alpha_{1B\text{-}2}$ fused to GFP (GFP-C2; aa 1768–2237) and the GFP protein alone did not translocate to the plasma membrane after stimulation with galanin (Fig. 4*c,d,f*). In addition, when cells were transfected with the G_i/G_o-coupled μOR rather than GalR2, no translocation was noted after receptor activation with the μOR selective agonist [D-Ala^2,*N*-Me-Phe^4, Gly^5-ol]-enkephalin (DAMGO) (Fig. 4*e*), suggesting that translocation of C1-GFP is Gαq-specific.

Immunostaining with an antibody against rat Gαq that does not recognize endogenous human Gαq demonstrated that, after receptor activation, Gαq was localized principally at the membrane and co-localized with GFP-C1 but not with GFP-C2 (Fig. 4*a, ii, c*). Translocation of these C-terminal fragments to the membrane and co-localization with immunohistochemically localized Gαq therefore correlate with the sensitivity of $\alpha_{1B\text{-}1}$ and $\alpha_{1B\text{-}2}$ to occlusion of G-protein modulation by Gαq. Although the C terminus is not free to undergo such translocation in the intact channel, our data suggest that the C terminus of $\alpha_{1B\text{-}1}$ may be tethered to the membrane proximally by virtue of its connection to transmembrane domain IV of the channel as well as distally by virtue of a Gαq-dependent mechanism. $\alpha_{1B\text{-}2}$ may on the other hand be tethered to the membrane only proximally. These differences in arrangement with respect to the membrane may have important implications for signaling (discussed below).

Maximov et al. (1999) have shown that the C-terminal end of the $\alpha_{1B\text{-}1}$ but not the $\alpha_{1B\text{-}2}$ C terminus contains a PDZ interacting domain that interacts with the PDZ domain of Mint-1 in neurons. To test the hypothesis that such interactions are responsible for the differential interaction of $\alpha_{1B\text{-}1}$ and $\alpha_{1B\text{-}2}$ with the membrane after receptor activation, we attempted to amplify Mint-1 from HEK-293 cells by reverse transcription-PCR but were unable to do so, although human fetal brain yielded robust PCR products (data not shown), consistent with a primarily neuronal distribution of expression as previously described (Okamoto and Sudhof, 1997). In addition, overexpression of the PDZ domain of human Mint-1 failed to alter the cellular distribution of the C1-GFP molecule before or after GalR2 activation by galanin (data not shown).

Gαq binds directly to the C terminus of α_{1B}

In an attempt to understand how Gαq influences the channel in a manner that depends on the C terminus, we sought to determine whether Gαq interacts directly with the C terminus of α_{1B}. Gαq*

