Rapid Report

Role of domain I of neuronal Ca^{2+} channel $\alpha 1$ subunits in G protein modulation

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

Department of Pharmacology, University College London, London WC1E 6BT, UK

(Received 11 February 1998; accepted 30 March 1998)

1. We studied the G protein inhibition of heteromultimeric neuronal Ca^{2+} channels by constructing a series of chimeric channels between the strongly modulated $\alpha 1$B subunit and the $\alpha 1$E(rbEII) subunit, which showed no modulation.

2. In parallel studies, $\alpha 1$ subunit constructs were co-expressed together with the accessory Ca^{2+} channel $\alpha 2$-δ and $\beta 2$a subunits in mammalian (COS-7) cells and *Xenopus* oocytes. G protein inhibition of expressed Ca^{2+} channel currents was induced by co-transfection of G$\beta 1$ and G$\gamma 2$ subunits in COS-7 cells or activation of co-expressed dopamine (D2) receptors by quinpirole (100 nM) in oocytes.

3. The data indicate that transfer of the $\alpha 1$B region containing the N-terminal, domain I and the I–II loop (i.e. the $\alpha 1B_{1\text{-}483}$ sequence), conferred G protein modulation on $\alpha 1$E(rbEII), both in terms of a slowing of activation kinetics and a reduction in current amplitude.

4. In contrast, the data are not consistent with the I–II loop and/or the C-terminal forming a unique site for G protein modulation.

G protein inhibition of neuronal N ($\alpha 1$B) and P/Q type ($\alpha 1$A) Ca^{2+} currents is mediated by G$\beta\gamma$ subunits (Herlitze, Garcia, Mackie, Hille, Scheuer & Catterall, 1996; Ikeda, 1996). Both the intracellular loop that links Ca^{2+} channel transmembrane domains I and II (DeWaard, Liu, Walker, Scott, Gurnett & Campbell, 1997; Zamponi, Bourinet, Nelson, Nargeot & Snutch, 1997) and a C-terminal sequence (Qin, Platano, Olcese, Stefani & Birnbaumer, 1997) have been implicated as sites at which G$\beta\gamma$ subunits bind to $\alpha 1$ subunits.

Functionally, the site of G protein action remains controversial. Mutations within the I–II loop, and specifically to the arginine residue in a QxxER consensus sequence proposed to be involved in G$\beta\gamma$ binding (Chen *et al.* 1995), abolish G$\beta\gamma$ binding and prevent the slowing of activation induced by GTPγS (De Waard *et al.* 1997). In contrast, the same mutation actually enhanced modulation in a different study (Herlitze, Hockerman, Scheuer & Catterall, 1997); whereas conversion of the entire $\alpha 1$A consensus sequence (QIEER) to that in $\alpha 1$C (QQLEE) did attenuate modulation. Transfer of the IS6/I–II loop from $\alpha 1$B to the non-modulated $\alpha 1$E(rbEII) causes some slowing of current activation kinetics in the presence of GTPγS, but does not result in current amplitude modulation (Page, Stephens, Berrow & Dolphin, 1997). In contrast, $\alpha 1$B was reported to retain G protein sensitivity when its I–II loop was replaced by the corresponding sequence from non-modulated $\alpha 1$C (Zhang, Ellinor, Aldrich & Tsien, 1996); their study implicated a role of domain I together with the C-terminal in G protein modulation. However, the G protein inhibition of human $\alpha 1$E appears to be due to G$\beta\gamma$ binding solely at the C-terminal site (Qin *et al.* 1997).

Here, we examine the potential contribution of regions implicated in G protein modulation using a series of constructs between the strongly modulated $\alpha 1$B subunit and the rat $\alpha 1$E(rbEII) subunit (Soong, Stea, Hodson, Dubel, Vincent & Snutch, 1993), which shows no modulation (Bourinet, Soong, Stea & Snutch, 1996*a*; Page *et al.* 1997). The results suggest that the $\alpha 1B_{1\text{-}483}$ sequence contains important determinants of G protein modulation.

METHODS

Materials

The following cDNAs were used: rat $\alpha 1$E (rbEII, GenBank accession number L15453); rabbit $\alpha 1$B (D14157); rat $\beta 2$a (M80545); rat $\alpha 2$-δ (M86621); rat $D2_{long}$ receptor (X77458, N5→G); bovine G$\beta 1$ (M13236), bovine G$\gamma 2$ (M37183) and Mut3-Green fluorescent protein (GFP) (U73901).

Production of Ca^{2+} channel constructs

Individual constructs were produced by the polymerase chain reaction (PCR) methodology as detailed previously (Page *et al.* 1997), using lower case letters for C termini and I–II loops and upper case for each of the four transmembrane domains as follows.

$\alpha 1$EbEEE. The pMT2 forward primer, pMT2F (AGC TTG AGG TGT GGC AGG CTT) and the chimeric reverse primer, TCC TGA GAG CAC ACC CAG GAC AAG GTT G, were used with the

α1E(rbEII) template; the resulting fragment was used as a primer and extended on the α1EBE1–pMT2 template using the reverse primer, GAC TTC ATG GAG CTC ATC AAG G. The product was digested with *Xba*I and *Acc*B7I and subcloned into the corresponding region of the α1E(rbEII)–pMT2 vector. The α1EbEEE construct differs from the chimera (termed EBE) used previously (Page *et al.* 1997); α1EbEEE substitutes only the I–II loop, whilst EBE exchanged both the 1S6 region and I–II loop.

