Identification of Residues in the N Terminus of $\alpha 1B$ Critical for Inhibition of the Voltage-Dependent Calcium Channel by $G\beta\gamma$

Carles Cantí, Karen M. Page, Gary J. Stephens, and Annette C. Dolphin

Department of Pharmacology, University College London, London WC1E 6BT, United Kingdom

To examine the role of the intracellular N terminus in the G-protein modulation of the neuronal voltage-dependent calcium channel (VDCC) $\alpha 1B$, we have pursued two routes of investigation. First, we made chimeric channels between $\alpha 1B$ and $\alpha 1C$, the latter not being modulated by $G\beta\gamma$ subunits. VDCC $\alpha 1$ subunit constructs were coexpressed with accessory $\alpha 2\delta$ and $\beta 2a$ subunits in *Xenopus* oocytes and mammalian (COS-7) cells. G-protein modulation of expressed $\alpha 1$ subunits was induced by activation of coexpressed dopamine (D2) receptors with quinpirole in oocytes, or by cotransfection of $G\beta 1\gamma 2$ subunits in COS-7 cells. For the chimeric channels, only those with the N terminus of $\alpha 1B$ showed any G-protein mod-

ulation; further addition of the first transmembrane domain and I-II intracellular linker of $\alpha 1B$ increased the degree of modulation. To determine the amino acids within the $\alpha 1B$ N terminus, essential for G-protein modulation, we made mutations of this sequence and identified three amino acids (S48, R52, and R54) within an 11 amino acid sequence as being critical for G-protein modulation, with I49 being involved to a lesser extent. This sequence may comprise an essential part of a complex $G\beta\gamma$ -binding site or be involved in its subsequent action.

Key words: calcium channel; neuronal; G-protein; $\alpha 1$ subunit; $G\beta\gamma$ subunit; modulation

The inhibition of N- ($\alpha 1B$) and P/Q-type ($\alpha 1A$) calcium currents by receptors, usually acting through pertussis toxin-sensitive G-proteins, appears to be mediated by $G\beta\gamma$ subunits (Herlitze et al., 1996; Ikeda, 1996). There has been some controversy concerning whether the $\alpha 1E$ calcium channel is G-protein-modulated (Page et al., 1998). We have now established that, whereas an N-terminally truncated isoform of rat $\alpha 1E$ is not subject to modulation, an isoform with a full-length N terminus is G-protein-modulated, either by coexpression of $G\beta\gamma$ subunits or by activation of a G-protein-coupled receptor (Page et al., 1998), which would agree with results obtained previously for full-length human $\alpha 1E$ (Qin et al., 1997).

A number of recent studies have established the importance of the intracellular loop that links transmembrane domains I and II, both in binding $G\beta\gamma$ and in mediating its effects to produce inhibition of the channel (Herlitze et al., 1997; Zamponi et al., 1997). However, this result is controversial, and several studies have suggested either that the I-II loop plays no role in G-protein modulation of $\alpha 1B$ (Zhang et al., 1996) or $\alpha 1E$ (Qin et al., 1997), or that alone it cannot mediate the effects of the $G\beta\gamma$ subunits (Page et al., 1997, 1998; Simen and Miller, 1998). Nevertheless it is not disputed that the I-II loops of $\alpha 1A$, B, and E comprise a major binding site or sites for $G\beta\gamma$ and contain a QxxER amino

acid consensus sequence common to many $G\beta\gamma$ -binding sites (De Waard et al., 1997; Herlitze et al., 1997; Zamponi et al., 1997; Dolphin et al., 1999). Secondly, a C-terminal $G\beta\gamma$ -binding site has recently been identified and proposed to be a region responsible for G-protein inhibition of human $\alpha 1E$ (Qin et al., 1997). However, it is clear that there are also a number of other sites in the $\alpha 1$ subunit of G-protein-modulated calcium channels that are involved in expression of the inhibition by $G\beta\gamma$. First, we have found that part of the intracellular N terminus of $\alpha 1B$ and $\alpha 1E$ is essential for their G-protein modulation (Page et al., 1998). Second, the transmembrane domain I has been found to have an important role (Zhang et al., 1996; Stephens et al., 1998b).

In the present study we have examined the critical nature of the intracellular N terminus of $\alpha 1B$, by making chimeric channels between $\alpha 1B$, which is strongly G-protein-modulated and $\alpha 1C$, which is not G-protein-modulated by this mechanism, and has a completely different N-terminal sequence. We have shown an absolute requirement for the $\alpha 1B$ N terminus for observation of G-protein modulation in all the chimeric constructs. Second, we have made specific deletions and point mutations to identify the sequence in the N terminus of $\alpha 1B$ that is responsible for conferring G-protein modulation.

MATERIALS AND METHODS

Materials

The following cDNAs were used: rat $\alpha 1C$ (isoform CII, GenBank accession number M67515), rabbit $\alpha 1B$ (D14157), rat $\beta 2a$ (M80545), rat $\alpha 2\delta$ -1 (neuronal splice variant, M86621), rat D2_{long} receptor (X17458, N5→G), bovine $G\beta 1$ (M13236), bovine $G\gamma 2$ (M37183), the C-terminal minigene of β -ARK (M34019), and mut-3 green fluorescent protein (GFP; U73901). All cDNAs were subcloned into the expression vector pMT2 (Swick et al., 1992).

Construction of chimeras

Chimeras were created using PCR following the methods described previously (Page et al., 1998; Stephens et al., 1998b). All constructs were subcloned into the pMT2 vector, and the sequences of the PCR products

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Correspondence should be addressed to Prof. A. C. Dolphin, Department of Pharmacology (Medawar Building), University College London, Gower Street, London WC1E 6BT, UK.