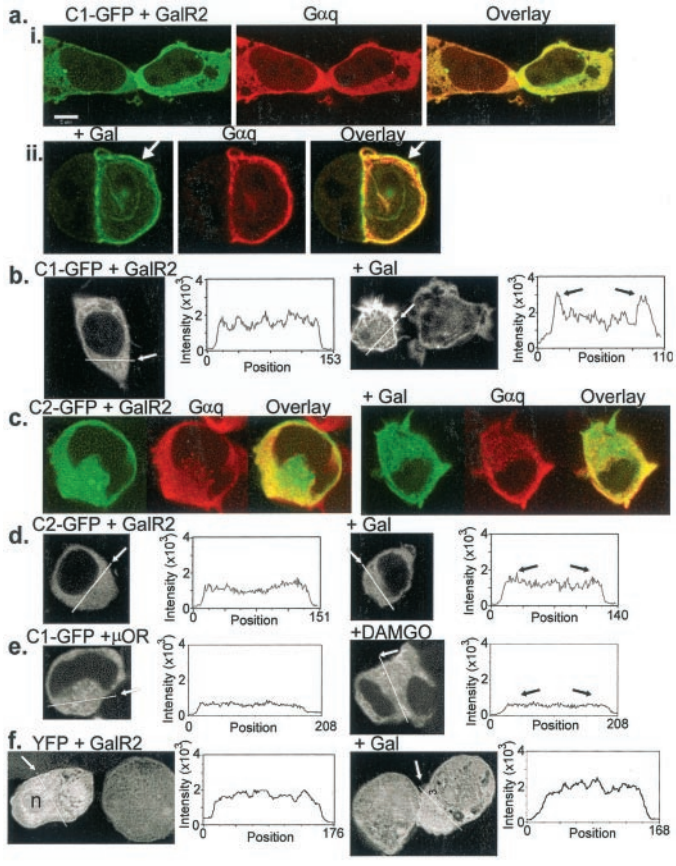

Figure 4. The influence of GalR2 receptor activation on the localization of C terminal fragments of α1B. tsA-201 cells were transfected with GFP-C1, GFP-C2, or a vector expressing the GFP protein alone, GalR2 or μOR, Gαq, or Gαi and were then immunostained with an anti-Gαq antibody and a Cy5-conjugated secondary antibody. Data were obtained from two to five separate transfections in each case. *a*, In the absence of galanin (*a, i*), GFP-C1 as well as Gαq were seen to be randomly distributed throughout the cytoplasm ($n = 24$ cells examined). After the application of galanin (*a, ii*), both Gαq and GFP-C1 were seen to translocate to the plasma membrane ($n = 30$ cells examined). *b, Left*, Line scan of GFP fluorescence through a cell expressing C1-GFP and GalR2 without galanin application. *b, Right*, Line scan of GFP fluorescence through a cell expressing C1-GFP and GalR2 with galanin application. Note the enhancement near the plasma membrane (*arrows*). *c*, In the absence or presence of galanin, GFP-C2 as well as Gαq were seen to be randomly distributed throughout the cytoplasm. *d, Left*, Line scan of GFP fluorescence through a cell expressing C2-GFP and GalR2 without galanin application ($n = 8$ cells examined). *d, Right*, Line scan of GFP fluorescence through a cell expressing C2-GFP and GalR2 with galanin application. Note the lack of enhancement near the plasma membrane (*arrows*; $n = 24$ cells examined). *e, Left*, Line scan of GFP fluorescence through a cell expressing C1-GFP, μOR, and Gαi without DAMGO application ($n = 11$ cells examined). *e, Right*, Line scan of GFP fluorescence through a cell expressing C1-GFP, μOR, and Gαi with DAMGO application ($n = 17$ cells examined). *f, Left*, Line scan of GFP fluorescence through a cell expressing GFP and GalR2 without galanin application ($n = 10$ cells examined). *f, Right*, Line scan of GFP fluorescence through a cell expressing GFP and GalR2 with galanin application ($n = 9$ cells examined). *YFP*, Yellow-shifted GFP.

and various α_{1B} C-terminal fragments fused to GFP were co-expressed in tsA-201 cells, and immunoprecipitation experiments were performed (Fig. 5). These fragments included GFP-C1, GFP-C2, aa 1871–2339 of $\alpha_{1B\text{-}1}$ (GFP-CC1), aa 1871–2237 of $\alpha_{1B\text{-}2}$ (GFP-CC2), aa 2164–2339 of $\alpha_{1B\text{-}1}$ (GFP-CCC1), aa 1768–2109 of $\alpha_{1B\text{-}1}$ (GFP-N), aa 1768–2009 of $\alpha_{1B\text{-}1}$ (GFP-NN), and aa 1768–1875 of $\alpha_{1B\text{-}1}$ (GFP-NNN). These constructs are illustrated