α1EbEEEb and α1EEEEb. An *Xho*I site was removed from position 5433 of α1B using the forward primer, AAG TGC CCT GCA CGA GTC GCG TA and the reverse primer, GCA CTC GAG CGC GGA AGA TGA AGC. The product was extended on the α1B–pMT2 template using the forward primer, TTA CTC GAG ACT CTT CCA TCT TAG G, to introduce the *Xho*I site. This product was digested and subcloned into α1E(rbEII) to give α1EEEEb, and into α1EbEEE to give α1EbEEEb.

α1BbEEE and α1BbEEEb. A *Mfe*I (pMT2) to *Kpn*I α1B digestion was used to swap the first domain of α1EbEEE with that of α1B to make α1BbEEE. α1BbEEEb was made by using the same *Kpn*I site in α1EbEEEb.

PCR was carried out using the proof-reading enzyme, *Pfu* (Stratagene). The sequences of the sub-cloned PCR products were verified by cycle-sequencing using SequiTherm Excel™ II (Epicentre Technologies, Madison, USA).

Expression of constructs

COS-7 cells. Cells were transfected by electroporation as described (Campbell, Berrow, Brickley, Page, Wade & Dolphin, 1995). Fifteen, 5, 5 and 1 μg of the pMT2–α1, α2-δ, β2a or β1b and green fluorescent protein (GFP) constructs, respectively, were used for transfection. When used, Gβ1 and Gγ2 were included at 2·5 μg each. Cells were maintained at 37 °C, then replated and maintained at 25 °C prior to recording.

***Xenopus* oocytes**. Adult *Xenopus laevis* females were anaesthetized by immersion in 0·2% tricaine then killed by decapitation and pithing, oocytes were surgically removed and defolliculated with 2 mg ml^{-1} collagenase type Ia in a Ca^{2+}-free ND96 saline (containing (mM): NaCl, 96; KCl, 2; $MgCl_2$, 1; Hepes, 5; pH adjusted to 7·4 with NaOH) for 2 h at 21 °C. cDNAs for the different α1, β2a and α2-δ subunits and D2 receptors were co-injected at a ratio of 3:1:1:3 into the nuclei of stage V and VI oocytes using a Drummond microinjector. Oocytes were incubated at 18 °C for 3–7 days in ND96 saline (as above plus 1·8 mM $CaCl_2$) supplemented with 100 μg ml^{-1} penicillin and 100 i.u. ml^{-1} streptomycin (Gibco) and 2·5 mM sodium pyruvate.

Electrophysiology

COS-7 cells. Recordings were made from fluorescent cells expressing the GFP reporter gene, replated between 1 and 16 h previously, using a non-enzymatic cell dissociation medium (Sigma). Borosilicate glass electrodes of resistance 2–4 MΩ were filled with a solution containing (mM): caesium aspartate, 140; EGTA, 5; $MgCl_2$, 2; $CaCl_2$, 0·1; K_2ATP, 2; Hepes, 10; pH 7·2; osmolarity adjusted to 310 mosmol l^{-1} with sucrose. GDPβS (2 mM) was included where stated. The external solution contained (mM): tetraethylammonium (TEA) bromide, 160; KCl, 3; $NaHCO_3$, 1·0; $MgCl_2$, 1·0; Hepes, 10; glucose, 4; $BaCl_2$, 1; pH 7·4; osmolarity adjusted to 320 mosmol l^{-1} with sucrose.

Whole cell currents were recorded using an Axopatch 1D amplifier. Data were filtered at 2 kHz and digitized at 5–10 kHz and analysed using pCLAMP6 and Origin 3.5. 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 capacitance current subtraction (*P*/4 or *P*/8 protocol) and series resistance compensation up to 85%.

***Xenopus* oocytes**. Whole cell recordings from oocytes were made in the two-electrode voltage clamp configuration with a chloride-free solution containing (mM): $Ba(OH)_2$, 40; TEA-OH, 50; KOH, 2; niflumic acid, 0·4; Hepes, 5; pH adjusted to 7·4 with methanesulphonic acid). In some experiments, niflumic acid was omitted and oocytes injected with 30–40 nl of 100 mM BAPTA to suppress endogenous chloride currents. Data were filtered at 1 kHz using a Geneclamp 500 amplifier, digitized through a Digidata 1200 interface (Axon Instruments) and stored using data acquisition software pCLAMP6. Currents were leak subtraction on line (*P*/4 protocol)

Experiments were performed at room temperature (20–24 °C). Data are expressed as means ± S.E.M. Statistical analysis was performed using Student's paired or unpaired *t* test as appropriate.