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were confirmed using cycle-sequencing. The constructs were assembled as follows: bCCCC, amino acid residues 1–95 of $\alpha 1B$, 125–2143 of $\alpha 1C$; bBcCCC 1–359 $\alpha 1B$, 409–2143 $\alpha 1C$; bBbCCC 1–483 $\alpha 1B$, 525–2143 $\alpha 1C$; cBcCCC 1–124 $\alpha 1C$, 96–359 $\alpha 1B$, 409–2143 $\alpha 1C$; cBbCCC 1–124 $\alpha 1C$, 96–483 $\alpha 1B$, 525–2143 $\alpha 1C$; cCbCCC 1–408 $\alpha 1C$, 360–483 $\alpha 1B$, 525–2143 $\alpha 1C$; and cCbCCC 1–95 $\alpha 1B$, 125–408 $\alpha 1C$, 360–483 $\alpha 1B$, 525–2143 $\alpha 1C$. Chimeric primers were used with the reverse primer CCA CCA GCA GGT CCA GGA TAT TGA (R1). The resulting PCR product was extended against a template using a forward primer (pMT2F2) directed against the vector TCT CCA CAG GTG TCC ACT. The following chimeric primers were used: GTG CTG GGT GTG CTG AGC GGA GAG TTT for bBcCCC; CAG CCA GTA GAA GAC CTG TGC CTT CAC CAT (reverse primer R2) for bBbCCC; CAC CGA GTG GCC TCC ATT TGA AAT AAT T for bCCCC. These chimeras were used as templates to make others. The primers TTT GAG CGG AGA GTT TGC TAA GG and R2 were used to make the first PCR product, which was then extended on bCCCC to give bCbCCC. The chimeras cBbCCC and cCbCCC were made using bBbCCC and bBcCCC as templates. In each case, the PCR product made using the primers TGT TGA ATG GAA ACC GTT CGA GTA CAT G and R1 was extended on $\alpha 1C$ pMT2 template to add the N terminus of $\alpha 1C$. For cCbCCC, restriction digestion of an *MfeI* site in domain I was used to substitute the N terminus of bCbCCC with that of $\alpha 1C$.

Construction of N-terminal deletion and point mutations

The $\alpha 1B$ N terminus was truncated at the 5' end by introducing a start codon before amino acid E7 to make $\alpha 1B$ $\Delta 2$ –6, Y45 ($\alpha 1B$ $\Delta 2$ –44), and Q51 ($\alpha 1B$ $\Delta 2$ –50). The following primers were used: CGC ACT AGT ATG GAG CTG GGC GGC CGC TAT ($\Delta 2$ –6), CAG ACT AGT ATG TAC AAA CAG TCG ATC GCG ($\Delta 2$ –44), and CAG ACT AGT ATG CAG CGC GCG CGG ACC AT ($\Delta 2$ –50). The $\alpha 1B$ $\Delta 45$ –55 construct was made by using the primer GGC CAG CGG GTC CTC ATG GCG CTG TAC AAC to delete the 11 amino acids, YKQSIQRART. For all of the $\alpha 1B$ point mutations, primers were designed so that single residues were mutated to alanines or so that a number of residues were mutated within the same primer. The following primers were used: R52A–R54A, TCG ATC GCG CAG GCC GCG GCG ACC ATG GCG CT; Y45A, CAG CGG GTC CTC GCC AAA CAG TCG ATC; K46A, CGG GTC CTC TAC GCA CAG TCG ATC GCG; Q47A, GTC CTC TAC AAA GCG TCG ATC GCG CAG; S48A, CTC TAC AAA CAG GCG ATC GCG CAG C; I49A, TAC AAA CAG TCG GCC GCG CAG CGC GCG; Q51A, CAG TCG ATC GCG GCG GCG GCG CGG ACC; R52A, TCG ATC GCG CAG GCC GCG GCG ACC ATG; R54A, GCG CAG CGC GCG GCG ACC ATG GCG CTG; 45YKQSIQA→AAAAA, GCC GCA GCA GCT GCC GCG CAG GCG GCG GCG (forward) and GGC AGC TGC TGC GCG GAG GAC CCG CTG (reverse); and 45YKQ→AAA, CGG GTC CTC GCC GCA GCG TCG ATC GCG CAG. The reverse primer used in each case was GTC GCT TCT GCT CTT CTT GG. For the PCR extension reactions, the forward primer used was AGC ACT AGT ATG GTC CGC TTC GGG GAC. The sequences of all constructs were verified.

Expression of constructs and electrophysiological recording

Xenopus oocytes. Adult female *Xenopus laevis* were killed by anesthetic overdose in a 0.25% solution of tricaine, decapitated, and pithed. Oocytes were removed and defolliculated by treatment with 2 mg/ml collagenase type Ia in a Ca^{2+} -free ND96 saline containing (in mM): NaCl, 96; KCl, 2; $MgCl_2$, 1; and HEPES, 5, pH-adjusted to 7.4 with NaOH for 2 hr at 21°C. Plasmid cDNAs for the different $\alpha 1$ subunits, plus accessory $\beta 2a$ and $\alpha 2\delta$ subunits and rat D2 receptors, were mixed in a ratio of 3:4:1:3 (except where stated), and ~10 nl was injected into the nuclei of stage V or VI oocytes. Injected oocytes were incubated at 18°C for 3–7 d in ND96 saline (as above plus 1.8 mM $CaCl_2$) supplemented with 100 μ g/ml penicillin, 100 IU/ml streptomycin (Life Technologies, Gaithersburg, MD), and 2.5 mM Na pyruvate. Whole-cell recordings from oocytes were made in the two-electrode voltage-clamp configuration with a chloride-free solution containing (in mM): Ba(OH)₂, 5; TEA-OH, 80; NaOH, 25; CsOH, 2; and HEPES, 5 (pH 7.4 with methanesulfonic acid). In all experiments, oocytes were injected with 30–40 nl of a 100 mM solution of K₃-1,2-bis (aminophenoxy) ethane-*N,N,N',N'*-tetra-acetic acid (BAPTA) in order to suppress endogenous Ca^{2+} -activated Cl^- currents. Electrodes contained 3 M KCl and had resistances of 0.3–2 M Ω . The holding potential (V_H) was –100 mV, and the test potential (V_t) used for time course studies was 0 mV. All illustrated traces are at this

potential, and the current amplitude was always measured 20 msec after the start of the test pulse. Membrane currents were recorded every 15 sec, amplified, and low-pass filtered at 1 KHz using a Geneclamp 500 amplifier and digitized through a Digidata 1200 interface (Axon Instruments, Foster City, CA). In all cases currents were leak subtracted on-line by a P/4 protocol.