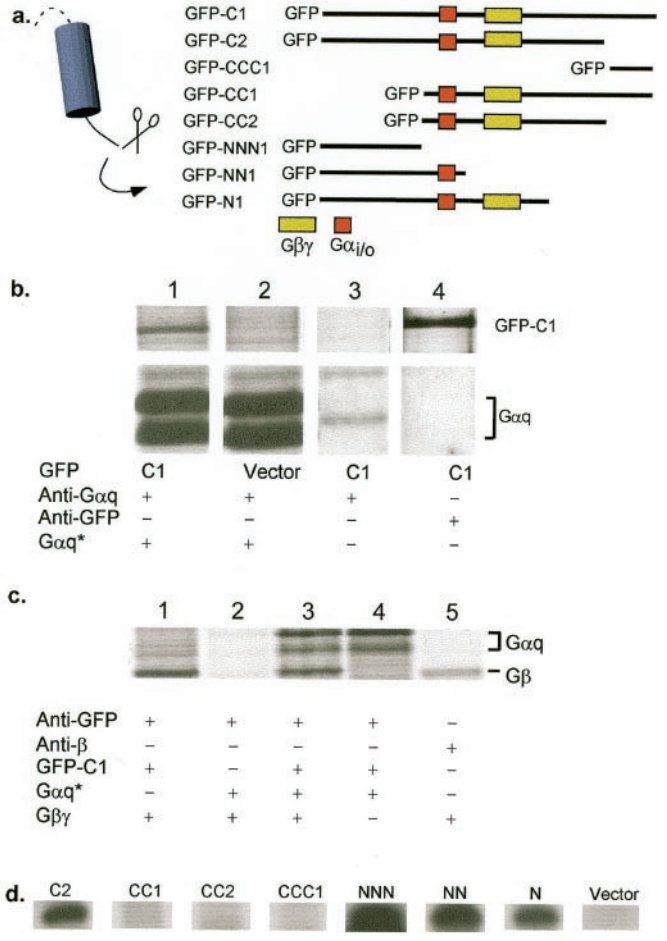

Figure 5. Immunoprecipitation experiments demonstrate Gαq binding to the C terminus of α1B. tsA-201 cells were co-transfected with various GFP fusion constructs and constitutively active rat Gαq (Gαq*). Immunoprecipitations were then performed as described in Materials and Methods. *a,* Graphical summary of the GFP fusion constructs used in this study. Portions of the C terminus of $\alpha_{1B\text{-}1}$ and $\alpha_{1B\text{-}2}$ were expressed as fusions with the GFP protein. Regions implicated in Gβγ (Qin et al., 1997) and Gαi/Gαo (Furukawa et al., 1998a,b) binding are shown. *b*, An anti-rat Gαq antibody that recognizes rat but not human Gαq immunoprecipitated a GFP fusion of the C terminus of $\alpha_{1B\text{-}1}$ (GFP-C1; *lane 1*) but not GFP alone (*lane 2*). No similar bands were immunoprecipitated in cells transfected with GFP-C1 but not Gαq* (*lane 3*). An immunoprecipitation of GFP-C1 with an anti-GFP antibody from cells transfected with GFP-C1 alone is shown for size comparison (*lane 4*). *c*, Immunoprecipitations were also performed with an anti-GFP antibody. The anti-GFP antibody co-immunoprecipitated Gαq* from cells expressing Gαq* and GFP-C1 (*lane 4*) but not from cells expressing Gαg* in the absence of GFP-C1 (*lane 2*). The anti-GFP antibody also co-immunoprecipitated Gβy from cells transfected with GFP-C1 as well as Gβγ (*lane 1*). Coexpression of Gαq* did not block Gβγ association with the C terminus (*lane 3*). An immunoprecipitation of Gβ with an anti-Gβ antibody is shown for size comparison (*lane 5*). *d*, Immunoprecipitation of Gαq in cells overexpressing Gαq*, Gβγ, and various GFP fusion molecules created from the C terminus of the α_{1B} channel. The construct *NNN* bound Gαq*, suggesting that aa 1768–1875 of the channel are sufficient for Gαq* binding.

in Figure 5*a*. Note that the GFP-CCC1 construct corresponds to the region of $\alpha_{1B\text{-}1}$ that differs from $\alpha_{1B\text{-}2}$. Also note that the GFP-CC1 construct corresponds to the portion of $\alpha_{1B\text{-}1}$ that was deleted in the Δ1875–2339 construct.