RESULTS

Effect of Gβ1γ2 co-expression on Ca^{2+} channel constructs

A series of chimeras between Ca^{2+} channel α1B and α1E(rbEII) subunits was constructed as shown in Fig. 1*A* to investigate the role of domain I, the I–II loop and the C-terminal in G protein regulation. α1 subunits were co-expressed together with accessory α2-δ and β2a subunits in COS-7 cells and modulation was studied by co-expressing Gβ1γ2 subunits. In controls, Gβ1γ2 was replaced by pMT2 vector and 2 mM GDPβS was included in the patch pipette to limit tonic facilitation (Stephens, Brice, Berrow & Dolphin, 1998). Current–voltage profiles were constructed (Fig. 1*B*) and the ability of co-expressed Gβ1γ2 subunits to slow current activation, a characteristic of G protein inhibition, was examined. Time constants of activation (τ_{act}) were derived from single exponential fits to the rising phase of currents (Fig. 1*C*). In the absence of exogenous Gβγ subunits, all currents activated rapidly and showed little inactivation over the time course used (as expected for co-expression of the β2a subunit which retards voltage-dependent inactivation of Ca^{2+} channels (Olcese *et al.* 1994)). In the presence of Gβ1γ2, α1B currents showed a marked slowing of activation kinetics in comparison to controls; in contrast, α1E(rbEII) currents showed no difference in activation with or without Gβ1γ2. Of the other constructs, those containing the N-terminal sequence/domain I/I–II loop of the α1B subunit sequence ($\alpha1B_{1\text{-}483}$) exhibited currents showing clear kinetic slowing in the presence of Gβ1γ2. Time constants of activation showed no significant differences for the effects of Gβ1γ2 on these responsive constructs: at −10 mV τ_{act} values were 32 ± 9 ms (α1B, $n = 8$), 29 ± 6 ms (α1BbEEE, $n = 11$) and 26 ± 5 ms (α1BbEEEb, $n = 7$). Thus, the subsequent exchange of the C-terminal, in addition to the $\alpha1B_{1\text{-}483}$ sequence, had no further effect on current activation kinetics.

Constructs in which either the I–II loop alone (α1EbEEE), the C-terminal sequence alone (α1EEEEb) or both elements

(α1EbEEEb) were exchanged exhibited currents showing no significant slowing in activation kinetics in the presence of Gβ1γ2 (Figs 1*B* and *C*).

Receptor-mediated G protein inhibition of Ca^{2+} channel constructs

In parallel studies, we reconstructed receptor-mediated inhibition of constructs (together with α2-δ and β2a subunits) in *Xenopus* oocytes. Inhibitory coupling of dopamine (D2) receptors was assessed in terms of the reduction in current amplitude by a saturating concentration of quinpirole (100 nM) (Fig. 2*A*). Only constructs containing the $\alpha1B_{1\text{-}483}$ sequence were modulated by receptor activation. There were no significant differences in inhibition by quinpirole amongst the responsive chimeras; however, quinpirole did cause a higher percentage inhibition in α1B than in α1BbEEE ($P < 0{\cdot}005$) or α1BbEEEb ($P < 0{\cdot}005$). No additional modulation to that seen in α1BbEEE was apparent in α1BbEEEb. Inhibition by quinpirole was absent in all of the other chimeras.

Quinpirole-induced inhibition in constructs containing the $\alpha1B_{1\text{-}483}$ sequence was accompanied by a depolarizing shift in the midpoint of activation ($V_{½}$) of current–voltage curves (Fig. 2*B*). Modified Boltzmann functions fitted to the data shown gave similar shifts in $V_{½}$ with quinpirole: α1B, +7·4 mV; α1BbEEE, +5·6 mV; α1BbEEEb, +6·5 mV. No such shift was seen for α1E(rbEII) (Fig. 2*B*).

Effects of facilitating prepulses on Gβ1γ2-induced inhibition on Ca^{2+} channel constructs

The voltage-dependent G protein modulation of Ca^{2+} channels can be reversed by the application of a large depolarizing prepulse prior to an activating pulse. Figure 3*A* illustrates that a depolarizing prepulse reversed both the inhibition of current amplitude and the slowed activation kinetics induced by Gβ1γ2 overexpression in COS cells expressing constructs containing the $\alpha1B_{1\text{-}483}$ sequence. Gβ1γ2-induced slowing of activation kinetics was maximal at just supra-threshold potentials and was reversed by prepulses to the control levels observed in the presence of