COS-7 cells. Cells were cultured and transfected, using the electroporation technique, essentially as described previously (Campbell et al., 1995a). The $\alpha 1$, $\alpha 2\delta$, $\beta 2a$, and GFP cDNAs were used at 15, 5, 5, and 1 μ g, respectively. When used, $G\beta 1$ and $G\gamma 2$ were included at 2.5 μ g each, or β -ARK was included at 5 μ g. Blank pMT2 vector was included where necessary to maintain the total cDNA at 31 μ g/transfection. Cells were replated using nonenzymatic cell dissociation medium (Sigma, St. Louis, MO), and then maintained at 25°C for between 1 and 16 hr before electrophysiological recording. Maximum GFP fluorescence and voltage-dependent calcium channel (VDCC) expression were observed between 2 and 4 d after transfection (Brice et al., 1997). Ca^{2+} channel currents were recorded using the whole-cell patch technique. Borosilicate glass 2–5 M Ω electrodes were used. The internal (electrode) and external solutions were similar to those described previously (Campbell et al., 1995b). The patch pipette solution contained in mM: Cs aspartate, 140; EGTA, 5; $MgCl_2$, 2; $CaCl_2$, 0.1; K₂ATP, 2; and HEPES, 10; pH 7.2, 310 mOsm with sucrose. GDP β S (2 mM) was included where stated. The external solution contained in mM: tetraethylammonium (TEA) bromide, 160; KCl, 3; $NaHCO_3$, 1.0; $MgCl_2$, 1.0; HEPES, 10; glucose, 4; and $BaCl_2$, 1 or 10, pH 7.4, 320 mOsm with sucrose. Whole-cell currents were elicited from V_H of –100 mV and recorded using an Axopatch 1D amplifier. Data were filtered at 2 kHz and digitized at 5–10 kHz. The junction potential between external and internal solutions was 6 mV, the values given in the figures and text have not been corrected for this. Current records are shown following leak and residual capacitance current subtraction (P/4 or P/8 protocol) and series resistance compensation up to 85%. Current amplitudes were measured 50 msec after the start of depolarization.

All experiments were performed at room temperature (18–20°C). Analysis was performed using pClamp6 and Origin software. Data are expressed as mean $\pm$ SEM. Statistical analysis was performed using paired or unpaired Student's *t* test, as appropriate.

RESULTS

In a previous study we made chimeras between the rat brain $\alpha 1E$ (rbEII) clone, which is not G-protein-modulated, and the strongly modulated $\alpha 1B$. The results of this study showed that rbEII was not modulated because it was N terminally truncated, and a full-length rat $\alpha 1E$ isoform showed clear G-protein modulation, although not to such a great extent as $\alpha 1B$. We further showed the importance of the first domain of $\alpha 1B$ in increasing the extent of G-protein modulation of $\alpha 1B/\alpha 1E$ chimeras (Page et al., 1998; Stephens et al., 1998b), as has another recent study (Simen and Miller, 1998). In the present study, we wished to examine the distinct role of the N terminus of $\alpha 1B$ in G-protein modulation. To do this we have taken two approaches. First, we have made chimeras between $\alpha 1B$ and the $\alpha 1C$ channel, which is not modulated by a $G\beta\gamma$ -mediated pathway under any conditions. Second, we have produced selective deletions and mutations of the $\alpha 1B$ N-terminal sequence. With such constructs we can determine the domains necessary for the expression of G-protein modulation.

G-protein modulation of $\alpha 1B/\alpha 1C$ chimeras by activation of the dopamine D2 receptor

In this part of the study, all channels were expressed with the accessory subunits $\alpha 2\delta$ and $\beta 2a$ (unless stated) in *Xenopus* oocytes, where they were coexpressed with the dopamine D2 receptor. A series of chimeras were made, in which the N terminus, first transmembrane domain, and I-II loop of $\alpha 1B$ were systematically substituted for those in $\alpha 1C$, in different permutations. Figure 1A shows the chimeras that were made, and the nomenclature employed, which uses capital letters for the transmembrane domains and small letters for the intracellular N-terminal and I-II loop. All

Table 1. Biophysical properties and G-protein modulation of calcium channel $\alpha 1$ subunit chimeras in *Xenopus* oocytes

	$\alpha 1B BBB$	$\alpha 1B BbCCC$	$\alpha 1B bC CCC$	$\alpha 1B CbCCC$	$\alpha 1B CcCCC$	$\alpha 1C bB CCC$	$\alpha 1C bC CCC$	$\alpha 1C CbCCC$	$\alpha 1C CCC$
With $\beta 2a$									
I_{Ba} peak amplitude, nA (n)	1195 $\pm$ 155 (8)	1054 $\pm$ 130 (12)	605 $\pm$ 90 (7)	760 $\pm$ 103 (10)	766 $\pm$ 267 (10)	744 $\pm$ 139 (6)	392 $\pm$ 82 (4)	1355 $\pm$ 115 (11)	657 $\pm$ 87 (10)
V_{50} for control I_{Ba} (mV)	−12.0 $\pm$ 1.2	−14.2 $\pm$ 0.8	3.7 $\pm$ 0.9	−8.5 $\pm$ 1.3	+0.4 $\pm$ 1.6	−21.4 $\pm$ 1.3	−8.3 $\pm$ 0.9	−14.8 $\pm$ 1.3	−8.7 $\pm$ 1.8
V_{50} for I_{Ba} plus quinpirole (mV)	−7.8 $\pm$ 1.4	−12.0 $\pm$ 0.9	6.2 $\pm$ 1.0	−7.0 $\pm$ 1.3	1.8 $\pm$ 1.5	−21.7 $\pm$ 1.2	−8.2 $\pm$ 0.4	−14.7 $\pm$ 1.4	−8.9 $\pm$ 1.8
Without $\beta 2a$									
I_{Ba} peak amplitude, nA	381 $\pm$ 46 (8)	805 $\pm$ 212 (6)	824 $\pm$ 161 (6)	636 $\pm$ 138 (7)	No expression	662 $\pm$ 199 (5)	681 $\pm$ 104 (4)	661 $\pm$ 104 (11)	470 $\pm$ 82 (4)
V_{50} for control I_{Ba} (mV)	−1.9 $\pm$ 0.1	−9.1 $\pm$ 1.0	2.5 $\pm$ 1.7	3.5 $\pm$ 1.5		−18.6 $\pm$ 2.0	−7.0 $\pm$ 0.8	−4.7 $\pm$ 0.8	+9.9 $\pm$ 1.9
V_{50} for I_{Ba} plus quinpirole (mV)	11.2 $\pm$ 1.6	−5.4 $\pm$ 1.0	5.2 $\pm$ 1.6	5.4 $\pm$ 1.6		−18.3 $\pm$ 2.4	−6.7 $\pm$ 0.4	−5.0 $\pm$ 0.7	+9.2 $\pm$ 1.7
ΔV_{50} for I_{Ba} due to $\beta 2a$ (mV)	−10.1	−5.1	−3.7	−12.0	Not determined	−2.8	−1.3	−10.1	−18.6