As shown in Figure 5*b, lane 1*, a rat-specific anti-Gαq antibody co-immunoprecipitated GFP-C1 in cells expressing rat Gαq* and GFP-C1. Anti-$G\alpha_q$ did not co-immunoprecipitate GFP in cells expressing Gαq* and GFP (Fig. 5*b, lane 2*) and did not co-immunoprecipitate C1-GFP in cells that were not expressing rat Gαq* (Fig. 5*b, lane 3*). As a molecular weight comparison, the GFP-C1 fragment was immunoprecipitated by an anti-GFP antibody (Fig. 5*b, lane 4*).

Similar results were obtained when immunoprecipitations were performed with an anti-GFP antibody (Fig. 5*c*). In cells expressing Gαq*, Gβ1, Gγ3, and GFP-C1, an anti-GFP antibody immunoprecipitated Gαq* as well as Gβγ (Fig. 5*c, lane 3*). The antibody co-immunoprecipitated Gβγ alone in cells expressing Gβγ and GFP-C1 (Fig. 5*c, lane 1*) and Gαq* alone in cells expressing Gαq* and GFP-C1 (Fig. 5*c, lane 4*). Neither Gαq* nor Gβγ was immunoprecipitated in cells expressing Gαq* and Gβγ but no GFP-C1 (Fig. 5*c, lane 2*). Gβ1 was co-immunoprecipitated with an anti-Gβ antibody for molecular weight comparison (Fig. 5*c, lane 5*).

To identify the region of the C terminus that interacts with Gαq, various portions of the C terminus (see Fig. 5*a*) were expressed as GFP fusion molecules in tsA-201 cells along with rat Gαq*, and cell lysates were subjected to immunoprecipitations with an anti-rat Gαq antibody as well as an anti-GFP antibody for molecular weight determination. As shown in Figure 5*d*, The GFP-C2, GFP-NNN, GFP-NN, and GFP-N constructs co-immunoprecipitated with Gαq*, but the GFP, GFP-CC1, GFP-CC2, and GFP-CCC1 constructs did not. These results suggest that Gαq binds to the C terminus of $\alpha_{1B\text{-}1}$ as well as $\alpha_{1B\text{-}2}$ and that the N-terminal portion of the C terminus is sufficient for Gαq binding. The interaction of Gαq* with the C terminus of α_{1B} is similar to the findings of Furukawa et al. (1998a,b), who showed that Gαi interacts with the C terminus of α_{1B}. This is the first demonstration that Gαq binds to Ca channels. We were unable to directly assess the role of this portion of the C terminus by electrophysiology, because deletion (construct Δ1768–2339) rendered the channel nonfunctional (data not shown).

Immunoprecipitation of Gβγ in cells transfected with GFP-C1 and Gβ1γ3 confirms the findings of Qin et al. (1997) and Furukawa et al. (1998a,b), who showed that Gβγ interacts with the C terminus of α_{1B}. Overexpression of Gαq* failed to block the ability of an anti-GFP antibody to immunoprecipitate Gβγ. These data suggest that although Gαq and Gβγ both bind to the C terminus of $\alpha_{1B\text{-}1}$, displacement of Gβγ binding to the C terminus by Gαq is unlikely to be taking place. Our electrophysiology experiments suggest that truncation of the C terminus could block the ability of Gαq to inhibit modulation, suggesting that regions not required for Gαq binding are also involved in producing these effects.