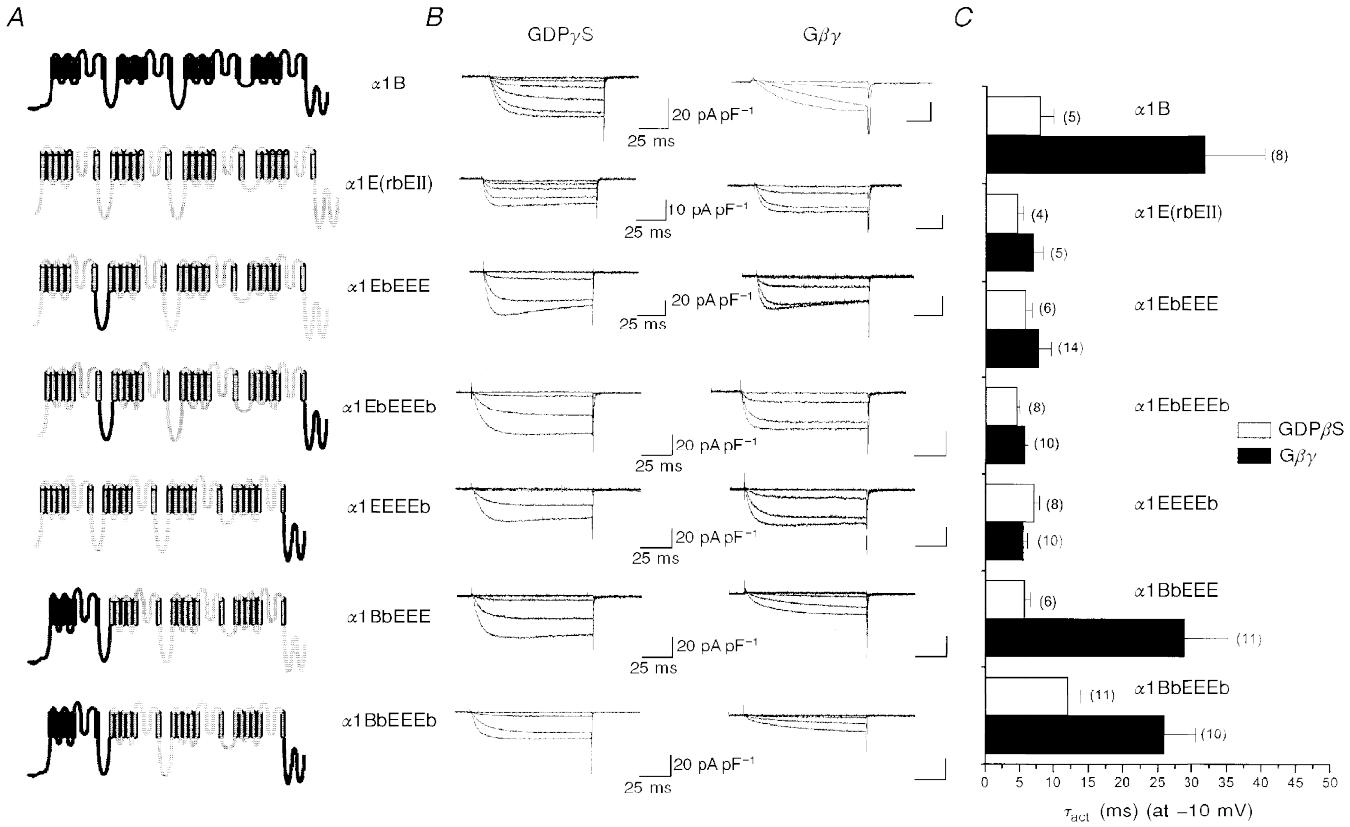

Figure 1. Effect of Gβ1γ2 on Ca^{2+} channel constructs in COS-7 cells

Ca^{2+} channel constructs between α1B and α1E(rbEII) subunits (as shown in *A*) were transfected together with cDNA coding for α2-δ and β2a subunits. *B*, example current density–voltage profiles for control cells (in the presence of GDPβS) and in the presence of Gβ1γ2. The initial test potential (V_t) shown was always −50 mV and was increased in 10 mV increments; holding potential $V_H = -100$ mV. Values for scale bars on the left apply also to scale bars on the right. *C*, time constant of activation (τ_{act}) at −10 mV for Ca^{2+} channel constructs coexpressed with Gβ1γ2 (black columns) or in control conditions in the presence of GDPβS (open columns); number of experiments, *n*, is given in parentheses. Only currents resulting from constructs containing the $\alpha1B_{1\text{-}483}$ sequence showed a clear slowing of activation kinetics.

GDPβS (Fig. 3*B*). The degree of prepulse-induced current amplitude facilitation (P2 : P1) in the presence of G$\beta1\gamma2$ was also maximal over a similar voltage range in responsive constructs (Fig. 3*C*). No facilitation was observed in constructs lacking the first domain of $\alpha1$B.

Prepulses also partially reversed the quinpirole-induced inhibition of current amplitude in constructs containing the $\alpha1B_{I\text{-}483}$ sequence in oocytes (Fig. 4*A* and *B*). An incomplete reversal of inhibition by a large depolarizing prepulse is a characteristic of G protein inhibition and might be indicative of additional non-voltage-dependent mechanisms (Luebke & Dunlap, 1994) or due partially to the rebinding of G$\beta\gamma$ subunits during the 10 ms interpulse interval. Prepulses caused a significant facilitation of quinpirole-inhibited currents in comparison to control levels (prepulses applied in the absence of quinpirole) for responsive constructs (Fig. 4*A*). There was no clear difference in the percentage facilitation between responsive constructs. No facilitation was observed in constructs lacking the first domain of $\alpha1$B.

Therefore, the transfer of the $\alpha1B_{I\text{-}483}$ sequence conferred full G protein modulation onto $\alpha1$E(rbEII) which was reversed by depolarizing prepulses. No additional effects were seen with the subsequent exchange of the C-terminal sequence.

DISCUSSION

Taking clues from our previous studies and those of others, we constructed chimeric channels to study the role of several regions of the Ca^{2+} channel $\alpha1$ subunit implicated in G protein regulation. The data indicate that the $\alpha1B_{I\text{-}483}$ sequence, representing the N-terminal, domain I and the I–II loop, contain important determinants. In contrast, the data are not consistent with the I–II loop and/or the

