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β Subunits of Voltage-Gated Calcium Channels

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Calcium channel β subunits have marked effects on the trafficking and on several of the biophysical properties of all high voltage activated calcium channels. In this article I shall review information on the different genes, on the structure of the β subunits, and on their differential expression and post-translational modification. Their role in trafficking and assembly of the calcium channel heteromultimer will be described, and I will then review their effects on voltage-dependent and kinetic properties, stressing the differences between palmitoylated β2a and the other β subunits. Evidence for effects on calcium channel pharmacology will also be examined. I shall discuss the hypothesis that β subunits can bind reversibly to calcium channels, and examine their role in the G protein modulation of calcium channels. Finally, I shall describe the consequences of knock-out of different β subunit genes, and describe evidence for the involvement of β subunits in disease.

KEY WORDS: Calcium channel; β subunit;, palmitoylation; phosphorylation; trafficking; gene targeting; G-protein; assembly; inactivation.

INTRODUCTION

Voltage-dependent calcium channels (VDCCs) play a major role both in the normal functioning and also in the pathophysiology of neurons and other excitable cells. Because of the unique property of Ca^{2+} to regulate many cellular processes, the correct trafficking and localization of VDCCs is of great importance for cells. VDCCs consist of a transmembrane α1 subunit ($Ca_v\alpha1$), which associates with a number of auxiliary subunits, first identified by their association with the purified dihydropyridine (DHP) receptor (Takahashi *et al.*, 1987; Tanabe *et al.*, 1987). In the case of all the high voltage activated (HVA) calcium channels in which it has been studied, the $Ca_v\alpha1$ subunit copurifies with an intracellular β subunit ($Ca_v\beta$) and an extracellular $Ca_v\alpha2$ subunit, that is attached by S−S bonds to a transmembrane δ subunit (Chang and Hosey, 1988; Liu *et al.*, 1996; Tanabe *et al.*, 1987; Witcher *et al.*, 1993). Skeletal muscle calcium channels also copurify with a γ subunit (γ1) (Takahashi *et al.*, 1987), but whether the recently cloned novel γ-like subunits (γ2–8) are tightly associated with other types of VDCCs remains controversial (Black and Lennon, 1999; Kang *et al.*, 2001; Klugbauer *et al.*, 2000; Letts *et al.*, 1998; Moss *et al.*, 2002).

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VOLTAGE-DEPENDENT CALCIUM CHANNEL β SUBUNIT GENES

The first $Ca_v\beta$ subunit to be identified, now called $Ca_v\beta$1a, was observed as a 54 kDa subunit in the purified skeletal muscle DHP receptor calcium channel complex (Takahashi *et al.*, 1987), and the gene was cloned following partial sequencing of the protein (Ruth *et al.*, 1989). Three other β subunit genes were then cloned by homology with $Ca_v\beta$1 (Castellano *et al.*, 1993a,b; Hullin *et al.*, 1992; Perez-Reyes *et al.*, 1992). All β subunits show a number of splice variants (Fig. 1, for reviews see Birnbaumer *et al.*, 1998; Perez-Reyes and Schneider, 1994). In humans the β subunit genes are all on different chromosomes: 17q21 for β1, (Gregg *et al.*, 1993), 10p12 for β2 (Taviaux *et al.*, 1997), 12q13 for β3 (Park *et al.*, 1997), and 2q22 for β4 (Taviaux *et al.*, 1997). The gene structure of β1, β2, and β3 has been investigated in detail (Colecraft *et al.*, 2002; Murakami *et al.*, 1996; Powers *et al.*, 1992; Takahashi *et al.*, 2003). β3 was found to contain 13 exons spanning 8 kb (Murakami *et al.*, 1996; see Fig. 2 in Birnbaumer *et al.*, 1998). More recently the

