Facilitation of rabbit α_{1B} calcium channels: involvement of endogenous G$\beta\gamma$ subunits

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1. The α_{1B} (N-type) calcium channel shows strong G protein modulation in the presence of G protein activators or G$\beta\gamma$ subunits. Using transient expression in COS-7 cells of α_{1B} together with the accessory subunits α_2–δ and β_{2a}, we have examined the role of endogenous G$\beta\gamma$ subunits in the tonic modulation of α_{1B}, and compared this with modulation by exogenously expressed G$\beta\gamma$ subunits.

2. Prepulse facilitation of control α_{1B}/α_2–δ/β_{2a} currents was always observed. This suggests the existence of tonic modulation of α_{1B} subunits. To determine whether endogenous G$\beta\gamma$ is involved in the facilitation observed in control conditions, the βARK1 G$\beta\gamma$-binding domain (amino acids 495–689) was overexpressed, in order to bind free G$\beta\gamma$ subunits. The extent of control prepulse-induced facilitation was significantly reduced, both in terms of current amplitude and the rate of current activation. In agreement with this, GDPβS also reduced the control facilitation.

3. Co-expression of the G$\beta_1\gamma_2$ subunit, together with the α_{1B}/α_2–δ/β_{2a} calcium channel combination, resulted in a marked degree of depolarizing prepulse-reversible inhibition of the whole-cell I_{Ca} or I_{Ba}. Both slowing of current activation and inhibition of the maximum current amplitude were observed, accompanied by a depolarizing shift in the mid-point of the voltage dependence of activation. Activation of endogenous G$\beta\gamma$ subunits by dialysis with GTPγS produced a smaller degree of prepulse-reversible inhibition.

4. The rate of reinhibition of α_{1B} currents by activated G protein, following a depolarizing prepulse, was much faster with G$\beta_1\gamma_2$ than for the decay of facilitation in control cells. Furthermore, βARK1 (495–689) co-expression markedly slowed the control rate of reinhibition, suggesting that the kinetics of reinhibition depend on the concentration of free endogenous or exogenously expressed G$\beta\gamma$ in the cells. In contrast, the rate of loss of inhibition during a depolarizing prepulse did not vary significantly between the different conditions examined.

5. These findings indicate that, in this system, the voltage-dependent facilitation of α_{1B} that is observed under control conditions occurs as a result of endogenous free G$\beta\gamma$ binding to α_{1B}.

Voltage-dependent G protein-mediated inhibition of neuronal Ca^{2+} channels is characterized by an inhibition of current amplitude and a slowing of the activation kinetics (for review, see Dolphin, 1996). G protein-mediated inhibition of neuronal N-type (α_{1B}) and P/Q-type (α_{1A}) calcium currents is mediated by G$\beta\gamma$, and not Gα, subunits (Herlitze, Garcia, Mackie, Hille, Scheuer & Catterall, 1996; Ikeda, 1996). The main binding sites for G$\beta\gamma$ subunits on α_{1A} and α_{1B} have been localized to two domains on the intracellular loop that links transmembrane domains I and II (De Waard, Liu, Walker, Scott, Gurnett & Campbell, 1997; Zamponi, Bourinet, Nelson, Nargeot & Snutch, 1997). Although a binding site for G$\beta\gamma$ has also been found on the C-terminal tail of human α_{1E} (Qin, Platano, Olcese, Stefani & Birnbaumer, 1997), this was not observed for α_{1B} (Zamponi *et al.* 1997).

G protein modulation is most pronounced at potentials just supra-threshold for activation; this has been interpreted as G protein-modulated channels requiring stronger depolarizations to open (Bean, 1989). Thus, inhibition is weaker at more depolarized potentials. Early models involved binding of activated G protein to both closed and open states of the channel (Elmslie & Jones, 1994; Boland & Bean, 1993). More recently, it has been suggested that these phenomena are due to binding of activated G protein to only the closed state of the channel (Patil, de Leon, Reed, Dubel, Snutch & Yue, 1996). The finding that G protein modulation is voltage dependent provided the basis for the use of large

depolarizing prepulses to reverse G protein-mediated inhibition (Elmslie & Jones, 1994). Enhancement of calcium currents in response to prior depolarization is termed facilitation, but it does not necessarily involve reversal of G protein modulation (for review, see Dolphin, 1996). It has also been observed for both cloned and native cardiac calcium channels (Sculptoreanu, Rotman, Takahashi, Scheuer & Catterall, 1993*a*; Sculptoreanu, Scheuer & Catterall, 1993*b*; Bourinet, Charnet, Tomlinson, Stea, Snutch & Nargeot, 1994), which do not show direct modulation by inhibitory G proteins.

In the present study, we have examined the G protein modulation of α_{1B} calcium channels in the presence of β_{2a}, a subunit that dramatically slows inactivation, in order to examine changes in the activation properties in isolation. Under these conditions, we observed facilitation of α_{1B} under control conditions, both in terms of an increase in activation kinetics following a depolarizing prepulse and an increase in current amplitude. We have therefore compared the effects of co-expressing exogenous $G\beta_1\gamma_2$ and of activating endogenous G proteins with GTPγS, and conversely of either reducing endogenous G protein activation with GDPβS or reducing free endogenous G$\beta\gamma$ concentrations by exogenous expression of βARK1 (495–689), which contains only the C-terminal G$\beta\gamma$-binding domain of βARK (Koch, Inglese, Stone & Lefkowitz, 1993; Pitcher, Touhara, Payne & Lefkowitz, 1995). Expression of βARK1 (495–689) has been shown previously to block several G$\beta\gamma$-mediated signalling pathways in COS-7 cells (Koch, Hawes, Inglese, Luttrell & Lefkowitz, 1994). We now show that the observed control facilitation of α_{1B} is reduced by expression of exogenous βARK1 (495–689), and is thus likely to be due to endogenous free G$\beta\gamma$ subunits.

METHODS

Materials

The following cDNAs were used: rabbit α_{1B} (accession number, D14157; Fujita *et al.* 1993) was provided by Dr Y. Mori (Seriken, Okazaki, Japan); rat β_{2a} (M80545; Perez-Reyes *et al.* 1992) was provided by Dr E. Perez-Reyes (Loyola University, Chicago, IL, USA); β_{1b} (X11394) was provided by Dr T. Snutch (UBC, Vancouver, Canada); the full-length rat α_2–δ (neuronal splice variant, M86621; Kim, Kim, Lee, King & Chin, 1992) was provided by Dr H. Chin (National Institutes of Health, Bethesda, MA, USA). Bovine $G\beta_1$ (M13236; Fong *et al.* 1986) and bovine $G\gamma_2$ (M37183; Gautam, Baetscher, Aebersold & Simon, 1989) were provided by Dr M. Simon (CalTech, Pasadena, CA, USA). The C-terminal minigene of βARK1 (*BTARKB*) was provided by Dr R. Lefkowitz (Duke University, Durham, NC, USA). The S65T mutant of green fluorescent protein (GFP) was a gift from Dr S. Moss (University College London, UK) and the mut-3 GFP mutant was a gift from Dr T. Hughes (Yale University, New Haven, CT, USA). All cDNAs were subcloned, using standard techniques, into the pMT2 expression vector (Genetics Institute, Cambridge, MA, USA) for transient expression in COS-7 cells, except that encoding βARK1 (495–689), which was expressed from pRK5 (Koch *et al.* 1994).

Transfection of COS-7 cells

COS-7 cells were cultured and transfected, using electroporation, essentially as described previously (Campbell, Berrow, Brickley, Page, Wade & Dolphin, 1995*a*). For transfection, 15, 5, 5 and 1 μg of α_1, α_2–δ, β_{2a} (or β_{1b}), and either S65T or mut-3 GFP constructs were used. When used, 2·5 μg of both $G\beta_1$ and $G\gamma_2$ cDNA or 5 μg βARK1 minigene (*BTARKB*) were included. Cells were maintained at 37 °C, then replated using a non-enzymatic cell dissociation medium (Sigma), and kept at 25 °C prior to electrophysiological recording. Successfully transfected cells were identified by expression of GFP. Maximum GFP fluorescence and Ca^{2+} channel expression were observed between 2 and 4 days post-transfection (Brice *et al.* 1997; Berrow, Brice, Tedder, Page & Dolphin, 1997; Stephens, Page, Burley, Berrow & Dolphin, 1997).

Antibodies

Polyclonal antisera to the calcium channel subunits α_{1B} and β were raised in rabbits using standard methodology. The calcium channel β_{common} antiserum was raised against a peptide corresponding to amino acids 65–79 of the rat brain β_{1b} sequence (SRPSDSDVSLEEDRE), as previously described (Berrow, Campbell, Fitzgerald, Brickley & Dolphin, 1995). The α_{1B} antiserum was raised against a peptide corresponding to amino acids 851–867 (RHHRHRDRKDKTSASTPA) of the rat brain α_{1B} sequence (rbB-1; Dubel, Starr, Ahlijanian, Enyeart, Catterall & Snutch, 1992). This epitope forms part of the intracellular loop between transmembrane segments S6 of domain II and S1 of domain III, and is 65% homologous to the corresponding region of the rabbit brain α_{1B} sequence used for this study (BIII, Fujita *et al.* 1993). Cross-reactivity of this antiserum with the rabbit α_{1B} was confirmed by expression and immunostaining of the rabbit α_{1B} subunit in COS-7 cells.