The parameters determined for the different $\alpha 1$ constructs (cotransfected with $\alpha 2\delta$, and either with or without $\beta 2a$) were measured as described in Materials and Methods and in the legend to Figure 1. Individual current density–voltage relationships were fitted with a Boltzmann equation $I = G_{max}/(1 + \exp[-(V - V_{50})/k])$, where G_{max} is the maximum conductance; V_{rev} is the reversal potential; k is the slope factor, and V_{50} is the voltage for 50% current activation. No systematic variation was seen in k , which was between 4.5 and 8 for all constructs, or in V_{rev} , which was between +45 and +51 mV for all constructs. For simplicity, these values are not given. The current amplitude is given at the peak of the I – V relationship, which is between 0 and +10 mV.

left panels), giving steps to potentials between −40 and +40 mV, before (P1) and 10 msec after (P2) a large depolarizing step to +120 mV (Page et al., 1998). The prepulse reverses $G\beta\gamma$ -mediated modulation, and hence P2 acts as an internal control. The $G\beta\gamma$ -mediated modulation was determined from the hyperpolarizing shift in the V_{50} of the current–voltage relationship in P2 compared to that in P1 (Fig. 3, right panels). For $\alpha 1B$, this shift was almost −10 mV (Figs. 3A, 4), and it was not significantly smaller for the chimeras $\alpha 1BbBbCCC$ and $\alpha 1BbCbCCC$ (Figs. 3B, 4). It was reduced, but still significantly different from $\alpha 1C$ for the $\alpha 1BbCbCCC$ and $\alpha 1BbCcCCC$ chimeras (Figs. 3C, 4). Of the other chimeras, all of which had the N terminus of $\alpha 1C$, none showed any greater shift in V_{50} than $\alpha 1C$ itself (Figs. 3D, 4). In control experiments recorded in the absence of coexpressed $G\beta 1\gamma 2$ and in the presence of intracellular GDP βS , the shift in V_{50} caused by a depolarizing prepulse was approximately −1.8 mV for $\alpha 1C$ ($n = 10$), very similar to the value for $\alpha 1C$ coexpressed with $G\beta 1\gamma 2$ (−2.1 mV; Fig. 4). A similar level of control facilitation was observed for $\alpha 1B$ ($n = 10$) (Fig. 4). Similar control results were obtained when the β -ARK1 $G\beta\gamma$ -binding domain was co-expressed, to act as a sink for endogenous $G\beta\gamma$ and prevent tonic modulation (Stephens et al., 1998a,b) (results not shown). This control prepulse potentiation is therefore likely to be caused by a mechanism other than G-protein modulation (Dolphin, 1996). The main discrepancy between the results examining direct $G\beta\gamma$ modulation and those examining receptor-mediated modulation involve the $\alpha 1BbCbCCC$ chimera, which is strongly modulated by overexpression of $G\beta 1\gamma 2$ (Figs. 3B, 4), and more weakly modulated by receptor-mediated inhibition (Figs. 1B, 2D). The reason for this may relate to differences in $G\beta\gamma$ subtype or concentration between the two systems.

Isolation of the amino acid residues of the N terminus essential for G-protein modulation

We have made a number of deletions to determine the amino acid sequences that are essential for G-protein modulation. From our previous study (Page et al., 1998), we found that the truncated $\Delta 1$ –55 $\alpha 1B$ construct was not G-protein modulated, in agreement with the N-terminally truncated $\alpha 1E$ (rbEII) isoform, that is also not G-protein-modulated. In the present series of experiments, the effect of quinpirole (100 nM) was determined during steps to 0 mV, because none of the constructs showed major shifts in voltage dependence of current activation, compared to $\alpha 1B$. Inhibition of wild-type $\alpha 1B$ was $35.3 \pm 2.2\%$ at 0 mV under these conditions ($n = 8$). We made a number of truncations: $\alpha 1B\Delta 2$ –6

and $\Delta 2$ –44, in line with regions of homology between the N-terminal sequences of all the G-protein modulated $\alpha 1$ subunits (Fig. 5A). These two constructs were as strongly G-protein-modulated as $\alpha 1B$ itself [respectively, $35.8 \pm 2.5\%$ ($n = 5$), and $36.1 \pm 5.3\%$ ($n = 6$) inhibition by quinpirole; Fig. 5B,C]. This identifies the 11 amino acid sequence of $\alpha 1B$ 45–55 (YKQSIQRART) (Fig. 5A) as being required for the G-protein modulation of $\alpha 1B$. To confirm this finding, deletion of only this sequence created a construct, $\alpha 1B \Delta 45$ –55, in which G-protein modulation was completely abolished [$-0.7 \pm 2.1\%$ inhibition by quinpirole ($n = 5$); Fig. 5B,C].

Point mutations to alanine (A) were then carried out to identify the specific amino acids in this 11 amino acid sequence that are essential for G-protein modulation. Mutation of both arginines to alanine (R52A, R54A) produced a construct that showed no G-protein modulation ($-2.5 \pm 2.5\%$ inhibition by quinpirole; $n = 8$). Point mutations of the individual amino acids in the QRART sequence (Q51A, R52A, and R54A) subsequently identified both arginines as being critical for G-protein modulation, because either mutation produced a construct that showed almost complete loss of inhibition by quinpirole (Fig. 5B,C).

The N-terminal part of this 11 amino acid sequence also contains residues that are critical for G-protein modulation. When YKQSI was mutated to AAAAA (Fig. 5A), the channel was not G-protein-modulated [$0.3 \pm 2.1\%$ inhibition by quinpirole ($n = 4$); Fig. 5C]. To confirm the importance of the amino acids 45–50 (YKQSIQA), an intermediate deletion $\alpha 1B \Delta N2$ –50 was made, to give a construct starting with methionine followed by Q51. This was also found not to be G-protein-modulated (Fig. 5C). Subsequent point mutations were made of the individual amino acids in the YKQSIQA sequence to A (Y45A, K46A, Q47A, S48A, and I49A). This identified only the serine and, to a lesser extent, isoleucine in the sequence as being involved in G-protein modulation. These mutations resulted in reduced quinpirole-induced inhibition of I_{Ba} to $4.5 \pm 1.0\%$ ($n = 6$) for S48A and $17.4 \pm 2.1\%$ ($n = 11$) for I49A, respectively (Fig. 5C). Although the individual point mutants Y45A, K46A, and Q47A were all strongly G-protein-modulated by quinpirole, the modulation of the construct containing the triple mutation YKQ→AAA was reduced ($18.8 \pm 3.9\%$ inhibition by quinpirole; $n = 5$; Fig. 5C), indicating an influence of these amino acids.