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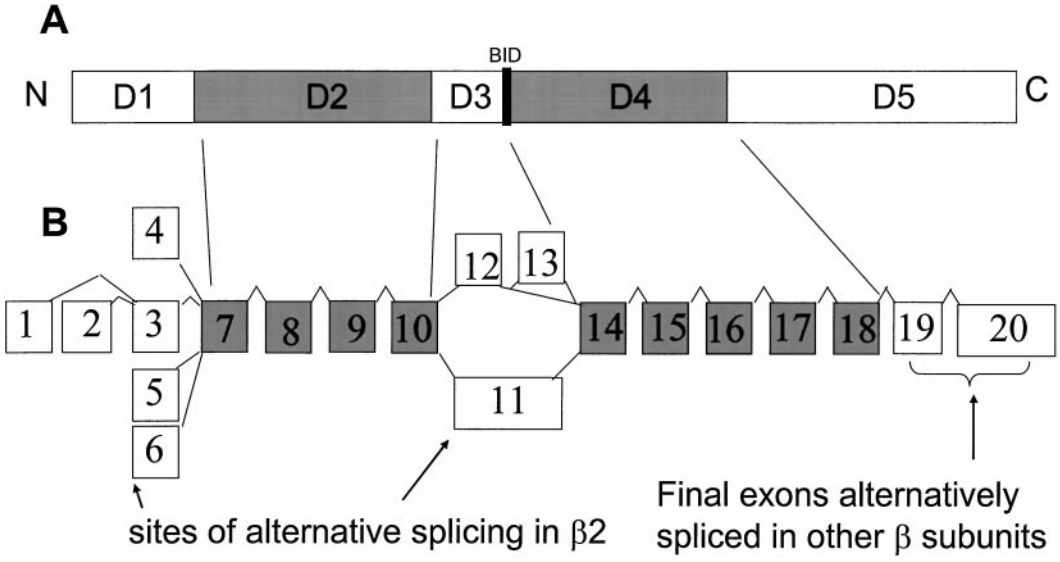

Fig. 1. Pattern of β subunit splice variants. (A) Domains of β subunits according to regions of high (shaded bars) and low (open bars) sequence homology between the four β subunits. (B) Approximate pattern of exons and alternative splicing for β2 derived from (Colecraft *et al.*, 2002) and (Takahashi *et al.*, 2003). For human β2, exons 11, 12, and 13 are 134, 20, and 62 bp, respectively. The first conserved exon (exon 7) is the equivalent of exon 2 for the β3 gene described in (Birnbaumer *et al.*, 1998).

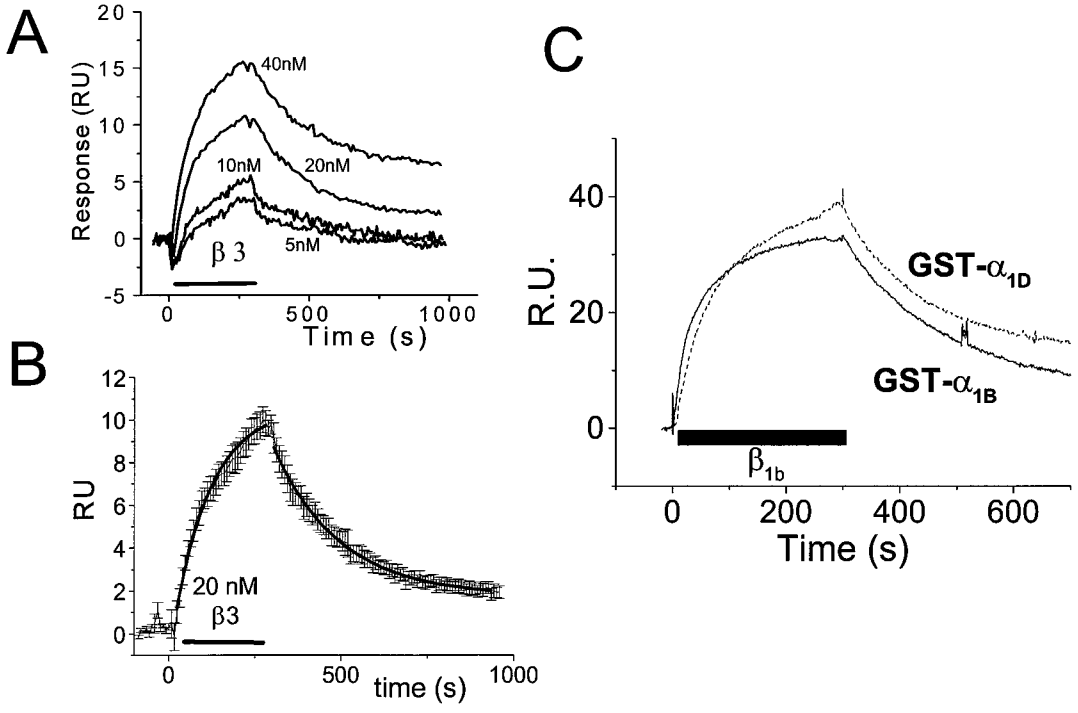

Fig. 2. Binding of β3 protein to GST fusion proteins of $Ca_v2.2$ I–II linker. (A) Examples of Biacore 2000 sensorgrams. Approximately 4 fmol. of the $Ca_v2.2$-GST fusion protein was immobilized via the anti-GST antibody on an individual flow cell of a CM5 dextran sensor chip. The VDCC β3 protein was diluted to the concentrations stated (5, 10, 20, and 40 nM β3), and injected over all flow cells at a flow rate of 50 μl.min^{-1} for 5 min as described in (Canti *et al.*, 2001). (B) Mean sensorgram for 20 nM β3 subunit binding to the I–II linker of $Ca_v2.2$. The data are the mean ± sem of six separate experiments. (C) Example sensorgrams for comparison of β1b binding to the I–II linker of $Ca_v2.2$ and $Ca_v1.3$.

gene structure of $\beta 2$ has been found to be more complex (Colecraft *et al.*, 2002).