The role of PKC

Binding of Gαq to the N-terminal portion of the C terminus (aa 1768–1875) suggests that Gαq is probably not directly involved in the differential translocation of the C terminus of $\alpha_{1B\text{-}1}$ and $\alpha_{1B\text{-}2}$ in our imaging experiments or the differential susceptibility of the two channels to modulation by GalR2. However, our overexpression studies clearly suggest that Gαq is capable of occluding modulation and that this effect of Gαq is lost when regions C-terminal to this Gαq binding site are disrupted. Gαq may therefore exert its effects indirectly. Because Gαq-coupled receptors can activate PKC, and PKC can block Gβγ effects by phosphorylation of Thr-422 on the I/II loop of the channel (Hamid et al., 1999), we tested the hypothesis that PKC activation by GalR2 was involved in blocking Gβγ effects. When HEK-293 cells ex-

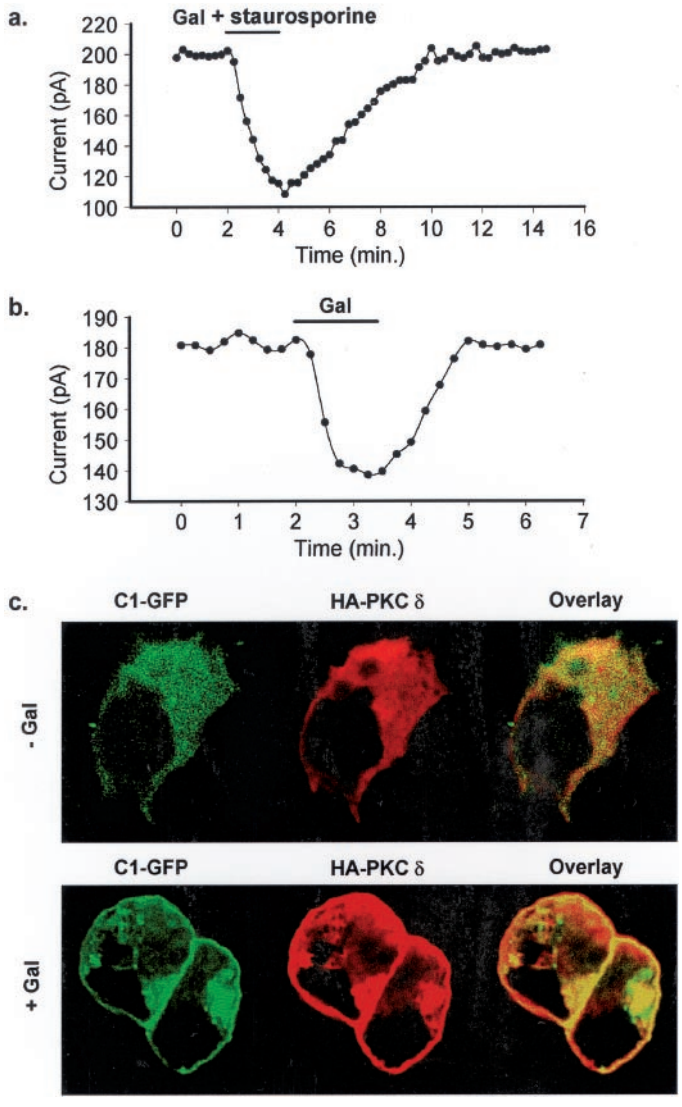

Figure 6. Involvement of PKC. *a*, HEK-293 cells were transfected with GalR2, α_{1B-1}, β1b, α2/δ, and CD8α. Galanin (100 nM) was applied simultaneously with staurosporine (1 μM) at the times indicated. In contrast to observations in the absence of staurosporine, staurosporine and galanin caused marked current inhibition. *b*, HEK-293 cells were transfected with GalR2, α_{1B-1}, β1b, α2/δ, CD8α, and cDNA for a kinase-inactive PKC-δ [HA-PKC-δ-kinase dead (Kd)]. Expression of HA-PKC-δ-kd was verified by immunostaining (data not shown). When galanin (100 nM) was applied, marked current inhibition was observed. *c*, tSA-201 cells were transfected with GalR2, C1-GFP, and HA-PKC-δ. In the absence of galanin, C1-GFP and HA-PKC δ were found evenly distributed throughout the cytoplasm (*top panel*). After exposure to galanin, both proteins translocated to the plasma membrane (*bottom panel*).

pressing α_{1B-1} and GalR2 were exposed to galanin and the PKC inhibitor staurosporine (1 μM) simultaneously, marked voltage-dependent inhibition was observed (Fig. 6*a*). Currents were inhibited by 49.3 ± 8.3% (n = 12), in contrast to the lack of inhibition observed in the absence of staurosporine (Fig. 1*d*). Currents after a prepulse were inhibited by 21.7 ± 1.9% (n = 12), significantly less than the inhibition observed before a prepulse ($p < 0.05$). These results suggest that the inhibition seen in the presence of staurosporine is substantially but not completely voltage-dependent.