Immunocytochemistry

COS-7 cells used for immunocytochemistry were transfected as for electrophysiological experiments, but omitting the GFP. Immunocytochemistry was performed essentially as described previously (Brice *et al.* 1997). Briefly, the cells were fixed in 4% paraformaldehyde 2–3 days post-transfection and permeabilized with 0·02% Triton X-100. The cells were then incubated overnight with α_{1B} antiserum (1 : 500 dilution), calcium channel β_{common} antiserum (1 : 500 dilution), or $G\beta_{common}$ antibody (Santa Cruz, California, USA; 250 μg ml^{-1}, used at 1 : 500 dilution). β_{1b} was used in this study rather than β_{2a}, as the antibody, although raised against a peptide common to all β subunits, recognizes β_{1b} better than β_{2a} (Brice *et al.* 1997). Bound antibodies were detected using biotin-conjugated goat anti-rabbit IgG antibody and streptavidin-conjugated Texas Red (Molecular Probes). Immunostaining was observed using an MRC 600 confocal microscope (Biorad, Hemel Hempstead, UK). Arbitrary optical density units were calculated using the Kontron KS400 program (Kontron Elektronik, Eshing, Germany).

Electrophysiology

Recordings were made at room temperature (20–22 °C) from COS-7 cells which had been replated between 1 and 16 h previously. Cells were viewed briefly using a fluorescein filter block, and only fluorescent cells expressing GFP were used in experiments. The internal (pipette) and external solutions and recording techniques are similar to those described previously (Campbell, Berrow, Fitzgerald, Brickley & Dolphin, 1995*b*). The patch pipette solution contained (mM): caesium aspartate, 140; EGTA, 5; $MgCl_2$, 2; $CaCl_2$, 0·1; K_2ATP, 2; GTP, 0·1 (when used); and Hepes, 10; pH 7·2, 310 mosmol l^{-1} with sucrose. GTPγS (100 μM) or GDPβS

(0·5–2 mM) were included, where stated, in place of GTP. The external solution contained (mM): TEA-Br, 160; KCl, 3; $NaHCO_3$, 1·0; $MgCl_2$, 1·0; Hepes, 10; glucose, 4; and $BaCl_2$ or $CaCl_2$, 1; pH 7·4, 320 mosmol l^{-1} with sucrose. Pipettes of resistance 2–4 MΩ were used. Whole-cell currents were elicited from holding potentials (V_h) of −100 mV. Cells used had a capacitance of 31·9 ± 4·1 pF ($n = 10$), 27·1 ± 1·8 pF ($n = 15$) and 37·3 ± 2·1 pF ($n = 10$) for control, G$\beta_1\gamma_2$ cDNA-transfected and βARK1 (495–689) cDNA-transfected cells, respectively. Cells were only used where series resistance was compensated to 80 %, and space clamp was adequate as judged by graded activation of I_{Ca} or I_{Ba}. The voltage errors from the residual uncompensated series resistance were less than 1 mV for the largest currents, and no further correction was made. An Axopatch-1D amplifier (Axon Instruments) was used, and data were filtered at 0·5–5 kHz and digitized at 1–10 kHz. Analysis was performed using pCLAMP 6 (Axon Instruments) and Origin 3·5 (Microcal Software, Northampton, MA, USA). 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). Data are expressed as means ± S.E.M. Statistical analysis was performed using Student's paired or unpaired *t* tests, as appropriate.

RESULTS

Biophysical properties of α_{1B} subunits co-transfected with β_{2a} and α_2–δ

Transient transfection of α_{1B} cDNA together with β_{2a} and α_2–δ cDNAs resulted in inward I_{Ca} with a similar current density to that which we have previously described for I_{Ba}. In most experiments described here, Ca^{2+} was used as the charge carrier, but a number of the experiments were performed with Ba^{2+} (where stated), and no major differences were observed. A minor difference was that the mid-point of voltage dependence of activation ($V_{1/2}$) (see legend to Fig. 1) was −10·5 ± 1·8 mV ($n = 7$) in 1 mM Ba^{2+}, whereas it was −6·6 ± 2·1 mV ($n = 11$) in 1 mM Ca^{2+}, as expected for differences in charge screening. The β_{2a} subunit acts to retard entry of channels into the inactivated state (Olcese *et al.* 1994), and was used here to limit any confounding effects of voltage-dependent inactivation on the observation of G protein modulation (see Jones & Elmslie, 1997, for discussion of this problem). Co-expression of β_{2a} dramatically slowed the voltage-dependent inactivation of α_{1B}, compared with that previously observed when these subunits were co-expressed with β_{1b} subunits (Page, Stephens, Berrow & Dolphin, 1997). The inactivation kinetics of α_{1B}/α_2–δ/β_{2a} I_{Ba} at −10 mV, measured using 8 s voltage steps, could usually be described by a single exponential, with a time constant of 4980 ± 800 ms ($n = 4$), together with a plateau phase representing approximately 30 % of the current.

Control I_{Ca} recorded in an intracellular solution without added GTP was normally rapidly activating (Fig. 1*A*). A few examples of currents with a clearly distinguishable additional slowly activating component were also observed (3/13 cells), and this was also observed when I_{Ca} was recorded with GTP in the intracellular medium (2/4 cells) (Fig. 1*B*). In contrast, in the presence of co-transfected G$\beta_1\gamma_2$ subunits, the inward currents usually consisted of a slowly activating current only (Fig. 1*C*, representing 12/14 cells, with 2/14 also having a rapidly activating component). To determine whether the slowed activation observed in some control cells was a result of the effect of endogenous free G$\beta\gamma$ subunits, currents were examined in the presence of co-expressed βARK1 (495–689) to bind free G$\beta\gamma$ (Koch *et al.* 1993), and in the absence of GTP. Under these conditions, currents were always rapidly activating ($n = 15$, Fig. 1*D*). From current–voltage relationships, current density was increased by βARK1 (495–689), compared with control currents (Fig. 1*E*), whereas G$\beta\gamma$ co-expression reduced current density and shifted the mid-point of activation to more depolarized potentials compared with the control (Fig. 1*E*).

Facilitation of α_{1B} following a large depolarizing prepulse

Current facilitation was examined by comparing current amplitudes and activation rates during a 50 ms test pulse before (P1) and 10 ms after (P2) application of depolarizing prepulses to +120 mV (Fig. 2, see left panel for protocol). The depolarizing prepulse produced a small but significant effect on control α_{1B} I_{Ca} current density (Fig. 2*A*, middle) and activation kinetics (Fig. 2*A*, right). The facilitation ratio of the current amplitudes was also calculated as P2/P1 at 11 ms after the start of the step (Fig. 3). This was designed to measure only rapidly activating current, as an estimate of current that was not modulated at −100 mV. Control facilitation ratios were greater than 1 particularly at the voltages that are just supra-threshold for activation (Fig. 3*A*, □; e.g. P2/P1 = 2·2 ± 0·4 at −20 mV). Currents were also examined using Ba^{2+} as the charge carrier (Fig. 3*B*) to determine whether the facilitation was Ca^{2+} dependent, and in the presence of GDPβS to limit any tonic activation of endogenous G$\alpha\beta\gamma$ by endogenous GTP. Control facilitation was still present, although to a smaller extent, in the presence of Ba^{2+} (Fig. 3*B*, ■; P2/P1 = 1·5 ± 0·2 at −20 mV), and was reduced, particularly at −30 mV, by the inclusion of GDPβS (Fig. 3*B*, ▨).

As described above, expression of G$\beta_1\gamma_2$ with α_{1B} caused a prominent slowing of activation kinetics and a reduction in current amplitude (Fig. 2*B*). Both of these effects were readily reversed by a depolarizing prepulse (Fig. 2*B*, middle and right), and the facilitated current activated at more hyperpolarized potentials than that before the prepulse (Fig. 2*B*, middle). The facilitation ratio was 5·9 ± 2·0 ($n = 6$) at −20 mV (Fig. 3*A*, ▨). The effect of GTPγS on facilitation was much less marked than that of G$\beta\gamma$ overexpression (Fig. 3*B*, ▩), the facilitation ratio being 3·0 ± 0·7 at −20 mV. In contrast, co-expression of βARK1 (495–689) almost completely abolished prepulse facilitation (Fig. 2*C*), both of the current amplitude (Fig. 2*C*, middle), and of the activation kinetics (Fig. 2*C*, right). Only a non-significant degree of facilitation remained at submaximal activation potentials (Fig. 3*A*, ▨; P2/P1 = 1·2 ± 0·4 at −20 mV).