Comparison of the $Ca_v\beta$ subunit sequences has led to the description of five domains (D1–D5), based on sequence similarities, with D2 and D4 being highly conserved between the four β subunits (Fig. 1(A)). The $Ca_v\beta$ subunit genes show alternative splicing, particularly in the three variable domains at the N and C termini and in the sequence between the two conserved domains (see Table I in Birnbaumer *et al.*, 1998 for a list of groups that have cloned various splice variants of the different β subunits). It was originally deduced that there was alternative splicing of exons 1, 5/6, and the final exon 13 in $\beta 3$, and this pattern was thought to extend to all β subunits (Fig. 1B). However, the exon structure for $\beta 2$ appears to be more complex than this (Colecraft *et al.*, 2002; Takahashi *et al.*, 2003). The variable N terminal D1 domain of $\beta 2$ results from alternative splicing of six exons, as identified from the human genomic DNA sequence. These are all associated with a common exon 7, equivalent to exon 2 in $\beta 3$. This gives rise to at least five different isoforms of $\beta 2$, with either short ($\beta 2$a, $\beta 2$b, or $\beta 2$e) or long ($\beta 2$c, $\beta 2$d) N terminal domains (Colecraft *et al.*, 2002). Only the $\beta 2$a isoform contains the two N terminal cysteines that represent palmitoylation sites (MQCCGL . . .) which is coded by $\beta 2$ exon 5 (Takahashi *et al.*, 2003). Alternative splicing of D3, the sequence between the two conserved domains, also occurs. This results in D3 regions which are either long (as in $\beta 1$a or $\beta 2$) or short with a highly conserved sequence (AKQKQKQ/S/V) present in $\beta 1$b, $\beta 3$ (exon 6) and $\beta 4$. This corresponds to exon 12 in the $\beta 2$ gene, but has not been found in any $\beta 2$ splice variants cloned to date. This D3 region is situated immediately before the highly conserved motif identified as the β interaction domain or BID (see below). The highly variable C terminus can also exist as a long form, present in $\beta 1$b, $\beta 2$, and $\beta 4$b and as a short form, present in $\beta 1$a, $\beta 3$, and $\beta 4$a (Fig. 1(B)).

Table I. Sequence of Known β Binding Site on the I–II Linker of HVA VDCCs and the Homologous Region in an LVA VDCC and a Primitive VDCC

Channel type	Consensus AID sequence
L type HVA	QQLEEDL-GY--WITQ-E
Non L type HVA	QQIERELNGY--WI-KAE
$\alpha 1$G LVA	GSCYEE**L**LKYLVY**I**LRKA
Jellyfish CyCa	HML<u>D</u>DAVK**G**YLD**WI**NQAS

Note. Residues in $\alpha 1$G and the jellyfish CyCa channel (Jeziorski *et al.*, 1998) that are identical to the consensus sequences in the HVA channels are shown in bold. A conserved charge in the jellyfish sequence is underlined.

Recently, several groups have identified truncated isoforms of β subunits (Hibino *et al.*, 2003; Hullin *et al.*, 2003). These appear to arise as a result of exon skipping in D3, causing a frame-shift and premature stop codon. In the case of the truncated $\beta 3$ found in heart, the skipped exon is the 20 bp exon 6 (Hullin *et al.*, 2003). A similar truncated $\beta 3$ mRNA was previously observed in mouse brain (Murakami *et al.*, 1996). This results in a protein lacking the β-interaction domain (BID) sequence. For the truncated $\beta 4$ identified in chicken cochlea and brain, a 59 bp exon is skipped resulting in the addition of 13 novel amino acids and truncation immediately after the BID sequence (Hibino *et al.*, 2003).

Phylogeny of β Subunits

Only a few nonmammalian $Ca_v\beta$ subunit genes have been identified. These include a *Xenopus laevis* $Ca_v\beta$ subunit (Tareilus *et al.*, 1997), which is highly homologous to mammalian $\beta 3$, a Drosophila $Ca_v\beta$ subunit (AAF21096), two *C. Elegans* $Ca_v\beta$ subunits (AAB53056 and AAK21500), a jellyfish $Ca_v\beta$ subunit (AAB87751), and two schistosome $Ca_v\beta$ subunits (Kohn *et al.*, 2001).