Modulation of N-terminal mutants of $\alpha 1B$ by $G\beta\gamma$

We have confirmed that the identified amino acids are similarly involved in direct $G\beta\gamma$ -induced modulation of $\alpha 1B$ by performing

Figure 2. Voltage dependence of modulation of the chimeras between $\alpha 1B$ and $\alpha 1C$ by activation of the dopamine D2 receptor. The percentage inhibition by quinpirole (100 nM) was determined at voltages between -20 and $+30$ mV, from current–voltage relationships performed in the absence and presence of quinpirole. Measurements were made isochronally, 20 msec after the start of the voltage step. *A*, $\alpha 1B$; *B*, $\alpha 1bBbCCC$; *C*, $\alpha 1bCcCCC$; *D*, $\alpha 1CbCCC$; and *E*, $\alpha 1CcCCC$. Experiments were performed both in the presence (white bars) and the absence (black bars) of overexpressed $\beta 2a$, except for $\alpha 1bCcCCC$, where no expression was seen in the absence of $\beta 2a$. The numbers of experiments (with, without $\beta 2a$) are 8, 6 (*A*); 12, 6 (*B*); 7, 6 (*C*); 10, 6 (*D*); and 9 (*E*). The statistical significance of the differences at each potential between inhibition in the presence and absence of $\beta 2a$ is indicated by $*p < 0.05$; $**p < 0.01$. Example currents in the presence of $\beta 2a$ are given as insets to parts *A–C* for $\alpha 1B$ and for all the chimeras shown. They were expressed as described in the legend to Figure 1. These traces were evoked by a pulse from -100 to 0 mV, and therefore do not show the maximum inhibition. Traces are shown before (*con*) and during quinpirole (100 nM) application (*quin*). *F*, Example traces showing the lack of effect of quinpirole on $\alpha 1C$, $\alpha 1CbCCC$, $\alpha 1CcCCC$, and $\alpha 1CbCCC$, all expressed with $\beta 2a$. The calibration bars are all 50 msec and 500 nA, unless otherwise stated.

experiments with coexpressed $G\beta 1\gamma 2$ in COS-7 cells. Examples of results obtained are shown in Figure 6. For the Q47A mutation, G-protein modulation was still observed, with slowly activating currents in P1 and a clear hyperpolarizing shift in the V_{50} for current activation resulting from the depolarizing prepulse (Fig. 6*A*). In contrast, for the R52A mutation, no G-protein modulation was observed (Fig. 6*B*). The mean results for all the constructs are given in Figure 7, expressed as P2/P1 facilitation ratio at -10 mV (Fig. 7*A*). The V_{50} for the I_{Ba} current–voltage relationship was also plotted, because this shows a depolarizing shift in G-protein-modulated channels, compared to the control $\alpha 1B$ expressed in the absence of $G\beta\gamma$ (Fig. 7*B*). These two sets of measurements are strongly correlated ($r = 0.76$, data not shown), and the depolarizing shift in activation V_{50} is also highly correlated to the percentage inhibition by quinpirole observed for the same constructs in the *Xenopus* oocyte experiments (Fig. 7*C*), suggesting that direct $G\beta\gamma$ modulation and quinpirole-induced modulation of these constructs are using the same mechanism. The I49A mutation stands out in both these systems as pro-

ducing a reduction, but not a complete inhibition of modulation (Fig. 7*C*).

Basis for the reduction in receptor-mediated modulation of the N-terminal point mutation I49A

G-protein modulation of calcium channels is strongly voltage-dependent, in that more inhibition is observed at low than at high depolarizations (Bean, 1989). To examine the basis for the reduced modulation of the partially modulated mutant ($\alpha 1B$ I49A) compared to $\alpha 1B$, we first examined, in *Xenopus* oocytes, the voltage dependence of the removal of inhibition by quinpirole, during a depolarizing prepulse (see Fig. 8*A* for voltage protocol). There was no significant effect of the I49A mutation on the voltage dependence of the prepulse-induced facilitation in the presence of quinpirole (Fig. 8*B*), or on the time course of removal of quinpirole-induced inhibition during a 100 mV depolarizing prepulse. Single exponential fits gave τ values for removal of inhibition (possibly representing dissociation of $G\beta\gamma$ at this depolarized potential) of ~ 20 msec for both constructs (Fig. 8*C*).

Figure 3. Examples of direct modulation by $G\beta 1\gamma 2$ of the chimeras between $\alpha 1B$ and $\alpha 1C$. The $\alpha 1$ subunits shown were coexpressed with $\alpha 2\delta$, $\beta 2a$, $G\beta 1$, and $G\gamma 2$ in COS-7 cells. *Left panel*, Traces obtained before and after a depolarizing prepulse (+120 mV, 100 msec). The prepulse protocol is above the top trace. *Right panel*, Current-voltage relationships (steps from -40 to +50 mV in 10 mV intervals, from a holding potential of -100 mV), measured 50 msec after the start of the step, for the currents in P1 (open circle) and P2 (filled circle). The current-voltage relationships were fitted (solid lines) with a modified Boltzmann equation as given in the legend to Table 1. The mean depolarizing shifts in V_{50} resulting from the depolarizing prepulse are given in Figure 4. *A*, Currents resulting from $\alpha 1B$ expression (currents shown resulting from steps -40 to 0 mV, and recorded in 1 mM Ba^{2+}). *B*, Currents resulting from $\alpha 1bCbCCCC$ expression (steps -40 to +20 mV shown, recorded in 10 mM Ba^{2+}). *C*, Currents resulting from $\alpha 1bCcCCCC$ expression (steps -40 to +20 mV shown, recorded in 10 mM Ba^{2+}). *D*, Currents resulting from $\alpha 1C$ expression (steps -40 to -10 mV shown, recorded in 1 mM Ba^{2+}). In this example the depolarizing prepulse was not preceded by a 10 msec step to the holding potential, but this had no effect on the subsequent results.

The only clear difference between I49A $\alpha 1B$ and wild-type $\alpha 1B$ was in the more rapid time course of reinstatement of G-protein modulation after its removal by a 100 msec prepulse to +100 mV (Fig. 8D). This could be fit to a single exponential with $\tau_{\text{reinstatement}}$ of 187 msec for $\alpha 1B$ and 85 msec for the I49A mutant (Fig. 8D, inset).