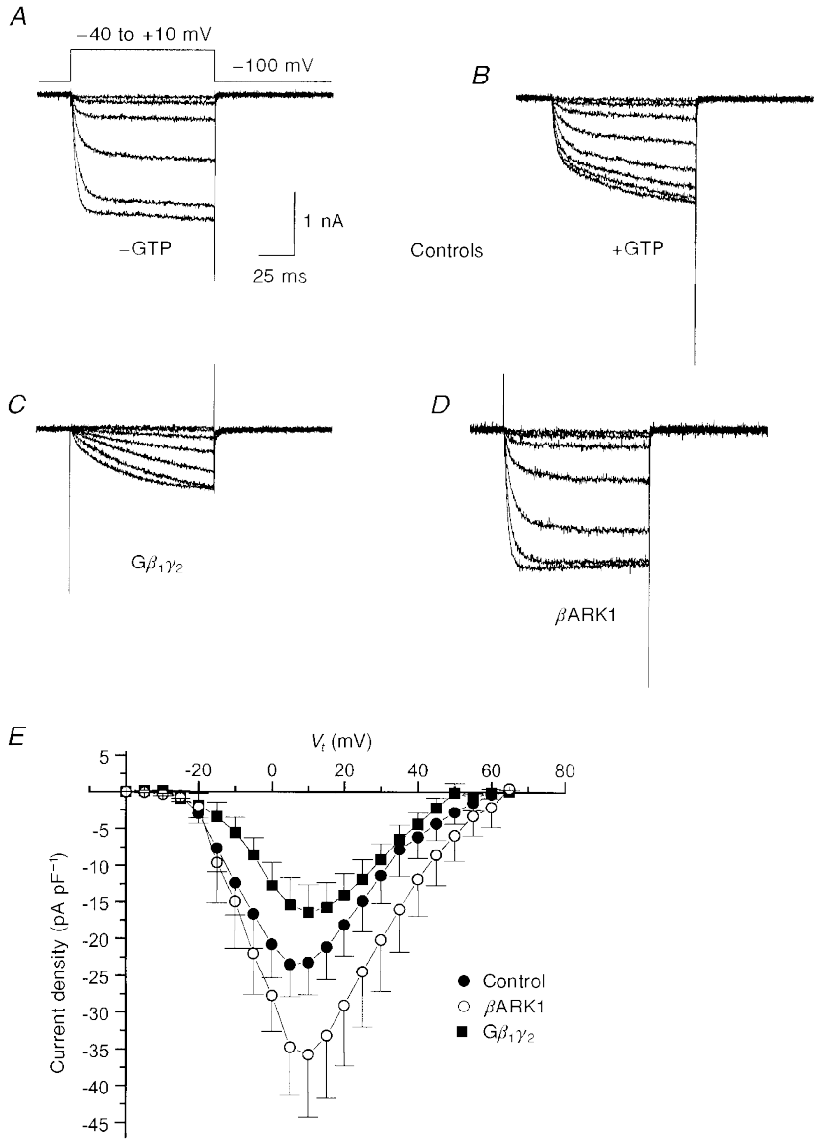

Figure 1. Calcium channel currents in COS-7 cells transfected with α_{1B}, and the effects of $G\beta_1\gamma_2$ and βARK1 (495–689) co-expression

A–D, examples of traces of I_{Ca} recorded from cells transfected with α_{1B} cDNA together with α_2–δ and β_{2a} cDNAs. Steps of 100 ms duration were applied to increasing test potentials (V_t) to maximally activate I_{Ca} from a holding potential (V_h) of −100 mV. V_t values are example traces between −40 and +10 mV. *A* and *B*, cells recorded with (*A*) or without (*B*) 0·1 mM GTP included in the intracellular medium. *C*, cells were co-transfected with $G\beta_1$ and $G\gamma_2$ cDNAs, and I_{Ca} was recorded in the absence of added intracellular GTP. *D*, cells were co-transfected with βARK1 (495–689) cDNA, and I_{Ca} was recorded in the absence of added intracellular GTP. *E*, current density–voltage relationships for cells recorded under conditions *A*, *C* and *D*. Results are given as means ± S.E.M. for 10, 11 and 15 cells, respectively. Individual current density–voltage plots were also fitted with the equation:

$$\text{Current density} = G_{max}(V - V_{rev})/\{1 + \exp[(V - V_{1/2})/k]\},$$

where G_{max} is maximum conductance (nS pF^{-1}), $V_{1/2}$ is the voltage at which 50 % of the current is activated, V_{rev} is the null potential and k is the slope factor. From this analysis, $V_{1/2}$ was −6·6 ± 2·1 mV for control, −4·9 ± 1·6 mV for βARK1 (495–689) and +0·1 ± 0·2 mV for $G\beta\gamma$ co-expression ($P < 0{\cdot}05$ *vs.* control); G_{max} was 0·6 ± 0·1 nS pF^{-1} for control, 0·8 ± 0·1 nS pF^{-1} for βARK1 (495–689) and 0·5 ± 0·1 nS pF^{-1} for $G\beta\gamma$ co-expression; k was 4·0 ± 0·4 mV for control, 3·1 ± 0·2 mV for βARK1 (495–689) and 4·5 ± 0·3 mV for $G\beta\gamma$ co-expression; and V_{rev} was 49·5 ± 3·5 mV for control, 53·1 ± 3·7 mV for βARK1 (495–689) and 51·8 ± 2·4 mV for $G\beta\gamma$ co-expression.

Kinetics of reinhibition of α_{1B} following a depolarizing prepulse

A prominent model of G protein interaction with α_1 subunits is one where channels exist in either a normal ('willing') or a 'reluctant' gating mode; the latter requiring stronger depolarization to open (Bean, 1989; Elmslie, Zhou & Jones, 1990). One possibility is that the transition between these states is determined by binding of activated G protein ($\beta\gamma$) subunits to the channel in a bimolecular reaction. Previous reports have examined the reversal of modulation (possibly representing actual dissociation of G$\beta\gamma$ from the channel), and the reinhibition following reversal (possibly representing re-association of G$\beta\gamma$ and the calcium channel), by using depolarizing prepulse protocols.

In order to characterize further the tonic- and G protein-mediated inhibition of α_{1B}, we examined the kinetics of reinhibition by increasing the delay between the depolarizing prepulse and the subsequent test pulse (P2). This analysis was performed for control facilitation, for that in the presence of GTPγS or co-expressed G$\beta_1\gamma_2$, and for the small degree of facilitation remaining in the presence of βARK1 (495–689).

Reinhibition (re-association) kinetics at −100 mV were examined using the protocols shown in Fig. 4. Following the prepulse, the time (Δt) before the subsequent test pulse (P2) was incremented until current was reinhibited to the same extent as during P1, prior to the prepulse. Data were recorded at levels of maximal prepulse potentiation (usually −20 mV). Under control conditions (Fig. 4*A*), reinhibition occurred with kinetics that were well described by a single exponential (Fig. 4*D*). The time constant of reinhibition ($\tau_{\text{reinhibition}}$), determined by exponential fits to data from individual cells, was 350 ± 35 ms ($n = 6$). This was not affected when Ba^{2+} was used as the charge carrier

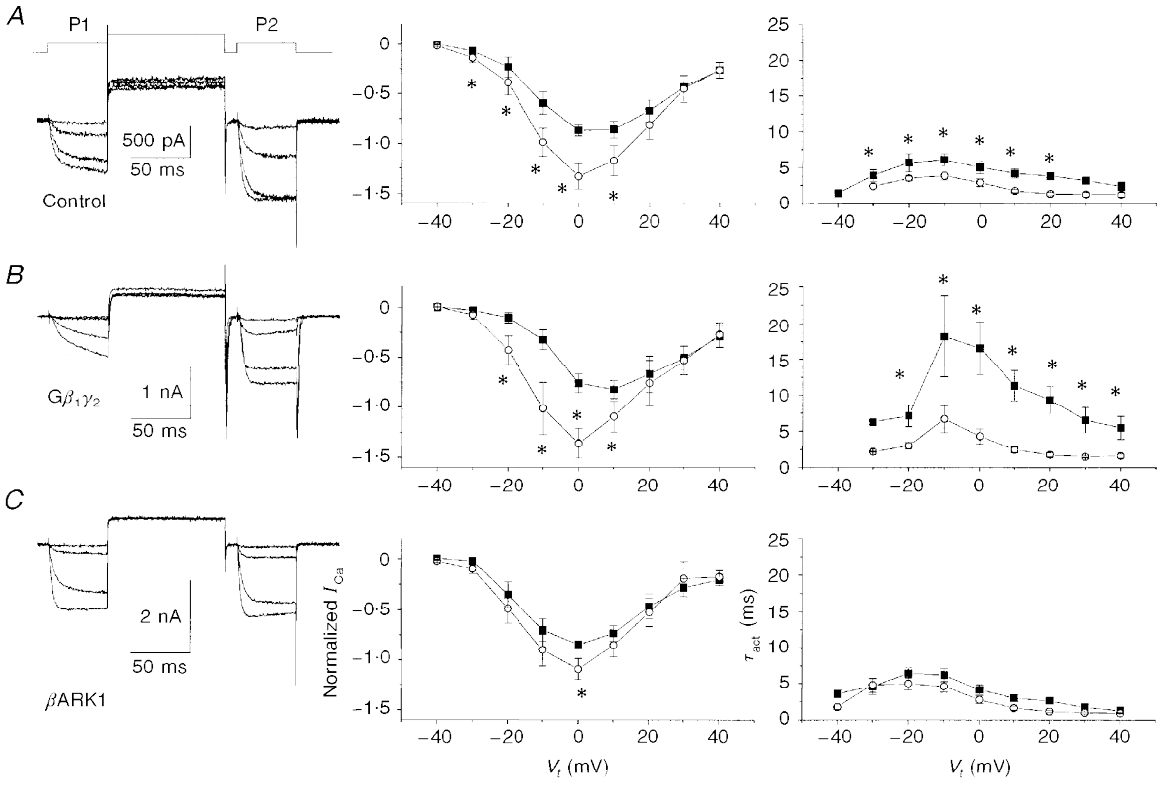

Figure 2. Prepulse potentiation of α_{1B} calcium channel currents

I_{Ca} was recorded from cells transfected with α_{1B} cDNA, together with α_2–δ and β_{2a} cDNAs in the absence of GTP in the intracellular solution, in control conditions (*A*, $n = 9$), with G$\beta_1\gamma_2$ co-expression (*B*, $n = 7$), or with βARK1 (495–689) co-expression (*C*, $n = 10$). Activation of I_{Ca} was examined from $V_h = -100$ mV, immediately before (P1) and 10 ms after (P2) application of a 100 ms depolarizing prepulse to +120 mV, according to the voltage protocol given. Example traces are given in the left panel: V_t values are between −40 and 0 mV. Current–voltage relationships are given in the middle panel from the amplitudes at 50 ms in P1 (■) and P2 (○). The currents have been normalized for each cell to the maximum current in P1 (normally at 0 mV in *A* and *C*, and +10 mV in *B*). Activation was described by a single exponential fit starting at the beginning of the negative current deflection for the currents in P1 and P2, to obtain the time constant of activation, τ_{act}. τ_{act}–voltage relationships are given in the right panel, with the same symbols as for the middle panel. All data are given as means ± S.E.M., and statistical significances were examined using Student's *t* test for paired data; * $P < 0{\cdot}05$.