A number of groups have demonstrated that phorbol esters can increase Ca currents and reduce G-protein modulation of Ca channels (Zhu and Ikeda, 1994; Stea et al., 1995; Hamid et al., 1999). Stea et al. (1995) observed that staurosporine applied at 5–10 μM blocked the effects of phorbol esters and metabotropic glutamate receptor activation on Ca currents. PKC-δ, a novel-type PKC, has been shown by a number of groups to be activated by Gq-coupled receptors. For example, PKC-δ has been implicated in the actions of α1-adrenergic receptors (Rohde et al., 2000), AT1-type angiotensin receptors (Muscella et al., 2000), and purinergic receptors (Shirai et al., 2000). When HEK-293 cells were transfected with a kinase-inactive PKC-δ, α_{1B-1}, and GalR2, galanin was observed to cause voltage-dependent inhibition of the currents. Galanin caused 31.6 ± 6.6% (n = 5) inhibition of currents before a prepulse and 17.6 ± 6.2% inhibition of currents after a prepulse, suggesting that the inhibition was substantially but not completely voltage-dependent and somewhat lower in magnitude than the inhibition observed in the presence of staurosporine (Fig. 6*b*).

To further confirm the involvement of PKC, tsA-201 cells were transfected with GalR2, C1-GFP, and a hemaglutinin (HA) tagged PKC-δ (HA-PKC δ). In the absence of galanin, C1-GFP and HA-PKC δ were seen to be distributed throughout the cytoplasm. When cells were exposed to galanin, both molecules translocated and were co-localized at the cell surface (Fig. 6*c*). These results are consistent with the notion that the C terminus of α_{1B-1} associates with PKC, possibly through a modular adapter protein (Jaken and Parker, 2000), and associates with the membrane through such an interaction. These experiments suggest that PKC-δ is involved, but we cannot exclude the involvement of other PKC isoforms on the basis of these experiments.

DISCUSSION

The experiments reported here seek to determine why activation of Gαq-coupled GPCRs fails to produce voltage-dependent inhibition of N-type Ca channels. The results suggest that the Gα subunit linked to such receptors may play an essential role in producing this selectivity and that the C terminus of the channel plays an important role in mediating these effects. Although Gαq binds to the proximal (N-terminal) portion of the C terminus, we also observed evidence for a functional role of the distal end of the C terminus in our electrophysiological and imaging experiments. Although this region of the channel appeared not to be necessary for binding Gαq, both the electrophysiological and imaging data suggest that it plays some role in mediating the effects of Gαq.

Perhaps the most compelling model to account for our results is a model in which Gαq that is associated with the proximal portion of the C terminus of the channel locally activates PKC, which in turn phosphorylates the channel and blocks G$\beta\gamma$-mediated inhibition (Fig. 7). PKC may indirectly associate with the distal portion of the C terminus of the channel, possibly through modular PDZ domain-containing adapter proteins (Jaken and Parker, 2000). This model can account for our finding that Gαq antagonizes G$\beta\gamma$ effects but does not directly bind to regions of the C terminus that appear to be necessary for such antagonism. The C terminus of the channel may act as a molecular scaffold, bringing the channel, Gαq, and PKC into close proximity.