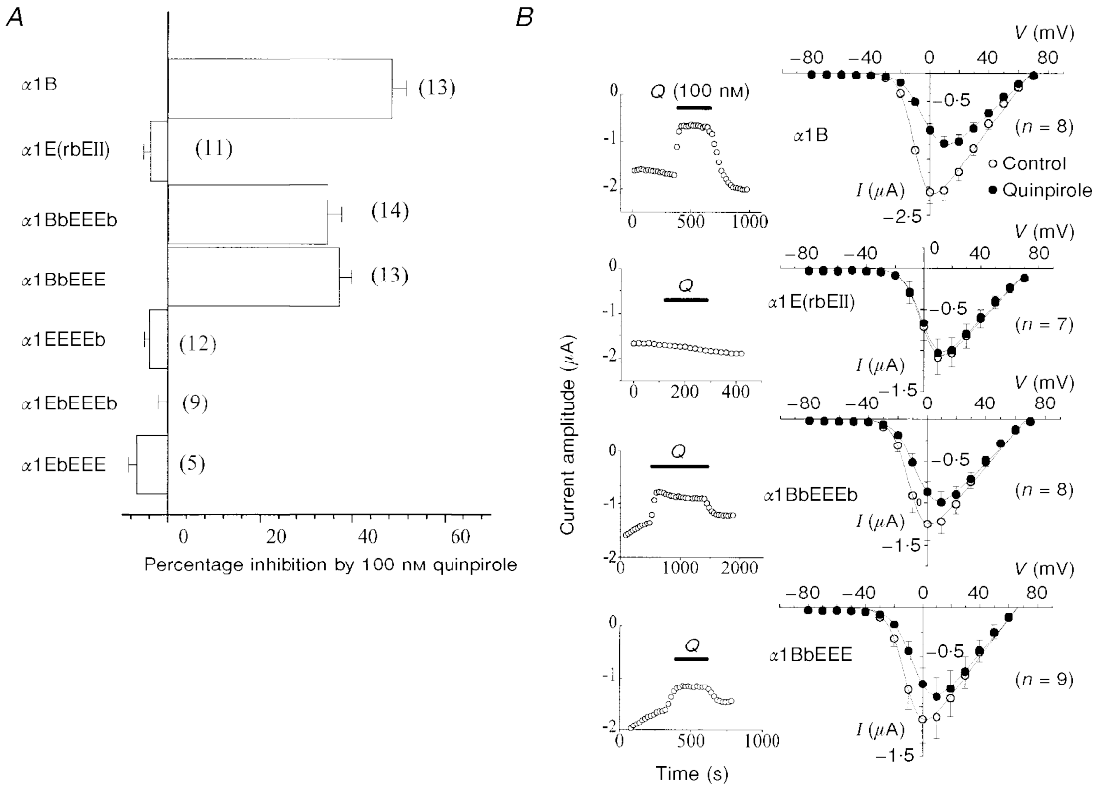

Figure 2. Effect of activation of D2 receptors on Ca^{2+} channel constructs in *Xenopus* oocytes

Ca^{2+} channel constructs were injected together with cDNA coding for $\alpha2\text{-}\delta$ and $\beta2$a subunits and dopamine (D2) receptors in all cases. *A*, activation of D2 receptors by quinpirole caused an inhibition of currents formed by constructs containing the $\alpha1B_{I\text{-}483}$ sequence; $V_t = 0$ mV and $V_H = -100$ mV; number of experiments, n, is given in parentheses. *B*, time course of barium current (I_{Ba}) inhibition by quinpirole (Q, 100 nM) in selected constructs ($V_t = 0$ mV, left panel) and corresponding effects of quinpirole on the current–voltage relationship (right panel). Current–voltage data were fitted with the equation:

$$\text{Current} = G_{max}(V - V_{rev})/\{1 + \exp[(V - V_{½})/k]\},$$

where G_{max} is maximum slope conductance, $V_{½}$ is the voltage at which 50 % of the current is activated, V_{rev} is the null potential and k is the slope factor. Quinpirole inhibition (●) was accompanied by a reduction in current amplitude, particularly at hyperpolarized potentials, with a corresponding shift in $V_{½}$ values.

C-terminal alone forming a unique site for G protein modulation.

Role of the I–II loop in G protein inhibition

Despite data demonstrating binding of radiolabelled Gβγ to the Ca^{2+} channel I–II loop (De Waard *et al.* 1997; Zamponi *et al.* 1997; Qin *et al.* 1997), the functional importance of this site is still controversial (see Dolphin, 1998). Substitution of the I–II loop of α1C, which does not bind Gβγ (De Waard *et al.* 1997; Zamponi *et al.* 1997), into α1B (Zhang *et al.* 1996) or α1E (Qin *et al.* 1997) produces constructs that retain G protein sensitivity. However, opposite results have also been reported (Herlitze *et al.* 1997); conversion of the Gβγ-binding QxxER consensus sequence in the I–II loop of α1A subunit to the corresponding α1C sequence greatly reduced G protein inhibition. Importantly, this conversion did not completely abolish inhibition (Herlitze *et al.* 1997). Furthermore, whilst replacement of the I–II loop of α1A with that of α1B did increase G protein inhibition, it did not fully account for all of the inhibition seen in parental α1B (Zamponi *et al.* 1997). These findings suggest that molecular determinants for G protein modulation additional to the I–II loop are likely to exist. In support of this, we have demonstrated that a chimera in which the IS6/I–II loop of α1E(rbEII) was replaced by that of α1B (termed EBE) exhibited a greater slowing of current activation kinetics with GTPγS than did α1E(rbEII), but that GTPγS had no effects on current amplitude (Page *et al.* 1997).

In the present study, the α1EbEEE construct, in which only the I–II loop and not IS6 was exchanged, was examined. In agreement with our previous study, G proteins had no effect on current amplitude. However, no significant changes in activation kinetics were seen. In our previous study the β subunit used was β1b, which produces less antagonism of Gβγ than the β2a subunit used here (Qin *et al.* 1997). However, it appears that the additional substitution of the 1S6 region of transmembrane domain I, which has been implicated as a determinant of voltage-dependent Ca^{2+} channel inactivation (Zhang, Ellinor, Aldrich & Tsien, 1994), can also subtly affect activation kinetics in the presence of Gβγ subunits.