($\tau_{reinhibition} = 361 \pm 124$ ms, $n = 3$). For $G\beta_1\gamma_2$ overexpression, the reinhibition kinetics of I_{Ca} were much more rapid (Fig. 4*B* and *D*), being 41·3 ± 8·9 ms ($n = 7$; $P < 0{\cdot}01$ compared with control). Similarly rapid reinhibition kinetics were obtained using Ba^{2+} as the charge carrier ($\tau_{reinhibition} = 25 \pm 5$ ms, $n = 5$; $P < 0{\cdot}05$ compared with control). Reinhibition kinetics of I_{Ba} in the presence of GTPγS were slower than for $G\beta_1\gamma_2$ ($\tau_{reinhibition} = 210 \pm 30$ ms, $n = 5$; $P < 0{\cdot}05$, compared with the $\tau_{reinhibition}$ in the presence of $G\beta\gamma$). To determine whether the slow reinhibition kinetics in control conditions were due to a low rate of reassociation with free $G\beta\gamma$ subunits, the time constant of reinhibition was further examined in the presence of expressed βARK1 (495–689) C-terminal polypeptide. Although the amount of facilitation was small, as shown above, reinhibition of I_{Ca} was extremely slow (Fig. 4*C*), $\tau_{reinhibition}$ being 653 ± 102 ms ($n = 9$; $P < 0{\cdot}05$ compared with control) (Fig. 4*D*).

Kinetics of loss of G protein-mediated inhibition of α_{1B} during a depolarizing prepulse

Dissociation of G protein subunits from α_{1B} at +120 mV was measured using the protocol shown in Fig. 5*A*. Two test pulses, P1 and P2, were given, with a depolarizing prepulse to +120 mV of 0·25–100 ms duration 10 ms before P2. In the absence of a prepulse, the P1 and P2 currents are identical. The length of the depolarizing prepulse was incremented until the current elicited by the subsequent voltage step had saturated at a maximal level; data were again taken at levels of maximal prepulse potentiation (usually −20 mV). Current amplitude was measured 11 ms after the start of the test pulse, to provide an estimate of only the rapidly activating current component. The time constant of dissociation (τ_{diss}) was then determined by single exponential fits to data from individual cells (see Fig. 5*B* for examples from three of the conditions: control, $G\beta_1\gamma_2$ and βARK1 (495–689) overexpression). The τ_{diss} did not differ significantly between the different conditions examined here (Fig. 5*C*), being between 18 and 24 ms, in agreement with the view that relief of block should be independent of free $G\beta\gamma$ subunit concentration at large depolarizations, where rebinding of $G\beta\gamma$ is minimal (Jones & Elmslie, 1997).

Immunolocalization of calcium channel and G protein subunits

The calcium channel β subunit and the $G\beta\gamma$ subunits both have binding sites located on the intracellular I–II loop of

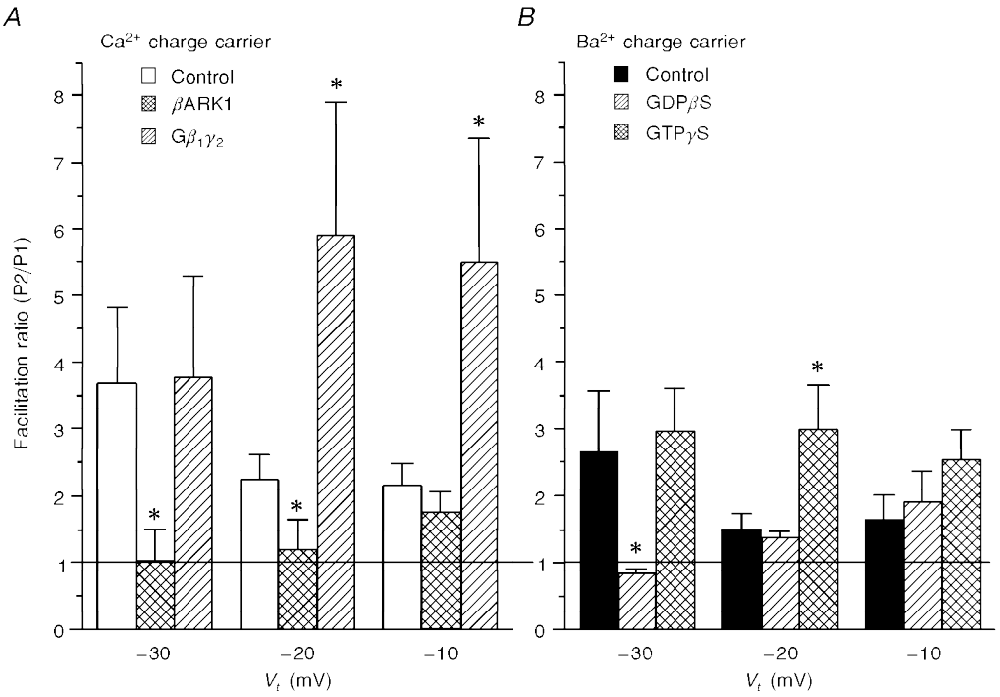

Figure 3. Comparison of facilitation ratios for α_{1B} calcium channel currents

The facilitation ratio at the stated potentials is the ratio P2/P1 of the current amplitudes in P1 and P2 measured 11 ms after the start of the step when the rapidly activating current component had saturated. This was to gain a measure of the steady-state inhibition at the holding potential. P2/P1 was determined for each cell under the conditions given. *A*, Ca^{2+} as charge carrier: control without GTP (□), βARK1 (495–689) co-expression (▩) and $G\beta_1\gamma_2$ co-expression (▨); $n = 9$, 10 and 6, respectively. *B*, Ba^{2+} as charge carrier: control without GTP (■), GDPβS (▨), GTPγS (▩); $n = 7$, 4 and 12, respectively. All data are given as means ± S.E.M., and statistical significances compared with respective controls were examined using Student's *t* test; * $P < 0{\cdot}05$.

the α_{1B} subunit (Pragnell, De Waard, Mori, Tanabe, Snutch & Campbell, 1994; De Waard *et al.* 1997; Zamponi *et al.* 1997). As one of the G$\beta\gamma$-binding sites overlaps directly with that of the calcium channel β subunit, it is important to demonstrate that in the present experiments the results obtained in experiments in which G$\beta\gamma$ is co-expressed are not due to interference with the role of the calcium channel β subunit in localizing the α_1 subunits to the plasma membrane (Chien *et al.* 1995; Brice *et al.* 1997). In support of the electrophysiological data, Fig. 6 shows that α_{1B} (and also the calcium channel β subunit) was still predominantly membrane localized in the presence of G$\beta_1\gamma_2$; in addition, a pan-specific antibody to Gβ indicated that significant amounts of this subunit also reached the cell membrane. Therefore, G$\beta\gamma$ co-expression does not suppress the calcium channel β subunit function to traffic α_1 subunits to the plasma membrane. This is in agreement with electrophysiological results that the maximum conductance (G_{max}) obtained from current density–voltage relationships (see legend to Fig. 1) was not significantly reduced in the presence of G$\beta\gamma$ compared with the control. In 1 mM Ca^{2+}, the control G_{max} was $0{\cdot}6 \pm 0{\cdot}1$ nS pF^{-1}, whereas for G$\beta\gamma$ overexpression it was $0{\cdot}5 \pm 0{\cdot}1$ nS pF^{-1} (Fig. 1*E*). In 1 mM Ba^{2+}, the control G_{max} was $0{\cdot}62 \pm 0{\cdot}11$ nS pF^{-1} ($n = 7$), whereas for G$\beta\gamma$ overexpression it was $0{\cdot}65 \pm 0{\cdot}13$ nS pF^{-1} ($n = 7$).