INTERACTION OF $CA_V\beta$ SUBUNITS AND $\alpha 1$ SUBUNITS: RELATIONSHIP TO THE DOMAIN STRUCTURE OF β SUBUNITS

Binding of $Ca_v\beta 1$ to the $\alpha 1$ I–II Linker

$Ca_v\beta$ subunits have been found to bind with very high affinity to the cytoplasmic intracellular linker between domains I and II of all HVA calcium channels, via an 18 amino acid motif called the α interaction domain (AID) on the I–II linker (Pragnell *et al.*, 1994). The consensus sequences present in $Ca_v1.x$ and $Ca_v2.x$ are given in Table I. The tryptophan in this sequence appears to be absolutely essential for β subunit interaction (Berrou *et al.*, 2002).

A 41 amino acid sequence (BID) on the β subunit was identified as the minimal sequence required to influence $\alpha 1$ subunit expression and to bind to the $\alpha 1$ subunit (De Waard *et al.*, 1994, 1996). The consensus sequence of BID is:

K--E---PYDVV**P**<u>S</u>MR**P**--LVG**P**<u>S</u>LKG**Y**EVTDMMKQ-ALFDF

The residues in bold have been identified as particularly important for binding to $\alpha 1$ subunits, and the two underlined serines are potential protein kinase C (PKC)

phosphorylation sites (minimal consensus sequence × S/T × R/K) (Walker and De Waard, 1998).

This small BID sequence alone can produce an increase in calcium current density, albeit not to the same extent as the full-length protein (De Waard *et al.*, 1994). The affinity between $Ca_v\beta$ subunits and a I–II linker fusion protein has been measured to be between 5 and 60 nM, in some cases with two affinities, depending on the β subunit (Canti *et al.*, 2001; De Waard *et al.*, 1994, 1995). In one study, no dissociation was seen for β1b from the Ca_v2.1 (α1A) I–II linker fusion protein after 10 h (De Waard *et al.*, 1995). However, in our own binding studies using surface plasmon resonance, the affinity of β3 for the a GST fusion protein of the I–II linker of Ca_v2.2 was about 20 nM (Fig. 2(A), (B)), and the k_{off} off was $5.2 \times 10^{-3}s^{-1}$ (Canti *et al.*, 2001). We found similar data for β1b binding to the I–II linker of both Ca_v2.2 and Ca_v1.3 (Bell *et al.*, 2001; Fig. 2(C)).

Binding of $Ca_v\beta$ Subunits to the N and C Termini of $Ca_v\alpha$1 Subunits

Two other β subunit interaction sites have been identified on various α1 subunits, on the C terminus (Qin *et al.*, 1997; Walker *et al.*, 1998) and the N terminus (Stephens *et al.*, 2000; Walker *et al.*, 1999). These appear to be of lower affinity and may be selective for certain β subunits.

Identification of Structural Folds in $Ca_v\beta$ Subunits

We have recently shown in a molecular modelling study that all $Ca_v\beta$ subunits consist of two conserved protein–protein interaction domains (Hanlon *et al.*, 1999). The first conserved domain in $Ca_v\beta$ subunits, corresponding to D2, is Src Homology-3 (SH3; Fig. 3). These are frequently found to bind to proline-rich motifs, such as