If we consider G-protein modulation as a simple bimolecular reaction, as has been done previously (Zhang et al., 1996; Stephens et al., 1998a):

Figure 4. Modulation by $G\beta 1\gamma 2$ of the chimeras between $\alpha 1B$ and $\alpha 1C$. Histogram giving the mean $\pm$ SEM of the hyperpolarizing shifts in V_{50} after a depolarizing prepulse for the same chimeras as in Figure 1. * $p < 0.05$; ** $p < 0.001$ compared to $\alpha 1C/G\beta 1\gamma 2$. All $\alpha 1B$ currents were recorded with 1 mM Ba^{2+} and all chimeras with 10 mM Ba^{2+} as charge carrier. It was checked for parental $\alpha 1B$ that the use of 1 or 10 mM Ba^{2+} did not affect the ΔV_{50} caused by a depolarizing prepulse. For the bars marked control, the parental constructs were expressed without $G\beta\gamma$ subunits, in the presence of GDP β S (1 mM), and a small prepulse-induced hyperpolarizing shift in V_{50} was observed for $\alpha 1B$ and $\alpha 1C$. A similar control shift was also observed for all the chimeras tested [for example for $\alpha 1bCbCCCC$ the control ΔV_{50} was -2.7 ± 0.8 mV ($n = 8$)]. This shift was not significantly different from that for $\alpha 1C$ coexpressed with $G\beta 1\gamma 2$. The number of experiments performed is given at the base of each bar.

$$C + G\beta\gamma \xrightleftharpoons[k_{-1}]{k_1} C \cdot G\beta\gamma$$

where C is one of the closed states of the calcium channel $\alpha 1B$ subunit, k_1 is the association rate constant, and k_{-1} is the dissociation rate constant for $G\beta\gamma$. At equilibrium, from the law of mass action:

$$k_1[G\beta\gamma][C] = k_{-1}[C \cdot G\beta\gamma]$$

at the holding potential, $1/\tau_{\text{reinstatement}} = k_1[G\beta\gamma] + k_{-1}$ (1)

and steady state inhibition = $k_1[G\beta\gamma]/(k_1[G\beta\gamma] + k_{-1})$ (2)

From our previous study (Stephens et al., 1998a), we estimated the $G\beta\gamma$ concentration to reach ~ 300 nM, when $G\beta\gamma$ was overexpressed. Taking an approximate value of 100 nM for the concentration of $G\beta\gamma$ resulting from quinpirole-induced receptor activation in the present study [a value of 130 nM can be calculated making the assumptions described in Stephens et al. (1998a)], we can obtain estimates by substitution of steady-state inhibition and $\tau_{\text{reinstatement}}$ values into Equations 1 and 2, of k_1 and k_{-1} . For $\alpha 1B$, k_1 is $25.2 \mu\text{M}^{-1}\text{sec}^{-1}$, and k_{-1} is 2.8 sec^{-1} , whereas for the I49A mutant k_1 is $21.0 \mu\text{M}^{-1}\text{sec}^{-1}$, and k_{-1} is 9.6 sec^{-1} . Clearly, the major difference is an apparent 3.4-fold increase in the off-rate for $G\beta\gamma$ in the I49A mutant. However, assuming that reassociation of $G\beta\gamma$ is very slow at +100 mV, the dissociation of $G\beta\gamma$ during the prepulse to +100 mV, found from Figure 8C, is 53.2 sec^{-1} for $\alpha 1B$ and 46.7 sec^{-1} for $\alpha 1B$ I49A, indicating that the apparent off-rate is more rapid for both constructs at this depolarized potential [as previously observed in Stephens et al. (1998a)], and the difference between the parental $\alpha 1B$ and the I49A mutant is lost.

Figure 5. The effect of various deletions and point mutations of the N terminus of $\alpha 1B$ on inhibition of I_{Ba} by the D2 agonist quinpirole. The sequence of the N terminus of $\alpha 1B$, with the 11 amino acid sequence identified as being involved in G-protein modulation in **bold**, and the points at which deletions were made shown by **arrows** beneath the sequence. Example traces, showing the effect of quinpirole (100 nM) on I_{Ba} in the $\alpha 1B$ $\Delta 2-44$ mutant (*left*), the $\alpha 1B$ $\Delta 45-55$ mutant (*center left*), the $\alpha 1B$ I49A mutant (*center right*), and the $\alpha 1B$ R54A mutant (*right*). Traces (100 msec duration) were obtained at a test potential of 0 mV, from a holding potential of -100 mV. *Con*, Control traces; *quin*, after perfusion of quinpirole. Histogram of the percentage inhibition by 100 nM quinpirole (mean $\pm$ SEM) of I_{Ba} in the various deletion and point mutants of the N terminus of $\alpha 1B$. The currents were activated at 0 mV, and the degree of inhibition was determined from the currents activated every 15 sec. The number of experiments for each condition is given in *parentheses*, and the significance of the differences compared to the inhibition of $\alpha 1B$ are given by $*p < 0.005$.

DISCUSSION

The molecular determinants for the inhibition of neuronal VDCC $\alpha 1$ subunits by $G\beta\gamma$ have been the subject of several studies. However, there remains no consensus of opinion concerning the functional importance of biochemically identified $G\beta\gamma$ -binding sites on the I-II loop and C terminus (De Waard et al., 1997; Page et al., 1997; Qin et al., 1997; Zamponi et al., 1997) (for review, see Dolphin, 1998). Furthermore, there has been little agreement on the extent of modulation of the E-type VDCCs (Bourinet et al., 1996; Toth et al., 1996; Yassin et al., 1996; Mehrke et al., 1997; Page et al., 1997; Qin et al., 1997). However, following our recent study

Figure 6. Examples of the effect of $\alpha 1B$ N-terminal mutations on $G\beta\gamma$ modulation in COS-7 cells. Coexpression of two $\alpha 1B$ N-terminal mutations with $\alpha 2\delta$, $\beta 2a$, and $G\beta 1\gamma 2$, recorded with 1 mM Ba^{2+} charge carrier. *Left panel*, Current traces are shown, evoked by the same protocol given in Figure 3. *Right panel*, Current-voltage relationships are given, from -40 to $+50$ mV, in 10 mV intervals, before (*open circles*) and after (*filled circles*) the depolarizing prepulse, fitted (*solid lines*) with the modified Boltzmann equation given in the legend to Table 1. *A*, The $\alpha 1B$ Q47A mutation (traces from -40 to 0 mV are shown). *B*, The $\alpha 1B$ R52A mutation (traces from -40 to $+10$ mV are shown).