DISCUSSION

Calcium channel facilitation is a phenomenon that has been observed in a number of different systems, for both cloned and native calcium channels (for review, see Dolphin, 1996). In the case of cloned α_{1C} calcium channels, facilitation has been shown to depend on the presence of a β subunit, and possibly to involve phosphorylation (Sculptoreanu *et al.* 1993*a*,*b*; Bourinet *et al.* 1994). In contrast, for native neuronal calcium channels, it has been suggested that facilitation represents the reversal of tonic G protein

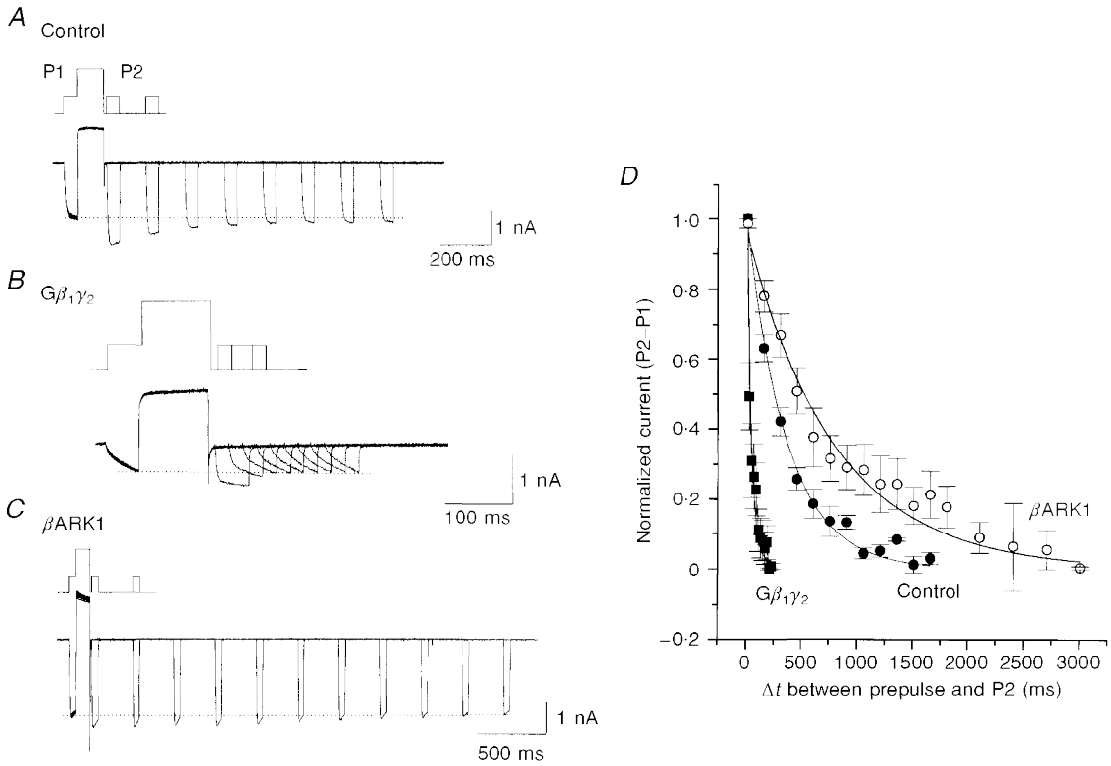

Figure 4. Reinhibition of α_{1B} at −100 mV following a depolarizing prepulse to +120 mV

I_{Ca} was recorded from cells transfected with α_{1B} cDNA together with α_2–δ and β_{2a} cDNAs in control conditions (no GTP in the patch pipette, *A*), and with G$\beta_1\gamma_2$ (*B*) or βARK1 (495–689) co-expression (*C*). *A*–*C*, facilitating prepulses to +120 mV were applied after a control depolarization (P1), and the time (Δt) between the prepulse and the second test pulse, P2, was incremented on successive recordings until reinhibition was complete. The Δt increments were 150, 20 and 300 ms in *A*, *B* and *C*, respectively, and V_t was to −20 mV where maximal prepulse potentiation was observed. In each case the first two voltage traces are shown above the set of current traces. A dotted line on the current traces indicates the current level at which reinhibition is complete. *D*, reinhibition of $\alpha_{1B}/\alpha 2$–δ/β_{2a} under control conditions (●, $n = 9$), with G$\beta_1\gamma_2$ (■, $n = 7$) or with βARK1 (495–689) (○, $n = 9$). Current amplitude was measured at the end of the 50 ms step at P2 for each Δt value and normalized to that at maximum potentiation (i.e. P2–P1 at $\Delta t = 10$ ms). The continuous lines represent single exponential fits to the group data.

modulation (Doupnik & Pun, 1994; Dolphin, 1996; Albillos *et al.* 1996). The present study has examined the role of endogenous and exogenous G$\beta\gamma$ subunits in the tonic and G protein-mediated modulation of α_{1B} calcium channels, which underlie the neuronal N-type current. In order to separate any effects on voltage-dependent inactivation (Zhang, Ellinor, Aldrich & Tsien, 1994; Herlitze, Hockerman, Scheuer & Catterall, 1997; Page *et al.* 1997) from voltage-dependent G protein-mediated slowing of activation kinetics, the auxiliary β_{2a} subunit was co-expressed in order to limit inactivation (Olcese *et al.* 1994). Under control conditions, amplitude facilitation (P2/P1) ratios significantly

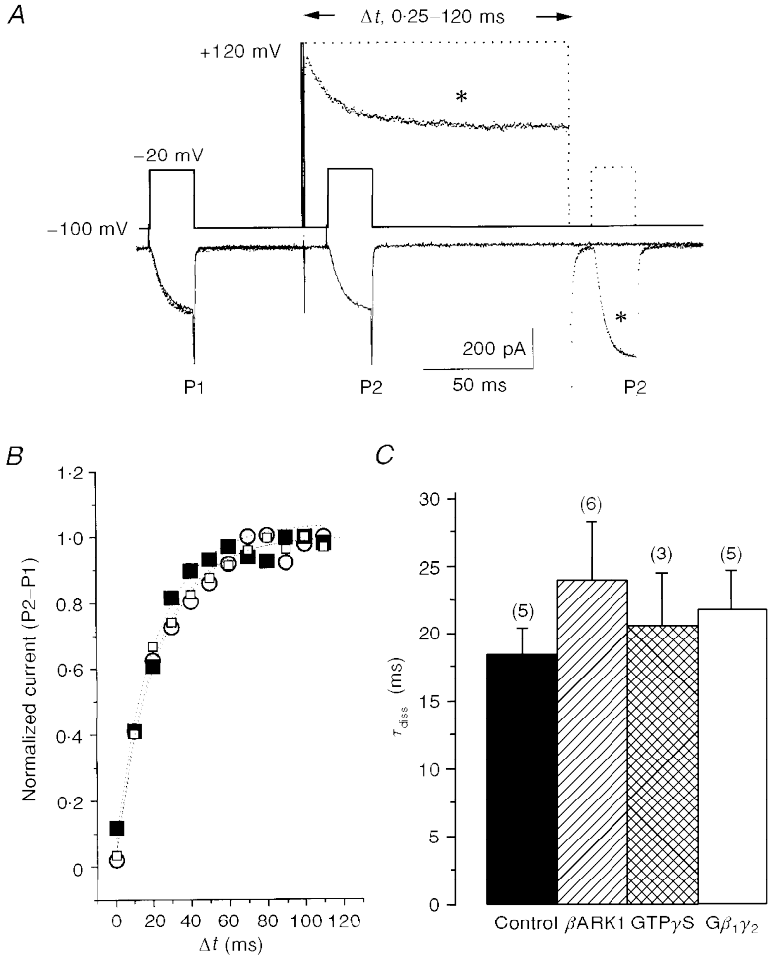

Figure 5. Relief of G protein inhibition of α_{1B} at +120 mV

Calcium channel currents were recorded from cells transfected with α_{1B} cDNA together with α_2–δ and β_{2a} cDNAs under control conditions (I_{Ca}), in the presence of 100 μM GTPγS in the patch pipette (I_{Ba}) or with G$\beta_1\gamma_2$ (I_{Ca}) or βARK1 (495–689) co-expression (I_{Ca}). *A*, two examples of the first (continuous line) and last (dotted line, trace marked with *) currents and the corresponding voltage protocols used are shown for α_{1B} under control conditions. Two test pulses, P1 and P2, were applied before and 10 ms after a depolarizing prepulse to +120 mV, respectively. The prepulse duration (Δt) was incremented from 0·25 ms (thick line) to 120 ms (dotted line). V_t was at levels of maximal prepulse potentiation; for this example, $V_t = -20$ mV. As the length of the prepulse (Δt) was incremented, current at P2 increased and saturated. *B*, relief of G protein-mediated block of α_{1B}/α_2–δ/β_{2a} in typical examples in control conditions (□), and with βARK1 (495–689) (○) or G$\beta_1\gamma_2$ (■) overexpression. Current amplitude was measured 11 ms after the start of the P1 and P2 steps for each Δt value, and normalized to that at maximum potentiation (i.e. maximum P2–P1 value). The time constant of dissociation (τ_{diss}) of the active G protein from the α_{1B} channel was determined from a single exponential fit to data from individual experiments (dotted lines). The τ_{diss} values for the examples given are 22·2, 21·2 and 23·3 ms for control, G$\beta_1\gamma_2$ and βARK1 (495–689), respectively. *C*, mean τ_{diss} ± S.E.M. for the different conditions shown, with the number of experiments given in parentheses. The rate of dissociation was similar for all conditions. There was no significant difference between control (■) and either decreasing free [G$\beta\gamma$] with βARK1 (495–689) expression (▨), increasing free [G$\beta\gamma$] with GTPγS (▩) or transfection of G$\beta_1\gamma_2$ (□).

less than 1 were never seen with β_{2a}, either in the presence or absence of a depolarizing prepulse between the two test pulses. This is in contrast to results obtained with β_{1b}, where the significant degree of inactivation during the prepulse protocol could mask facilitation (G. J. Stephens & A. C. Dolphin, unpublished observations).