Role of the intracellular C-terminal tail in G protein inhibition

The Ca^{2+} channel C-terminal has been implicated in G protein inhibition. Zhang *et al.* (1996) propose that both domain I and the C-terminal contribute elements to a multi-structural site, whilst Qin *et al.* (1997) suggest a unique site on the C-terminal. The thirty-eight amino acid Gβγ-binding site identified in the human α1E C-terminal sequence (Qin *et al.* 1997) is entirely conserved in

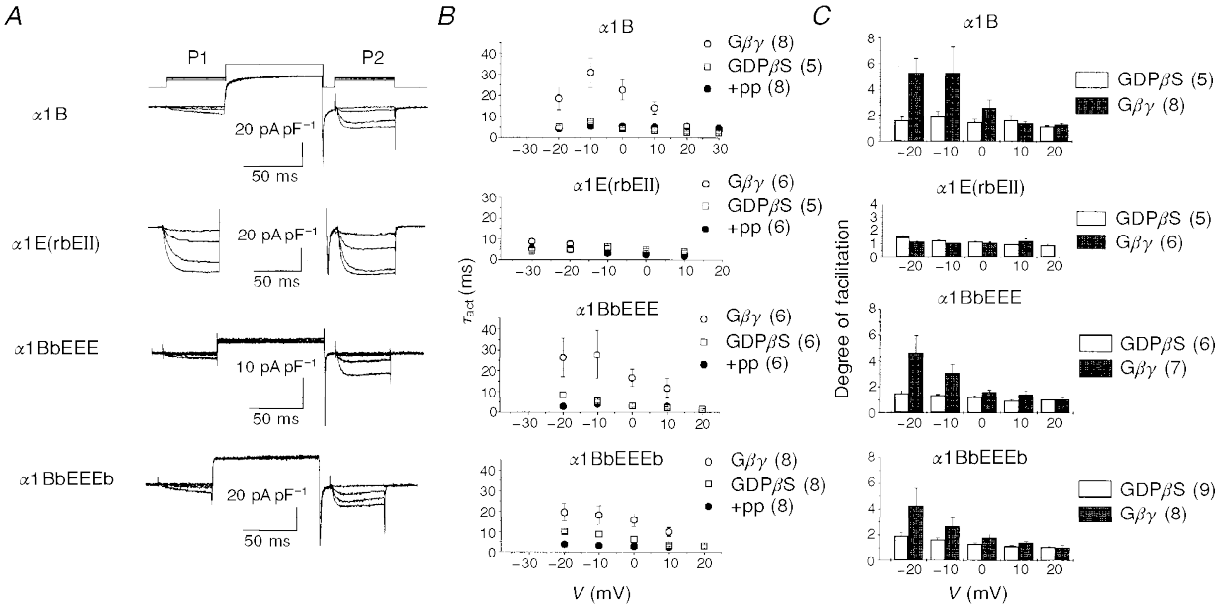

Figure 3. Reversal of Gβγ inhibition of Ca^{2+} channel constructs by large depolarizing prepulses in COS-7 cells

Application of depolarizing prepulses reversed Gβ1γ2-induced inhibition, as shown for selected constructs. *A*, I_{Ba} ($V_t = -40$ to 0 mV) was examined immediately before (P1) and 10 ms after (P2) application of a depolarizing prepulse to +120 mV; $V_H = -100$ mV. *B*, in constructs containing the $\alpha1B_{1\text{-}483}$ sequence, prepulses (pp) reversed Gβ1γ2-induced slowing of activation kinetics to control levels (recorded separately at P1 in control cells). *C*, in constructs containing the $\alpha1B_{1\text{-}483}$ sequence, prepulses reversed Gβ1γ2-induced inhibition of current amplitude (P2 : P1 measured at 50 ms). In all instances, note the lack of effects on α1E(rbEII). Number of experiments, *n*, is given in parentheses.

α1E(rbEII). Despite the presence of this site, we saw no evidence of inhibition of α1E(rbEII). A possible explanation for this is that β subunits may compete for Gβγ binding and effectively block any modulation. More specifically, the β2a subunit presence here was shown selectively to block Gβγ binding to the human α1E C-terminal site (Qin *et al.* 1997). However, this is unlikely to explain the lack of G protein effects here as we also see no receptor-mediated inhibition of α1E(rbEII) in the absence of any exogenous β subunits (C. Cantí and A. C. Dolphin, unpublished results), suggesting an inherent G protein insensitivity of the α1E(rbEII) subunit.

The $\alpha 1B_{1\text{-}483}$ sequence contributes to G protein inhibition

Transfer of the $\alpha 1B_{1\text{-}483}$ sequence to α1E(rbEII) conferred G protein-induced slowing of current activation kinetics and reduction in current amplitude. In another major study on the determinants of G protein modulation, Zhang *et al.* (1996) proposed a role for domain I together with the C-terminal, but not the I–II loop. In contrast, we find that for α1E(rbEII), inhibition was not further increased by the subsequent exchange of the C-terminal in combination with the $\alpha 1B_{1\text{-}483}$ sequence; this suggests that α1E(rbEII) lacks only molecular determinants within the $\alpha 1B_{1\text{-}483}$ sequence. Despite these discrepancies, it is clear that domain I contains important determinants of G protein modulation. In this regard, the reduction in G protein inhibition caused by exchanging both domain I and the C terminal of α1B for these regions of α1C (Zhang *et al.* 1996) may be interpreted not as a lack of Gβγ binding to the α1B I–II loop, but rather a failure of binding to be fully translated into a functional effect due to the lack of the α1B domain I. Such an effect is consistent with the present results (as discussed below).