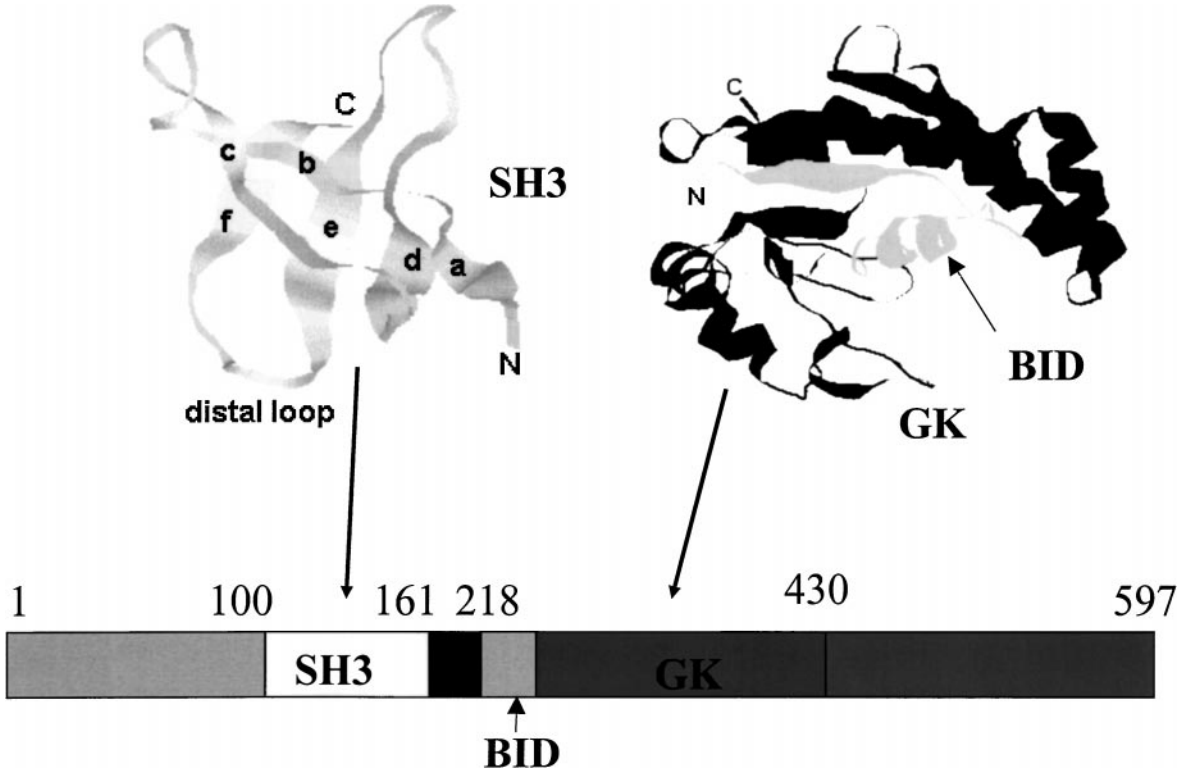

Fig. 3. Structural models of the SH3 and GK domains of β1b. Upper panel: Structural models for the SH3 and GK domains of β1b as described in Hanlon *et al.* (1999). Lower panel: Linear model of β1b, showing the numbers of amino acids involved in each domain, and the position of the BID.

PP$\times\times$P or $\times$PPP$\times$ (McPherson, 1999). In the $Ca_v\beta$ subunit SH3 domain, there is reasonable sequence homology of certain conserved residues which form the hallmark proline binding residues of all SH3 domains. These residues are Y111, Y140, W145, and F158 in β1b (Chien *et al.*, 1998; Hanlon *et al.*, 1999). The SH3 domain is attached by a flexible linker (corresponding to the D3 domain) leading into the BID, which is at the start of the second conserved domain, corresponding to D4. We identified this to be a Guanylate Kinase (GK) domain (Fig. 3), which has homology to guanylate kinase enzymes (Hanlon *et al.*, 1999). However, as in many other identified GK domains, the GK domain in $Ca_v\beta$ subunits is not predicted to have catalytic activity because the glycine-rich ATP binding motif present in true guanylate kinases (G$\times\times$G$\times$GK) (Kistner *et al.*, 1995) is not conserved in $Ca_v\beta$ subunits (e.g. it is GPSLKGY in β1b). The homology at the primary sequence level of the $Ca_v\beta$ subunit GK domain with other GK domains is fairly low, and the predicted structural homology in Fig. 3 was identified by a threading algorithm (Hanlon *et al.*, 1999). In $Ca_v\beta$1b there was also low homology in the N terminal 100 amino acids with a PDZ (Post-Synaptic Density protein 95 (PSD-95), Discs large protein (dlg), and Zona occludens-1 (ZO-1)) domain (Hanlon *et al.*, 1999). However, this homology is not conserved among all $Ca_v\beta$ subunits or splice variants, and there is extensive splicing in this region. Finally, there is also a nonconserved C terminal tail (corresponding to the D5 domain), which is highly divergent between different $Ca_v\beta$ subunits (Hanlon *et al.*, 1999), and predicted to have less secondary structure.

This finding potentially places the $Ca_v\beta$ subunits within the family of Membrane Associated Guanylate Kinase proteins (MAGUKs), which include PSD-95 and p55 (Chishti, 1998; Sheng and Wyszynski, 1997). These usually have between 1 and 3 PDZ domains at the N terminus, followed by an SH3 and a GK domain. These proteins often have a flexible linker between the SH3 and GK domains, termed the HOOK, that is involved in docking with another protein, another parallel with $Ca_v\beta$ subunits. In the case of p55, hDlg (Chishti, 1998), and CASK (Cohen *et al.*, 1998), a conserved sequence in this HOOK region binds to Band 4.1 protein, a spectrin binding protein that links to the actin cytoskeleton. Another motif in the HOOK region of SAP-102 binds to calmodulin (Masuko *et al.*, 1999). The protein p55 is also N-terminally palmitoylated (Chishti, 1998), as is the $Ca_v\beta$2a subunit.