G$\beta\gamma$ subunits mediate both G protein-induced and tonic inhibition of the α_{1B} calcium channel

The prepulse facilitation data for the calcium channel α_{1B} subunit under control conditions suggest some tonic facilitation in COS-7 cells (Fig. 3), as previously reported for expression in HEK 293 cells (Toth, Shekter, Ma, Philipson

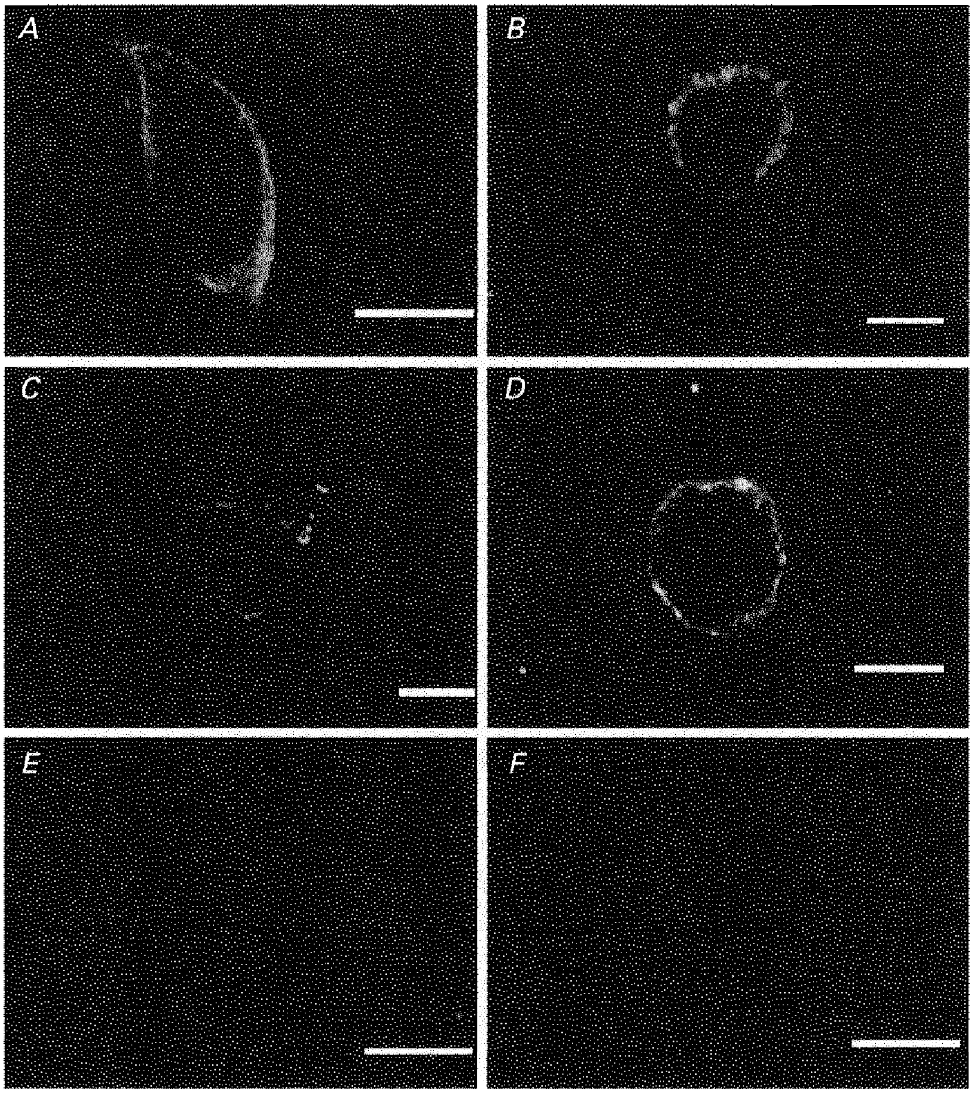

Figure 6. Immunolocalization of α_{1B}, β and Gβ subunits in COS-7 cells

Cells were transfected with α_{1B}, α_2–δ, β_{1b} and G$\beta_1\gamma_2$ cDNAs, except for *A* and *F* where the G$\beta_1\gamma_2$ subunits were omitted, and *E* which represents transfection of pMT2 vector alone. The cells were labelled with polyclonal antibodies directed against α_{1B} (*A*, *B* and *E*), β_{common} (*C*), G$\beta_{(common)}$ (a pan-specific antibody that recognizes all Gβ isoforms, *D*) or with α_{1B} pre-immune serum (*F*). Scale bars are 15 μm. The staining at the plasma membrane (M) and in the intracellular compartment (C) of a random sample of cells from each group were compared using confocal microscopy. The location of membrane and intracellular compartment were determined from an overlaid phase image. For each of the four groups, immunostaining against the underlined subunit (in arbitrary units (a.u.) determined using the Kontron KS400 program) was significantly higher for the plasma membrane than the intracellular compartment: *A*, $\underline{\alpha_{1B}}/\alpha_2$–$\delta/\beta_{1b}$ (M = 117 ± 10 a.u., C = 93 ± 10 a.u.; $n = 8$, $P < 0{\cdot}005$); *B*, $\underline{\alpha_{1B}}/\alpha_2$–$\delta/\beta_{1b}$, G$\beta_1$1$\gamma_2$ (M = 119 ± 13 a.u., C = 69 ± 9 a.u.; $n = 8$, $P < 0{\cdot}005$); *C*, α_{1B}/α_2–$\delta/\underline{\beta_{1b}}$, G$\beta_1\gamma_2$ (M = 98 ± 8 a.u., C = 62 ± 8 a.u.; $n = 17$, $P < 0{\cdot}005$); *D*, $\underline{\alpha_{1B}}/\alpha_2$–$\delta/\beta_{1b}$, $\underline{G\beta_1\gamma_2}$ (M = 98 ± 16 a.u., C = 64 ± 12 a.u.; $n = 8$, $P < 0{\cdot}05$). Values are means ± S.E.M. No staining was observed in cells transfected with pMT2 vector alone or cells stained using α_{1B} pre-immune serum.

& Miller, 1996). Dialysis with GDPβS reduced control facilitation at −30 mV, indicating that rather than being an intrinsic property of the α_{1B} calcium channel, as may be the case for α_{1C} (Scuptoreanu *et al.* 1993*a*), it may be a result of tonic modulation by endogenous free G$\beta\gamma$ subunits, whose level could be influenced by the endogenous GTP concentration, and possibly by tonic activation of endogenous receptors (Koch *et al.* 1994). It may also reflect the voltage dependence of the influence of the calcium channel β subunit, whose binding may compete with G$\beta\gamma$ (Campbell *et al.* 1995*b*; Dolphin, 1996; Qin *et al.* 1997). To examine further the possibility that α_{1B} is modulated by free G$\beta\gamma$ under control conditions, we examined the effect of co-expression of βARK1 (495–689) (Koch *et al.* 1994). This is the C-terminal G$\beta\gamma$-binding domain of βARK1, comprising amino acids 495–689, which has no kinase activity. It has an affinity for G$\beta\gamma$ sufficient to interfere with G$\beta\gamma$-mediated signalling in other systems (Koch *et al.* 1994). We observed a marked reduction in the amount of prepulse potentiation in cells co-expressing βARK1 (495–689), presumably because it lowered the amount of free G$\beta\gamma$ able to bind to α_{1B}.

In contrast, dialysis with GTPγS potentiated the observed facilitation of α_{1B}, in agreement with our previous studies in which the auxiliary β subunit was β_{1b} (Page *et al.* 1997). Co-expression of α_{1B} with G$\beta_1\gamma_2$ caused classical modulation of I_{Ca}, in which G protein inhibition is associated with a depolarizing shift in $V_{1/2}$. This inhibition was readily reversed by a large depolarizing prepulse, both in terms of facilitation of current levels and increase in current activation rate. These findings confirm the involvement of G$\beta\gamma$ in N-type calcium channel inhibition (Herlitze *et al.* 1996; Ikeda, 1996). It is likely that the action of GTPγS is to recruit endogenous G protein subunits, and in all cases co-expression of G$\beta_1\gamma_2$ led to a greater modulation of activation kinetics than GTPγS. These differences are probably due to a higher effective concentration of the active G protein subunits being present when G$\beta_1\gamma_2$ subunits are co-expressed, as discussed below. In addition, GTPγS produces, in equal proportion, Gα–GTPγS and free G$\beta\gamma$, whereas when G$\beta_1\gamma_2$ subunits are transfected they are presumably present in excess over Gα subunits.