The $\alpha 1B_{1\text{-}483}$ sequence represents the N-terminal, domain I and the I–II loop regions. The corresponding sequence of α1E(rbEII) shows only a few major regions of difference. The N-terminal shows the clearest difference, with the α1E(rbEII) N-terminal being fifty-five amino acids shorter than that of α1B. The involvement of the N-terminal region is currently under investigation. The entire 1S1–1S6 region shows a remarkable degree of homology (including complete conservation of the intracellular loops between IS2–IS3 and IS4–IS5); only the H5 linker between IS5 and IS6 in the putative pore region shows a clear divergence. However, this more likely reflects differences in pore properties such as ion

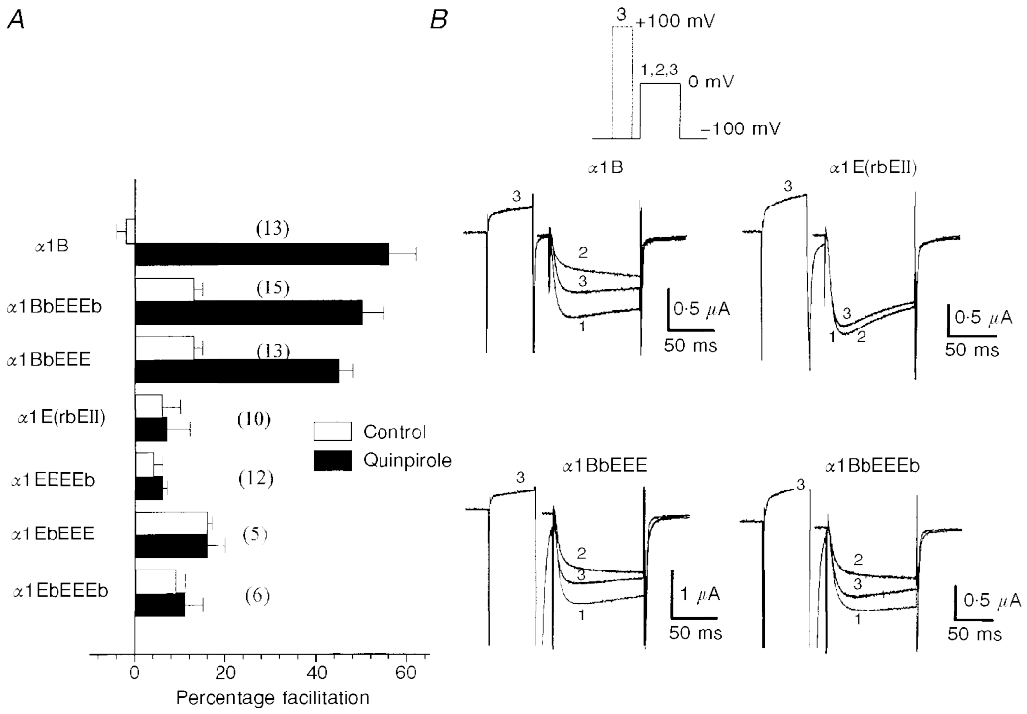

Figure 4. Reversal of D2 receptor-induced inhibition of Ca^{2+} channel constructs by large depolarizing prepulses in *Xenopus* oocytes

Application of depolarizing prepulses caused a reversal of quinpirole-induced inhibition of I_{Ba} for constructs containing the $\alpha 1B_{1\text{-}483}$ sequence. *A*, percentage facilitation of control (open columns) and quinpirole-inhibited I_{Ba} (black columns) induced by a prepulse to +100 mV. *B*, in constructs containing the $\alpha 1B_{1\text{-}483}$ sequence, inhibition of control I_{Ba} (1) by quinpirole (2) was partially reversed by prepulse to +100 mV (3). In all cases, $V_t = 0$ mV and $V_H = -100$ mV. Number of experiments, n, is given in parentheses.

permeation (Bourinet *et al.* 1996*b*) and single channel conductance (Dirksen, Nakai, Gonzalez, Imoto & Beam, 1997). Finally, the I–II loop shows sequence differences. However, as discussed previously (Page *et al.* 1997) and above, the I–II loop alone does not account for G protein inhibition.

Our findings suggest that the binding of Gβγ to either the I–II loop or the C-terminal alone is insufficient to mediate G protein inhibition. However, the data do not rule out a contribution of either region to a site composed of different elements capable of translating Gβγ binding into a functional effect. Whilst the I–II loop and/or the C-terminal may be the primary target for Gβγ binding, the functional changes which occur upon binding, both in terms of a slowing of current activation kinetics and a reduction in current amplitude, are mediated by important molecular determinants within the $\alpha 1B_{1\text{-}483}$ sequence.

Bourinet, E., Soong, T. W., Stea, A. & Snutch, T. P. (1996*a*). Determinants of the G protein-dependent opioid modulation of neuronal calcium channels. *Proceedings of the National Academy of Sciences of the USA* **93**, 1486–1491.

Bourinet, E., Zamponi, G. W., Stea, A., Soong, T. W., Lewis, B. A., Jones, L. P., Yue, D. T. & Snutch, T. P. (1996*b*). The α1E calcium channel exhibits permeation properties similar to low-voltage-activated calcium channels. *Journal of Neuroscience* **16**, 4983–4993.