The function of these protein–protein interaction domains in other members of the MAGUK family has been extensively studied. For example PSD-95 is involved in clustering of certain ion channels and receptors through its different domains (Blackstone and Sheng, 1999). An intramolecular interaction between the SH3 and GK domains has also been identified (Mcgee and Bredt, 1999). This has been borne out by the crystal structure of the combined SH3-GK domains of PSD-95 (Mcgee *et al.*, 2001; Tavares *et al.*, 2001). The identification of the domain structure of $Ca_v\beta$ subunits opens the possibility that they may be involved not only in trafficking $Ca_v\alpha$1 subunits to the plasma membrane but also in their localization with other proteins, via SH3 and GK domains, and that the SH3 and GK domains may also form intramolecular intractions. It is fascinating to speculate that the high affinity binding of the BID to the I–II linker of the α1 subunits, might subsequently alter this intramolecular interaction, and allow these two domains to interact with other parts of the calcium channel or to other proteins.

Only one such binding protein has so far been identified for full-length $Ca_v\beta$ subunits. A yeast two-hybrid screen revealed that $Ca_v\beta$3 binds to a small G protein, kir/Gem, that also binds to calmodulin (Beguin *et al.*, 2001). This protein is strongly expressed in pituitary, and inhibits the activity of HVA calcium channels, apparently by binding to the $Ca_v\beta$ subunit and preventing β-mediated trafficking of the $Ca_v\alpha$1 subunit to the plasma membrane. In another study the truncated β4 isoform identified in cochlea was found to bind to nuclear chromobox protein-2, involved in gene silencing, via its BID domain. This suggests an additional role for a β subunit, as truncated β4 is found in the nucleus and may influence transcription in the cell types in which it is expressed (Hibino *et al.*, 2003). Full-length β4 did not bind to this chromobox protein, possibly because its secondary structure prevented the interaction.

DISTRIBUTION OF NATIVE VDCC β SUBUNITS

The β1 subunit, as the β1a splice variant, appears to be the only β subunit expressed in skeletal muscle (for review see Hofmann *et al.*, 1994). In cardiac tissue there has been some controversy as to the nature of the major endogenous β subunit. The β2 subunit was originally detected in purified human cardiac DHP receptors containing Ca_v1.2 (Haase *et al.*, 1996). However, mRNAs for β1b, β2a, β2b, and β3 have been identified in human ventricular tissue (Hullin *et al.*, 2003), and β1b was identified as a major β subunit expressed in human heart (Hullin *et al.*, 1999). In contrast, only β2 protein was observed in rat heart (Ludwig *et al.*, 1997). However, heterologous expression of Ca_v1.2 with β2a does not mimic the inactivation properties of endogenous cardiac calcium currents (Wei *et al.*, 2000). Furthermore, overexpression of β2a in rat ventricular myocytes slows inactivation of the

endogenous currents suggesting that it is not the endogenous β subunit (Wei *et al.*, 2000), and it has recently been suggested that other $\beta 2$ isoforms $\beta 2$b and $\beta 2$c mimic more closely the endogenous cardiac calcium current (Colecraft *et al.*, 2002; Yamada *et al.*, 2001). In smooth muscle, although the calcium channel complement is also primarily L type, the main β subunit appears to be $\beta 3$, although $\beta 2$ is also present (Ludwig *et al.*, 1997).

The $\beta 1$b splice variant of $\beta 1$ is widely expressed in a number of tissues, including brain, where its distribution is rather diffuse (Ludwig *et al.*, 1997). The $\beta 2$ subunit is expressed at a fairly low level in brain but is present in specific neuronal cell types, including cerebellar Purkinje cells, hippocampal pyramidal neurons, and photoreceptors (Ball *et al.*, 2002; Ludwig *et al.*, 1997). Both $\beta 3$ and $\beta 4$ are strongly expressed in brain, with $\beta 3$ being predominant in olfactory bulb, cortex, hippocampus, and habenula, and $\beta 4$ prominent in the cerebellum (Ludwig *et al.*, 1997). The truncated $\beta 4$ isoform identified in chick cochlea was shown to be expressed at the protein as well as transcript level in both brain and cochlea, and in the latter it appeared to be the only $\beta 4$ isoform expressed (Hibino *et al.*, 2003).