Kinetics of reinhibition of α_{1B} by G$\beta\gamma$ subunits following a depolarizing prepulse

We further examined the interaction between α_{1B} and activated G protein subunits in terms of reinhibition characteristics for α_{1B} calcium current under control conditions, and compared this with α_{1B} current in the presence of GTPγS (as a measure of maximal modulation of endogenous G proteins), co-expressed with G$\beta_1\gamma_2$ (as a measure of a saturated system), or co-expressed with βARK1 (495–689) to bind endogenous free G$\beta\gamma$ subunits (Koch *et al.* 1994). Consistent with an increased concentration of active G protein subunits, G$\beta_1\gamma_2$ cDNA transfection produced a much faster reinhibition of α_{1B} current than GTPγS, and this in turn was faster than the reinhibition following facilitation under control conditions. Presumably the action of GTPγS is dependent on the concentration of endogenous G$\beta\gamma$ generated by GTPγS-induced activation of Gα subunits. There is no Gα_o in COS-7 cells, but they do contain Gα_{11} and Gα_q (see Lee, Park, Wu, Rhee & Simon, 1992) and also Gα_i and Gα_s (Daaka, Pitcher, Richardson, Stoffel, Robishaw & Lefkowitz, 1997), all of which could generate endogenous G$\beta\gamma$ subunits in this system. This suggests either that the level of G$\beta\gamma$ attainable from endogenous G$\alpha\beta\gamma$ heterotrimers is much lower than the level reached when G$\beta\gamma$ cDNA is co-transfected into this system, or that the particular endogenous G$\beta\gamma$ subunits generated do not couple well to the calcium channel α_{1B} subunit present. The former explanation seems more likely for our results with α_{1B}, in that N-type calcium channels seem to be fairly promiscuous in the G$\beta\gamma$ isoforms with which they couple (Ikeda, 1996); in agreement, we see similar effects on α_{1B} when G$\beta_1\gamma_2$ is replaced by the G$\beta_2\gamma_3$ combination (G. J. Stephens & A. C. Dolphin, unpublished results). The prolongation of the reinhibition time course in the presence of βARK1 (495–689) suggests that this has lowered the resting level of free G$\beta\gamma$. Thus, during the depolarizing prepulse, residual G$\beta\gamma$ that was bound to α_{1B} is removed, and rebinding is slow because it occurs from the bulk phase. The fact that rebinding does occur indicates that, at −100 mV, G$\beta\gamma$ binding to the α_{1B} calcium channel is of high affinity. The affinity of G$\beta\gamma$ binding to the interaction domain (AID) on the α_{1A} I–II loop *in vitro* has been measured to be 63 nM (De Waard *et al.* 1997). The present results provide evidence that actual dissociation of G$\beta\gamma$, rather than just a reduction in its effectiveness, occurs during depolarization.

The rate of calcium current reinhibition has been shown previously to be dependent on the concentration of agonist (Elmslie & Jones, 1994; Zhou, Shapiro & Hille, 1997). In oocytes, reinhibition of α_{1B} following a depolarizing prepulse during modulation by a receptor agonist had a time constant of about 77 ms (Zhang, Ellinor, Aldrich & Tsien, 1996). These studies were performed in the presence of both the agonist somatostatin and exogenously expressed G$\beta\gamma$, although G$\beta\gamma$ expression may not be maximal as the ligand was still able to inhibit currents. In general, reinhibition rates in native neurons following inhibition of the calcium current by agonist application have time constants of between 50 and 140 ms (see Dolphin, 1996; Zhou *et al.* 1997). It seems likely that transient overexpression of G$\beta_1\gamma_2$ in the present study causes maximal inhibition of the α_{1B} current.

Estimation of free G$\beta\gamma$ concentrations

For the reaction:

$$C + G\beta\gamma \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} CG\beta\gamma,$$

C is one of the closed states of the calcium channel α_{1B} subunit, k_1 is the association rate constant and k_{-1} is the

dissociation rate constant, both of which may be (indirectly or directly) voltage dependent. At equilibrium, from the law of Mass Action:

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

(where $[G\beta\gamma]$ and $[CG\beta\gamma]$ are the concentrations of free and channel-bound $G\beta\gamma$, respectively), at −100 mV

$$1/\tau_{\text{reinhibition}} = k_1[G\beta\gamma] + k_{-1}, \quad (1)$$

and

$$\text{Steady-state inhibition} = k_1[G\beta\gamma]/(k_1[G\beta\gamma] + k_{-1}); \quad (2)$$

therefore,

$$\text{P1/P2} = k_{-1}/(k_1[G\beta\gamma] + k_{-1}). \quad (3)$$

Substituting into eqn (1) the $\tau_{\text{reinhibition}}$ values for control, $G\beta\gamma$ or βARK1 (495–689) overexpression obtained from Fig. 4, and substituting into eqn (3) the facilitation ratios P2/P1 determined in Fig. 3*A* for a step to −20 mV, k_{-1} at −100 mV can be calculated to be 1·25 s^{-1} for control, 4·26 s^{-1} for $G\beta_1\gamma_2$ overexpression, and 1·28 s^{-1} for βARK1 (495–689) overexpression. It is of interest that the k_{-1} value is similar in the two cases involving endogenous $G\beta\gamma$, but is larger for co-expressed $G\beta_1\gamma_2$, suggesting that the dissociation rate from the α_{1B} channel for different $G\beta\gamma$ subunits may not be identical. However, if we assume the affinity of $G\beta\gamma$ binding to α_{1B} at −100 mV to be similar to that calculated for $G\beta\gamma$ binding to the α_{1A} I–II intracellular loop (63 nM; De Waard *et al.* 1997), for all the conditions described here, we can estimate k_1 to be 19·8, 67·6 and 20·3 μM^{-1} s^{-1} for control, $G\beta_1\gamma_2$ and βARK1 (495–689) overexpression, respectively, at −100 mV. Taking these values, the free $[G\beta\gamma]$ under these three conditions is 50, 295 and 11·8 nM, respectively.

Kinetics of dissociation of $G\beta\gamma$ from α_{1B} during a depolarizing prepulse

The time constants for relief of G protein-mediated inhibition of α_{1B} currents by varying the depolarizing prepulse are very similar under control conditions, or in the presence of GTPγS, or with $G\beta_1\gamma_2$ or βARK1 (495–689) co-expression (τ_{diss} = 18–24 ms). The similarity of these dissociation rates under conditions of varying free $G\beta\gamma$ concentration indicates that there is little reassociation of $G\beta\gamma$ during the depolarizing prepulse. Thus, there is a strong voltage dependence of k_1, and dissociation will be favoured at this potential (see Patil *et al.* 1996). Assuming k_1 to be negligible at +120 mV, then k_{-1} under control conditions is approximately 50 s^{-1} at this potential, about 50-fold more rapid than at −100 mV.

Dissociation time constants, measured by varying the depolarizing prepulse, of between 5 and 12 ms have been reported for neuronal N-type calcium channels in the presence of GTPγS (Currie & Fox, 1997; Jones & Elmslie, 1997), and 3 ms for somatostatin inhibition of α_{1B} currents in oocytes (Zhang *et al.* 1996), which are somewhat faster than those measured in the present study. One major difference between this and the study of cloned α_{1B} expressed in oocytes (Zhang *et al.* 1996) is that α_{1B} was co-expressed with the β_{2a} subunit here. The role of this subunit in the kinetics of G protein modulation requires further comparative investigation; however, it may be speculated that the kinetics of interaction between $G\beta_1\gamma_2$ and α_{1B} are dependent on the β subunit present, since there are binding sites for both on the I–II loop. Some preliminary data support this hypothesis, in that transmitter-induced slowing of calcium channel kinetics was prominent with β_{2a}, but not seen with β_{1b} (Patil, Brody, Snutch & Yue, 1997). The molecular composition of neuronal calcium channels, and in particular β subunit diversity, may thus have important consequences not only for voltage-dependent inactivation, but also in the fine-tuning of modulation by $G\beta\gamma$ subunits.

We also show that $G\beta\gamma$ co-expression does not suppress calcium channel β subunit function to traffic α_1 subunits to the plasma membrane. Conversely, current amplitude and activation rates were clearly inhibited by $G\beta_1\gamma_2$ in the presence of β subunits; and the reversal of inhibition by depolarizing prepulses suggests that the nature of the interaction of $G\beta\gamma$ with the channel is highly dynamic. In contrast, the α_1–β interaction might be expected to be much more stable with very high affinity binding reported *in vitro* (De Waard, Witcher, Pragnell, Liu & Campbell, 1995). Nevertheless, it is likely that this binding is also voltage dependent, and it will be of interest to determine the effects of the β subunit on $G\beta\gamma$ binding to the I–II loop, to examine more fully the hypothesis that β subunits and G proteins may interact competitively with calcium channel α_1 subunits (Campbell *et al.* 1995*b*; Bourinet, Soong, Stea & Snutch, 1996; Qin *et al.* 1997).

In conclusion, the data shown here indicate that under control conditions there is sufficient free $G\beta\gamma$ present in the vicinity of the α_{1B} calcium channel to produce a significant tonic block of α_{1B} current. If this also occurs in native neurons and neurosecretory cells, it would lead to frequency- or depolarization-dependent facilitation of calcium entry through N-type channels, as has been observed in several cell types (e.g. Doupnik & Pun, 1994; Albillos *et al.* 1996).

Albillos, A., Gandía, L., Michelena, P., Gilabert, J. A., Del Valle, M., Carbone, E. & García, A. G. (1996). The mechanism of calcium channel facilitation in bovine chromaffin cells. *Journal of Physiology* **494**, 687–695.

Bean, B. P. (1989). Neurotransmitter inhibition of neuronal calcium channels by changes in channel voltage dependence. *Nature* **340**, 153–156.

Berrow, N. S., Brice, N. L., Tedder, I., Page, K. & Dolphin, A. C. (1997). Properties of cloned rat α1A calcium channels, transiently expressed in the COS-7 cell line. *European Journal of Neuroscience* **9**, 739–748.