Campbell, V., Berrow, N., Brickley, K., Page K., Wade, R. & Dolphin, A. C. (1995). Voltage-dependent calcium channel β-subunits in combination with alpha1 subunits have a GTPase activating effect to promote hydrolysis of GTP by G alpha$_o$ in rat frontal cortex. *FEBS Letters* **370**, 135–140.

Chen, J., DeVivo, M., Dingus, J., Harry, A., Li, J., Sui, J., Carty, D. J., Blank, J. L., Exton, J. H., Stoffel, R. H., Inglese, J., Lefkowitz, R. J., Logothetis, D. E., Hildebrandt, J. D. & Iyengar, R. (1995). A region of adenylyl cyclase 2 critical for regulation by G protein beta gamma subunits. *Science* **268**, 1166–1169.

DeWaard, M., Liu, H., Walker, D., Scott, V. E., Gurnett, C. A. & Campbell, K. P. (1997). Direct binding of G-protein βγ complex to voltage-dependent calcium channels. *Nature* **385**, 446–450.

Dirksen, R. T., Nakai, J., Gonzalez, A., Imoto, K. & Beam, K. G. (1997). The S5-S6 linker of repeat I is a critical determinant of L-Type Ca^{2+} channel conductance. *Biophysical Journal* **73**, 1402–1409.

Dolphin, A. C. (1998). Mechanisms of modulation of voltage-dependent calcium channels by G-proteins. *Journal of Physiology* **506**, 3–11.

Herlitze, S., Garcia, D. E., Mackie, K., Hille, B., Scheuer, T. & Catterall, W. A. (1996). Modulation of Ca^{2+} channels by G-protein βγ subunits. *Nature* **380**, 258–262.

Herlitze, S., Hockerman, G. H., Scheuer, T. & Catterall, W. A. (1997). Molecular determinants of inactivation and G protein modulation in the intracellular loop connecting domains I and II of the calcium channel α1A subunit. *Proceedings of the National Academy of Sciences of the USA* **94**, 1512–1516.

Ikeda, S. R. (1996). Voltage-dependent modulation of N-type calcium channels by G protein βγ subunits. *Nature* **380**, 255–258.

Luebke, J. I. & Dunlap, K. (1994). Sensory neuron N-type calcium currents are inhibited by both voltage-dependent and -independent mechanisms. *Pflügers Archiv* 428, 499–507.

Olcese, R., Qin, N., Schneider, T., Neely, A., Wei, X., Stefani, E. & Birnbaumer, L. (1994). The amino terminus of a calcium channel β subunit sets rates of channel inactivation independently of the subunit's effect on activation. *Neuron* **13**, 1433–1438.

Page, K. M., Stephens, G. J., Berrow, N. S. & Dolphin, A. C. (1997). The intracellular loop between domains I and II of the B-type calcium channel confers aspects of G protein sensitivity to the E-type calcium channel. *Journal of Neuroscience* **17**, 1330–1338.

Qin, N., Platano., Olcese, R., Stefani, E. & Birnbaumer, L. (1997). Direct interaction of Gβγ with a C-terminal Gβγ-binding domain of the Ca^{2+} channel α1 subunit is responsible for channel inhibition by G protein-coupled receptors. *Proceedings of the National Academy of Sciences of the USA* **94**, 8866–8871.

Soong, T. W., Stea, A., Hodson, C. D., Dubel, S. J., Vincent, S. R. & Snutch, T. P. (1993). Structure and functional expression of a member of the low voltage-activated calcium channel family. *Science* **260**, 1133–1136.

Stephens, G. J., Brice, N. L., Berrow, N. S. & Dolphin, A. C. (1998). Facilitation of α1B calcium channels: involvement of endogenous Gβγ subunits. *Journal of Physiology* **509**, 15–27.

Zamponi, G. W., Bourinet, E., Nelson, D., Nargeot, J. & Snutch, T. P. (1997). Crosstalk between G proteins and protein kinase C is mediated by the calcium channel α1 subunit I–II linker. *Nature* **385**, 442–446.

Zhang, J.-F., Ellinor, P. T., Aldrich, R. W. & Tsien, R. W. (1994). Molecular determinants of voltage-dependent inactivation in calcium channels. *Nature* **372**, 97–100.

Zhang, J.-F., Ellinor, P. T., Aldrich, R. W. & Tsien, R. W. (1996). Multiple structural elements in voltage-dependent Ca^{2+} channels support their inhibition by G proteins. *Neuron* **17**, 991–1003.

Acknowledgements

We thank the following for generous gifts of cDNAs: Dr T. Snutch (UBC, Canada) for α1E(rbEII) and β1b; Dr H. Chin (NIH, USA) for α2-δ; Dr Y. Mori (Seiriken, Japan) for α1B; Dr E. Perez-Reyes (Loyola, USA) for β2a; Dr M. Simon (CalTech, USA) for Gβ1 and Gγ2; Professor P. G. Strange (Reading, UK) for D2 receptor; Dr T. Hughes (Yale University, USA) for Mut3-GFP; Genetics Institute (CA, USA) for pMT2. C.C. was a recipient of a postdoctoral fellowship from the Ministerio de Educacion y Ciencia of Spain. We also gratefully acknowledge financial support from The Wellcome Trust, and thank Ms A Odunlami, Mr I. Tedder, Ms M. Li and Ms J. May for technical assistance. This work benefited from the use of the Seqnet facility (Daresbury, UK).

Corresponding author

G. Stephens: Department of Pharmacology (Medawar), University College London, London WC1E 6BT, UK.

Email: g.stephens@ucl.ac.uk