Our results are consistent with the findings of Kammermeier and Ikeda (1999), who showed that overexpression of regulator of G-protein signaling 2 (RGS2) in sympathetic neurons, a Gq/G11-specific RGS protein, caused PTX-resistant Ca channel inhibition by metabotropic glutamate to switch from voltage-

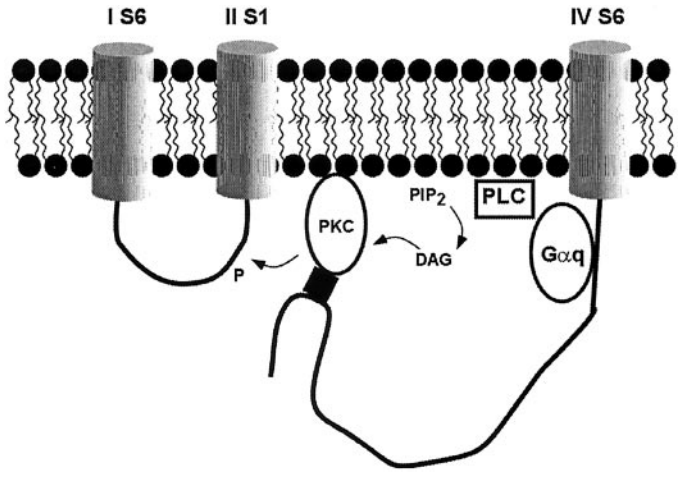

Figure 7. A proposed model accounting for the observed effects of Gαq and PKC on modulation by galanin receptors. Gαq associated with the proximal portion of the C terminus may activate phospholipase C proximal to the Ca channel complex. PKC may associate indirectly with the distal end of the C terminus of the Ca channel. When activated, this channel- and membrane-associated PKC may phosphorylate the I/II loop of the channel and may suppress modulation by Gβγ. *I S6*, Sixth membrane spanning segment of domain I; *II S1*, first membrane spanning segment of domain II; *IV S6*, sixth membrane spanning segment of domain IV; *PLC*, phospholipase C; PIP_2, phosphatidylinositol 4,5-bisphosphate; *DAG*, diacyl glycerol.

independent to voltage-dependent. The results described in that study are similar to ours in that both reports suggest that Gβγ released from Gq-coupled receptors can inhibit calcium channels. Both studies also suggest that Gq somehow occludes the actions of Gβγ. Kammermeier and Ikeda (1999) used RGS overexpression to directly antagonize Gq actions, and we have used calcium channel mutations to generate calcium channels that are less susceptible to the actions of Gq. We find that Gαq affects primarily the magnitude of the inhibition produced by receptor activation, whereas Kammermeier and Ikeda (1999) observe effects primarily on the voltage dependence of the inhibition.

Our electrophysiological data suggest that deletion of the Gβγ binding region (construct Δ2037–2087) in the C terminus "unmasks" G-protein modulation from Gαq-coupled receptors, similar to the effects of deletions of the C-terminal end of the C terminus. This may suggest that this region (CC14) mediates some of the affinity of Gαq for the C terminus. However, this seems unlikely, because the Gβγ binding region on the C terminus (Qin et al., 1997) does not overlap with the Gαq binding region we identified or the Gαi binding region identified by Furukawa et al. (1998a,b). Alternatively, disruption of the CC14 region may cause a conformational change in the C terminus that nonspecifically disrupts Gαq binding or other aspects of C-terminal function. These data are consistent with the notion that there is some critical spatial relationship between the proximal and distal portions of the C terminus that is required for occlusion. This model can account for the fact that deletion of distal portions of the C terminus as well as internal deletions in the C terminus block the masking effect, because both alterations would alter the structural relationship between the distal and proximal C terminus. The lower degree of voltage dependence of inhibition for this construct compared with Δ1875–2339 or α_{1B-2} may suggest that this smaller structural alteration partially but not completely disrupts a Gαq-dependent blocking mechanism. Our data also suggest that Gαq binding does not prevent Gβγ binding to the C terminus, and indeed the portion of the C terminus involved in Gαq binding was not implicated in Gβγ binding in previous studies (Qin et al., 1997; Furukawa et al., 1998a,b). Because the C terminus of α_{1B-1} as well as the truncated C terminus of α_{1B-2} appear to bind Gαq, but only the former shows occlusion of Gβγ effects, it appears that Gαq binding per se cannot block Gβγ effects. The N-terminal portion of the C terminus also appears to be necessary for channel expression.