Berrow, N., Campbell, V., Fitzgerald, E. M., Brickley, K. & Dolphin, A. C. (1995). Antisense depletion of β-subunits modulates the biophysical and pharmacological properties of neuronal calcium channels. *Journal of Physiology* **482**, 481–491.

Boland, L. M. & Bean, B. P. (1993). Modulation of N type calcium channels in bullfrog sympathetic neurons by luteinising hormone releasing hormone: kinetics and voltage-dependence. *Journal of Neuroscience* **13**, 516–533.

Bourinet, E., Charnet, P., Tomlinson, W. J., Stea, A., Snutch, T. P. & Nargeot, J. (1994). Voltage-dependent facilitation of a neuronal α_{1C} L-type calcium channel. *EMBO Journal* **13**, 5032–5039.

Bourinet, E., Soong, T. W., Stea, A. & Snutch, T. P. (1996). 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.

Brice, N. L., Berrow, N. S., Campbell, V., Page, K. M., Brickley, K., Tedder, I. & Dolphin, A. C. (1997). Importance of the different β subunits in the membrane expression of the α1A and α2 calcium channel subunits: studies using a depolarisation-sensitive α1A antibody. *European Journal of Neuroscience* **9**, 749–759.

Campbell, V., Berrow, N., Brickley, K., Page, K., Wade, R. & Dolphin, A. C. (1995*a*). 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.

Campbell, V., Berrow, N. S., Fitzgerald, E. M., Brickley, K. & Dolphin, A. C. (1995*b*). Inhibition of the interaction of G protein G_o with calcium channels by the calcium channel β-subunit in rat neurones. *Journal of Physiology* **485**, 365–372.

Chien, A. J., Zhoa, X., Shirokov, R. E., Puri, T. S., Chang, C. F., Sun, D., Rios, E. & Hosey, M. M. (1995). Roles of a membrane-localised β subunit in the formation and targeting of functional L-type Ca^{2+} channels. *Journal of Biological Chemistry* **270**, 30036–30044.

Currie, K. P. M. & Fox, A. P. (1997). Comparison of N- and P/Q-type voltage-gated calcium channel current inhibition. *Journal of Neuroscience* **17**, 4570–4579.

Daaka, Y., Pitcher, J. A., Richardson, M., Stoffel, R. H., Robishaw, J. D. & Lefkowitz, R. J. (1997). Receptor and G$\beta\gamma$ isoform-specific interactions with G protein-coupled receptor kinases. *Proceedings of the National Academy of Sciences of the USA* **94**, 2180–2185.

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

De Waard, M., Witcher, D. R., Pragnell, M., Liu, H. & Campbell, K. P. (1995). Properties of the α1-β anchoring site in voltage-dependent Ca^{2+} channels. *Journal of Biological Chemistry* **270**, 12055–12064.

Dolphin, A. C. (1996). Facilitation of Ca^{2+} current in excitable cells *Trends in Neurosciences* **19**, 35–43.

Doupnik, C. A. & Pun, R. Y. K. (1994). G-protein activation mediates prepulse facilitation of Ca^{2+} channel currents in bovine chromaffin cells. *Journal of Membrane Biology* **140**, 47–56.

Dubel, S. J., Starr, T. V. B., Ahlijanian, M. K., Enyeart, J. J., Catterall, W. A. & Snutch, T. P. (1992). Molecular cloning of the α1 subunit of an ω-conotoxin-sensitive calcium channel. *Proceedings of the National Academy of Sciences of the USA* **89**, 5058–5062.

Elmslie, K. S. & Jones, S. W. (1994). Concentration dependence of neurotransmitter effects on calcium channel kinetics in frog sympathetic neurones. *Journal of Physiology* **481**, 35–46.

Elmslie, K. S., Zhou, W. & Jones, S. W. (1990). LHRH and GTP-γ-S modify calcium current activation in bullfrog sympathetic neurons. *Neuron* **5**, 75–90.

Fong, H. K., Hurley, J. B., Hopkins, R. S., Miake-Lye, R., Johnson, M. S., Doolittle, R. F. & Simon, M. I. (1986). Repetitive segmental structure of the transducin beta subunit: homology with the CDC4 gene and identification of related mRNAs. *Proceedings of the National Academy of Sciences of the USA* **83**, 2162–2166.

Fujita, Y., Mynlieff, M., Dirksen, R. T., Kim, M.-S., Niidome, T., Nakai, J., Friedrich, T., Iwabe, N., Miyata, T., Furuichi, T., Furutama, D., Mikoshiba, K., Mori, Y. & Beam, K. G. (1993). Primary structure and functional expression of the ω-conotoxin-sensitive N-type calcium channel from rabbit brain. *Neuron* **10**, 585–598.

Gautam, N., Baetscher, M., Aebersold, R. & Simon, M. I. (1989). A G protein γ subunit shares homology with ras proteins. *Science* **244**, 971–974.

Herlitze, S., Garcia, D. E., Mackie, K., Hille, B., Scheuer, T. & Catterall, W. A. (1996). Modulation of Ca^{2+} channels by G-protein $\beta\gamma$ 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 $\beta\gamma$ subunits. *Nature* **380**, 255–258.

Jones, S. W. & Elmslie, K. S. (1997). Transmitter modulation of neuronal calcium channels. *Journal of Membrane Biology* **155**, 1–10.

Kim, H. L., Kim, H., Lee, P., King, R. G. & Chin, H. (1992). Rat brain expresses an alternatively spliced form of the dihydropyridine-sensitive L-type calcium channel α2 subunit. *Proceedings of the National Academy of Sciences of the USA* **89**, 3251–3255.

Koch, W. J., Hawes, B. E., Inglese, J., Luttrell, L. M. & Lefkowitz, R. J. (1994). Cellular expression of the carboxyl terminus of a G protein-coupled receptor kinase attenuates G$\beta\gamma$-mediated signalling. *Journal of Biological Chemistry* **269**, 6193–6197.

Koch, W. J., Inglese, J., Stone, W. C. & Lefkowitz, R. J. (1993). The binding site for the βgamma subunits of heterotrimeric G proteins on the β-adrenergic receptor kinase. *Journal of Biological Chemistry* **268**, 8256–8260.

Lee, C. H., Park, D., Wu, D., Rhee, S. G. & Simon, M. I. (1992). Members of the G_q alpha subunit gene family activate phospholipase C beta isozymes *Journal of Biological Chemistry* **267**, 16044–16047.

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.

Patil, P. G., Brody, D. L., Snutch, T. P. & Yue, D. T. (1997). β-subunit modulation of G-protein inhibition and voltage-dependent inactivation of P/Q type (A-class) neuronal calcium channels. *Biophysical Journal* **72**, A22.

Patil, P. G., de Leon, M., Reed, R. R., Dubel, S., Snutch, T. P. & Yue, D. T. (1996). Elemental events underlying voltage-dependent G-protein inhibition of N-type calcium channels. *Biophysical Journal* **71**, 2509–2521.

Perez-Reyes, E., Castellano, A., Kim, H. S., Bertrand, P., Baggstrom, E., Lacerda, A., Wei, X. & Birnbaumer, L. (1992). Cloning and expression of a cardiac/brain β subunit of the L-type calcium channel. *Journal of Biological Chemistry* **267**, 1792–1797.

Pitcher, J. A., Touhara, K., Payne, E. S. & Lefkowitz, R. J. (1995). Pleckstrin homology domain-mediated membrane association and activation of the beta-adrenergic receptor kinase requires co-ordinated interaction with G $\beta\gamma$ subunits and lipid. *Journal of Biological Chemistry* **270**, 11707–11710.

Pragnell, M., De Waard, M., Mori, Y., Tanabe, T., Snutch, T. P. & Campbell, K. P. (1994). Calcium channel β-subunit binds to a conserved motif in the I-II cytoplasmic linker of the α_1-subunit. *Nature* **368**, 67–70.

Qin, N., Platano, D., Olcese, R., Stefani, E. & Birnbaumer, L. (1997). Direct interaction of G$\beta\gamma$ with a C terminal G$\beta\gamma$ binding domain of the calcium 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.

Scuptoreanu, A., Rotman, E., Takahashi, M., Scheuer, T. & Catterall, W. (1993*a*). Voltage-dependent potentiation of the activity of cardiac L-type calcium channel α1 subunits due to phosphorylation by cAMP-dependent protein kinase. *Proceedings of the National Academy of Sciences of the USA* **90**, 10135–10139.

Sculptoreanu, A., Scheuer, T. & Catterall, W. A. (1993*b*). Voltage-dependent potentiation of L-type Ca^{2+} channels due to phosphorylation by cAMP-dependent protein kinase. *Nature* **364**, 240–243.

Stephens, G. J., Page, K., Burley, J. R., Berrow, N. S. & Dolphin, A. C. (1997). Functional expression of rat brain cloned α1E calcium channels in COS-7 cells. *Pflügers Archiv* **433**, 523–532.

Toth, P. T., Shekter, L. R., Ma, G. H., Philipson, L. H. & Miller, R. J. (1996). Selective G-protein regulation of neuronal calcium channels. *Journal of Neuroscience* **16**, 4617–4624.

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.

Zhou, J., Shapiro, M. S. & Hille, B. (1997). Speed of Ca^{2+} channel modulation by neurotransmitters in rat sympathetic neurons. *Journal of Neurophysiology* **77**, 2040–2048.

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