(Page et al., 1998), it now seems clear that all $\alpha 1E$ orthologues are G-protein-modulated when the long N terminus is present.

Requirement for the N terminus of $\alpha 1B$ for G-protein modulation

The present study was performed to further our understanding of the involvement of the N terminus of the VDCC $\alpha 1B$ in G-protein modulation, first identified by Page et al. (1998). We therefore made a series of chimeras between $\alpha 1B$, which is strongly G-protein-modulated, and $\alpha 1C$, which is not modulated by $G\beta\gamma$, in the systems studied. Our conclusions are that the N terminus of $\alpha 1B$ is absolutely essential for its G-protein modulation. No modulation was observed of any channel that contained the N terminus from $\alpha 1C$. The sequences of the intracellular N termini of $\alpha 1B$ and $\alpha 1C$ show little homology, and it is thus clear that the N terminus of $\alpha 1B$ plays a role in G-protein modulation that cannot be substituted by that of $\alpha 1C$.

Role of the I-II linker and first transmembrane domain of $\alpha 1B$ in G-protein modulation

In contrast to the results concerning the N terminus, the I-II linker of $\alpha 1B$ was not completely essential; significant G-protein modulation was observed in the chimeras $\alpha 1bBcCCC$ and $\alpha 1bCcCCC$, although the extent of modulation was less than for the control $\alpha 1B$. These results are of mechanistic interest because of the inability of the $\alpha 1C$ I-II linker to bind $G\beta\gamma$ (De Waard et al., 1997; Qin et al., 1997; Dolphin et al., 1999), presumably because of the lack of the QxxER-binding motif. It is possible that $G\beta\gamma$ binding to the I-II linker of $\alpha 1B$ increases its concentration close to its site of action, but is not directly involved in its functional effects.

Both the I-II linker and the first transmembrane domain of $\alpha 1B$ are, however, essential for the observation of a reduction of G-protein modulation by overexpression of exogenous $\beta 2a$ subunit in the *Xenopus* oocyte system. Whereas $\alpha 1B$ itself and $\alpha 1bBcCCC$ showed significantly greater modulation by quinpirole in the absence of coexpressed VDCC $\beta 2a$ subunit, the $\alpha 1bBcCCC$ and $\alpha 1bCcCCC$ chimeras exhibited a similar degree

Figure 7. Mean effect of $\alpha 1B$ deletions and mutations on $G\beta\gamma$ modulation in COS-7 cells. **A**, The P_2/P_1 ratio was determined in COS-7 cells overexpressing $G\beta 1\gamma 2$, from current amplitudes during steps to -10 mV before and after a depolarizing prepulse ($+120$ mV, 100 msec), for the same N-terminal deletions and point mutations shown in Figure 5. Comparison is made with $\alpha 1B$ in the absence of $G\beta\gamma$, recorded with 1 mM GDP β S in the patch pipette (1). The value $[(P_2/P_1) - 1]$ is plotted, which will be 0 if there is no facilitation. **B**, The activation V_{50} was determined for the same constructs coexpressed with $G\beta 1\gamma 2$, and compared to the value for $\alpha 1B$ in the absence of $G\beta\gamma$, recorded with 1 mM GDP β S in the patch pipette (1). The dashed lines are 1 SEM more positive than the mean value for $\alpha 1B$ (1), and 1 SEM more negative than the mean value for $\alpha 1B/\text{G}\beta\gamma$ (2). **C**, Correlation between Δ activation V_{50} (the data given in **B**, after subtraction of the V_{50} for $\alpha 1B$) on the y-axis, and the data from Figure 5C (percentage inhibition of I_{Ba} by 100 nM quinpirole), on the x-axis. The numbers identifying the constructs refer to the bars in **A** and **B**. Regression analysis (dotted line) gives a coefficient, r of 0.92 ($p < 0.001$). The data divide into a group of modulated and a group of nonmodulated constructs, as identified, except for constructs 14 (I49A) and 9 (YKQ $\rightarrow$ AAA).

of inhibition by quinpirole in the presence and absence of coexpressed $\beta 2a$. The mechanism of this partial antagonism by $\beta 2a$ remains unclear, but is not completely shared by other β subunits such as $\beta 1b$ (C. Canti and A. C. Dolphin, unpublished results).

The first transmembrane domain of $\alpha 1B$ clearly has a role in G-protein modulation, as suggested previously (Zhang et al., 1996; Stephens et al., 1998b). We have found that, although it can be substituted by that of $\alpha 1C$, the $\alpha 1b\text{CbCCC}$ chimera is less modulated than $\alpha 1b\text{BbCCC}$ by quinpirole in the *Xenopus* oocyte system. It is possible that the first transmembrane domain mediates the effects of $G\beta\gamma$ subunits to slow current activation, via interference with the function of its voltage sensor. Evidence suggests that only one $G\beta\gamma$ subunit binds per $\alpha 1$ subunit, in a voltage-dependent manner (Stephens et al., 1998a; Zamponi and Snutch, 1998). We previously estimated the off-rate (k_{-1}) of $G\beta\gamma$

subunits to be $\sim 1.3 \text{ sec}^{-1}$ at -100 mV and 50 sec^{-1} at $+120$ mV (Stephens et al., 1998a). Thus, the binding of $G\beta\gamma$ is probably of higher affinity to the channel with the voltage sensors in their resting state. The action of $G\beta\gamma$ subunits is to delay channel opening and to produce a depolarizing shift in the voltage dependence of activation (Patil et al., 1996). Presumably, this is achieved either by slowing the movement of the voltage sensors (and the IS4 sensor in particular), in response to a change in transmembrane voltage, or reducing the efficiency of coupling of the voltage sensor to channel opening (Jones et al., 1997).