There is some evidence in the literature that Gαq may preferentially couple with Gβ5 subunits (Fletcher et al., 1998). Some reports have suggested that Gβ5 may modulate Ca channels less effectively than other Gβ subunits (Garcia et al., 1998), although Gβ5γ2 can produce voltage-dependent inhibition (Zhou et al., 2000). Gβ5 may not always be effective in this regard, owing to the formation of heterodimers between Gβ5 and GGL domain-containing RGS proteins limiting the Gβ5 pool available for inhibition (Zhou et al., 2000). However, this mechanism is unlikely to account entirely for the lack of ability of GalR2 to modulate Ca currents in our experiments, because truncation of the C terminus allowed modulation to take place. Kammermeier and Ikeda (1999) also suggested that Gβγ subunits released on metabotropic Glu receptor activation were capable of producing voltage-dependent Ca channel inhibition when Gαq function was blocked. It is, however, possible that preferential association between Gβ5 and Gαq played some role in the reduced ability of GalR2, compared with opioid receptors, for example, to modulate Ca channels with C-terminal alterations in our experiments.

Gαq appears to be similar to Gαs, Gα12, and Gα13 and different from other Gα subunits in that it is palmitoylated but not myristoylated. Evanko et al. (2000) have recently shown that Gαq requires Gβγ for membrane association. This would imply that a fraction of Gβγ may be unavailable for interactions with Ca channels in Gαq-overexpressing cells by virtue of interactions with Gαq. Similarly, a greater capacity of Gαq to serve as a Gβγ "sink" may account for some of the effects of Gαq overexpression. Such an effect of Gα subunits has been suggested by Jeong and Ikeda (1999). However, neither model can account for some of the substantial voltage-dependent modulation that we observed after perturbing the C terminus of the channel, although again these mechanisms may contribute to the relatively smaller effect of G_q- compared with G_i/G_o-coupled receptor activation in our experiments.

Interestingly, the region of α_{1B} implicated in Gαq binding in our experiments has also been found to mediate the binding of calmodulin to the Ca channel (Lee et al., 1999; Peterson et al., 1999). Both Gα and Gβγ subunits (Liu et al., 1997) have been shown to interact with calmodulin, and calmodulin is thought to interact directly with G-protein receptors (Hishinuma et al., 1998; Wang et al., 1999). We cannot, therefore, rule out the possibility that calmodulin mediates the interaction between G-proteins and the C terminus of Ca channels. However, such interactions seem unlikely to require activated calmodulin, because we observed a blocking of Gβγ effects by Gαq in the presence of the Ca buffer BAPTA.

Other GPCR effectors such as adenylate cyclase, phospholipase C, and G-protein-coupled inward rectifier potassium channels (GIRKs) can also be dually modulated by both Gα and Gβγ subunits (Camps et al., 1992; Katz et al., 1992; Jhon et al., 1993; Schreibmayer et al., 1996; Huang et al., 1997). In the case of GIRKs, for example, it has been demonstrated that both Gα and Gβγ can bind to GIRK. Similar to our observations on Ca

channels, expression of certain Gα isoforms can occlude Gβγ activation of GIRKs (Peterson et al., 1999). It is therefore possible that occlusion of Gβγ effects by certain Gα isoforms is a mechanism that is conserved among a variety of G-protein effectors.

The fact that the α_{1B-2} splice variant is modulated by GalR2 receptor activation suggests that some neuronal Ca channels may be susceptible to modulation by Gαq-coupled receptors depending on the nature of their C terminus. Indeed, a recent report (Tanaka et al., 2000) suggesting that Gαq may be concentrated at neuronal terminals may imply that C-terminal splicing is one mechanism that determines the sensitivity of particular presynaptic Ca channels to G-protein-mediated inhibition.

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