A comparison of the distribution of $Ca_v\alpha 1$ and $Ca_v\beta$ subunits indicates that there is certainly not an exclusive association between particular pairs of $Ca_v\alpha 1$ and β subunits. However, the $\beta 3$ subunit associates with a majority of N type calcium channels in rabbit brain, as 56% of ω-conotoxin-GVIA binding sites were immunoprecipitated by an antibody to $\beta 3$ (Scott *et al.*, 1996). In the same study, $\beta 4$ was associated with 24% and $\beta 1$b with 10% of N type channels. Such heterogeneity may partially account for the diversity of properties of N type channels. The affinity of all β subunits for the $Ca_v2.2$ AID peptide was similar. In contrast rabbit brain P/Q type calcium channels were associated with all four β subunits in the following order $\beta 4$ (48%), $\beta 3$ (36%), $\beta 1$b (8%), and $\beta 2$ (7%). The relative amount of the different β subunits in rabbit brain is $\beta 3 = \beta 4 > \beta 1\text{b} > \beta 2$ (Witcher *et al.*, 1995), and the *in vitro* affinity of the different β subunits for the $Ca_v2.1$ I–II linker is $\beta 4 > \beta 2 > \beta 1\text{b} >> \beta 3$ (De Waard *et al.*, 1995). Taken together, these results suggest that the subunit composition of native N and P/Q type channels depends on a combination of the concentration of the different β subunits expressed in particular cell types, and their relative affinity for interaction with the I–Il linker of $Ca_v2.1$ and $Ca_v2.2$.

It will be of great interest in the future to determine the subcellular localization of particular $Ca_v\alpha 1$-β combinations. There is very little evidence concerning the types of $Ca_v\beta$ subunits associated with presynaptic calcium channels. Neurotransmitter release from hippocampal, cerebellar, and other neurons shows dependence on Ca^{2+} entry through P/Q and N-type channels (Huston *et al.*, 1995; Luebke *et al.*, 1993; Regehr and Mintz, 1994) implying a presynaptie localization for many $Ca_v2.1$ and $Ca_v2.2$ $\alpha 1$ subunits. In contrast, in rat cerebellar Purkinje cells, the $Ca_v2.1$ subunit is localized to the soma and distal dendrites. From immunohistochemical and *in situ* hybridization studies, although $\beta 2$ protein and mRNA was present only at a fairly low level in most brain areas, it was identified to be more strongly expressed in cerebellar Purkinje cells. $\beta 4$ was expressed both in Purkinje and granule cells (Ludwig *et al.*, 1997; Westenbroek *et al.*, 1995). Nevertheless, it is not known which of the accessory subunits are associated with the functional $Ca_v2.1$-containing VDCC complexes and whether this differs between terminals, cell bodies, and dendrites of Purkinje cells (Mintz *et al.*, 1992). Our results, discussed below, on differential β subunit trafficking obtained in polarized cells (Brice and Dolphin, 1999), would predict that if $\beta 2$a and $\beta 4$ are the predominant β subunits in Purkinje neurons, the somatodendritic $Ca_v2.1$ subunits might be associated largely with the $\beta 2$a subunit, mediating slow inactivation, whereas the presynaptic $Ca_v2.1$ channels might rather be bound to $\beta 4$. Supporting evidence that presynaptic $Ca_v2.1$ channel complexes may differ from those in cell bodies comes from electrophysiological data on Purkinje cells, which are unusual in their somatic current being predominantly P-type, with its characteristically slow inactivation kinetics (Mintz *et al.*, 1992). A unique property of rat $\beta 2$a, in contrast to other β subunits, is its ability to attenuate current inactivation, suggesting that the somatic P type currents in Purkinje cells represent $Ca_v2.1$ associated with a $\beta 2$a subunit (De Waard and Campbell, 1995). Furthermore, in the calyx of Held, where the presynaptic current has been recorded directly, it was classified pharmacologically as P type, but exhibited marked inactivation (Forsythe *et al.*, 1998). This suggests that it is likely to be associated with a β subunit other than $\beta 2$a, although clearly it could be associated with another $\beta 2$ splice variant showing greater inactivation, as is the case in heart (Colecraft *et al.*, 2002). Furthermore, the existence of splice variants of $Ca_v2.1$ with differing inactivation kinetics is also predicted to be a major determinant of the P type phenotype (Bourinet *et al.*, 1999).

ROLES OF β SUBUNITS IN CALCIUM CHANNEL ASSEMBLY AND TRAFFICKING

There has yet been little study of the mechanisms involved in the assembly of heteromeric VDCC complexes.