Some of our chimera results and conclusions do not agree with those of a previous work (Furukawa et al., 1998), which also made a chimera with the I-II linker of $\alpha 1B$ in $\alpha 1C$ and showed it to be G-protein-modulated, thus indicating that the I-II linker alone could mediate G-protein modulation. However, their chimera,

Figure 8. Voltage dependence of inhibition, rate of loss of inhibition, and reinhibition rate for $\alpha 1B$ and $\alpha 1B$ I49A in *Xenopus* oocytes. *A*, Voltage protocol, showing variation of the prepulse voltage (V), the prepulse duration (Δt_{dep}), and the interpulse interval (Δt_{inter}) between the prepulse and the test pulse. The prepulse potential was 100 mV and 50 msec duration, and the interpulse interval was 20 msec, unless these parameters were varied. *B*, Effect of increasing the 50 msec prepulse voltage (V) on prepulse facilitation in the presence of quinpirole. Facilitation was measured as $(I_{Ba}$ in P2) – $(I_{Ba}$ in P1) and normalized to the maximum facilitation observed (normalized ΔI). $\alpha 1B$ (open circles), $\alpha 1B$ I49A (filled circles). The inset histogram gives the V_{50} values (mean $\pm$ SEM, determined by fitting Boltzmann functions to the data from the number of individual experiments given above each bar) for $\alpha 1B$ (white bar) and $\alpha 1B$ I49A (black bar). *C*, Effect of increasing the duration of the 100 mV prepulse (Δt_{dep}) on prepulse facilitation in the presence of quinpirole. Facilitation was measured as described in *B*. $\alpha 1B$ (open circles), $\alpha 1B$ I49A (filled circles). The inset histogram gives the $\tau_{dissociation}$ values (mean $\pm$ SEM, determined by fitting a single exponential to the data from the number of experiments given above each bar) for $\alpha 1B$ (white bar) and $\alpha 1B$ I49A (black bar). *D*, Effect of increasing the interval between the 100 mV, 50 msec prepulse and the subsequent test pulse P2 on the facilitation in the presence of quinpirole. Facilitation was measured as described in *B*: $\alpha 1B$ (open circles), $\alpha 1B$ I49A (filled circles). The inset histogram gives the $\tau_{reinhibition}$ values (mean $\pm$ SEM, determined by fitting a single exponential to the data from the number of experiments given above each bar) for $\alpha 1B$ (white bar) and $\alpha 1B$ I49A (black bar).

together with other chimeras described in their paper, involved substitution of more than just the I-II linker of $\alpha 1B$. In the chimera in question, a region of $\alpha 1B$ from part of IS5 through to IIS2 was substituted into $\alpha 1C$, with in addition several amino acid substitutions and deletions, and the results are thus not directly comparable. Furthermore, in their study the reciprocal chimera, made up of $\alpha 1B$ with a region including the I-II linker of $\alpha 1C$, was also G-protein-modulated.

Our finding is that substitution into $\alpha 1C$ of the region from the N terminus to the end of the I-II linker of $\alpha 1B$ ($\alpha 1bBbCCCC$) does not produce a channel that is as strongly modulated as $\alpha 1B$ in the *Xenopus* oocyte assay, although in the $G\beta\gamma$ overexpression assay, there was no significant difference between $\alpha 1bBbCCCC$ and $\alpha 1B$. Thus, it is likely that other regions in the rest of $\alpha 1B$ may also contribute to the extent of G-protein modulation of $\alpha 1B$, possibly including the C terminus (Qin et al., 1997; Hamid et al., 1999), which may form part of a complex $G\beta\gamma$ -binding pocket.

Amino acids in the N terminus of $\alpha 1B$ that are critical for G-protein modulation

From our mutational study of the N terminus, we identified the sequence between amino acids 45 and 55 (YKQSIQRART) as being essential for G-protein modulation, because a deletion to amino acid 55 produced a construct that showed no G-protein modulation (Page et al., 1998), whereas a channel truncated to amino acid 44 was fully modulated, and a deletion of these 11 amino acids (45–55) resulted in a nonmodulated construct. Subsequently, we have identified three amino acids within this sequence, S48, R52, and R54, that when mutated to alanine, markedly reduce G-protein modulation of $\alpha 1B$, and a fourth amino acid (I49) that also shows an involvement. The substitution of just two amino acids (R52A, R54A) completely abolished G-protein modulation, whereas constructs containing the individual mutations still showed a small degree of modulation (4 and 9% inhi-

bition by quinpirole, respectively). The R52A and R54A constructs also individually showed some slowing of current activation when coexpressed with $G\beta\gamma$, whereas the double mutant did not (Fig. 6B; results not shown). The RAR motif is reminiscent of the RAK motif found in one of the $G\beta\gamma$ -binding sites on GIRK4 (Krapivinsky et al., 1998).

The I49A mutation stands out as producing a reduction in G-protein modulation in both systems (Fig. 6C). It is of interest that the 11 amino acid motif we have identified is identical in rat $\alpha 1E$ and $\alpha 1A$, except for I49, whose equivalent is lysine in $\alpha 1E$, and methionine in $\alpha 1A$. Furthermore, both $\alpha 1A$ and $\alpha 1E_{long}$ show less G-protein modulation than $\alpha 1B$ in a number of systems (Zamponi et al., 1997; Page et al., 1998), possibly involving this amino acid substitution. In the present study we have observed that the τ_{reinh} after a depolarizing prepulse is more than twice as fast for $\alpha 1B$ I49A (85 msec) than for $\alpha 1B$ (187 msec; Fig. 8D). However, in our previous study we observed that the τ_{reinh} for both $\alpha 1B$ and $\alpha 1E_{long}$ was ~ 95 msec (Page et al., 1998). We are currently re-examining the comparison between $\alpha 1B$ and $\alpha 1E_{long}$ under the present conditions (5 mM Ba^{2+} , BAPTA-injected oocytes), to investigate whether our previous lack of observation of any difference in τ_{reinh} between $\alpha 1B$ and $\alpha 1E_{long}$ was caused by an influence of niflumic acid, which we have subsequently found to affect G-protein modulation of $\alpha 1B$ currents.

It is possible that the N terminus forms a $G\beta\gamma$ or VDCC β -binding site, or it may be involved in the downstream effects of $G\beta\gamma$ binding. We have observed that inactivation is increased in a number of the $\alpha 1B$ mutants, suggesting an impairment of interaction with $\beta 2a$ (G. J. Stephens and A. C. Dolphin, unpublished results). However, one can consider that these mutations in the N terminus of $\alpha 1B$ may alter the binding affinity for $G\beta\gamma$ (see Results for I49A). From this analysis the major difference is an apparent 3.4-fold increase in the off-rate for $G\beta\gamma$ in the I49A mutant. Thus, YKQSIQRART may form part of a $G\beta\gamma$ -binding site, with I49 playing a modulatory role in binding affinity, or it may be involved in the interaction between $G\beta\gamma$ and VDCC β subunits.

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