It is assumed that the $\alpha 1$ subunit folds to form an immature channel in the endoplasmic reticulum, however, the mechanism whereby this occurs is not known. The accessory subunits, particularly the intracellular β subunit, have been shown to have marked effects on the properties of HVA $\alpha 1$ subunits, including modification of kinetics, amplitude, and targeting of the complex to the plasma membrane (Brice *et al.*, 1997; Singer *et al.*, 1991). We have shown that the converse also applies, in that antisense-induced depletion of $Ca_v\beta$ subunits from DRGs results in a reduction of the amplitude of endogenous calcium currents, and slowed kinetics of activation (Berrow *et al.*, 1995; Campbell *et al.*, 1995).

Most research indicates that all $Ca_v\beta$ subunits increase the functional expression of HVA $\alpha 1$ subunits (for review see Birnbaumer *et al.*, 1998). This could in theory be attributed to an increase in the open probability of the channel, an increase in single channel conductance, an increase in the number of functional channels inserted into the plasma membrane, or a combination of several processes.

Concerning the effect of $Ca_v\beta$ subunits on the number of channels in the plasma membrane, initial studies in *Xenopus* oocytes showed that for $Ca_v1.2$ and $Ca_v2.3$, the $Ca_v\beta$ subunits had no effect on the voltage-dependence of charge movement (visualized as gating current), and did not increase the total amount of charge transferred, which is a measure of the number of voltage sensors moving in the membrane, and therefore of channels inserted into the membrane (Neely *et al.*, 1993). However, the β subunits were found to hyperpolarize the voltage-dependence of the ionic current (Olcese *et al.*, 1996). Thus, the β subunits produced an increase in the ratio of charge movement to ionic current, and were said to improve the coupling between voltage sensor movement and channel opening (Neely *et al.*, 1993; Olcese *et al.*, 1996). However, in contrast to these studies, other groups have found that co-expression of a β subunit did increase the charge movement associated with $Ca_v1.2$ gating (Colecraft *et al.*, 2002; Josephson and Varadi, 1996). Furthermore, we, and others, have shown that $Ca_v\beta$ subunits have a chaperone-like effect, promoting functional expression of the $Ca_v2.1$, 2.2, and 2.3 subunits at the plasma membrane of mammalian cells, and increasing localisation of the channels at the plasma membrane (Bichet *et al.*, 2000; Brice *et al.*, 1997; Raghib *et al.*, 2001) (Fig. 4). Similar results have been obtained for cardiac $Ca_v1.2$ with $\beta 2a$ (Chien *et al.*, 1995). The reason for the difference between these results and those of Neely *et al.* (1993) is unclear, except that they were performed in *Xenopus* oocytes, which contain an endogenous β subunit, that may have been present in sufficient concentration to saturate the effect on trafficking, while not saturating the effect on voltage-dependent processes, since we have found there is a mismatch in the concentration-dependence of these two processes (Canti *et al.*, 2001). The consensus from a number of different expression systems now appears to be that $Ca_v\beta$ subunits do indeed increase the amount of functional HVA calcium channels expressed at the plasma membrane.

It has been proposed that the I–II linker of HVA calcium channels contains an endoplasmic reticulum retention signal, which is masked by binding of the β subunit to the I–II linker (Bichet *et al.*, 2000). This must happen at an early stage in channel assembly, allowing the heteromeric channel subsequently to be trafficked through the Golgi network to the plasma membrane. The consensus AID sequence for binding to the BID is shown in Table I for HVA channels (Walker and De Waard, 1998). However, these sequences do not contain a strong endoplasmic reticulum retention signal of the types identified in other studies (Schwappach *et al.*, 2000). Furthermore, the differences compared to the homologous sequence in the T type channel Cav3.1 or $\alpha 1G$ (Table I) which does not require a $Ca_v\beta$ subunit to reach the plasma membrane (Dolphin *et al.*, 1999) do not indicate that a known endoplasmie reticulum retention signal has been lost in the corresponding T channel sequence. Although there is in general a low level of conservation, in the C terminal part of the consensus sequence, the conserved W in the AID sequence of HVA Ca^{2+} channels is changed to Y in $\alpha 1G$, a conservative substitution, compared to the W to A mutation that originally identified this residue as essential for $Ca_v\beta$ subunit binding (De Waard *et al.*, 1996). Thus the degree of conservation of the consensus sequence in $\alpha 1G$ may be sufficient for some level of $\alpha 1$-β interaction, since at least one β subunit ($\beta 1b$) was observed to enhance functional expression of $\alpha 1G$ (Dolphin *et al.*, 1999).

It is of interest that a jellyfish $\alpha 1$ subunit (CyCa $\alpha 1$) has recently been cloned, and is a homologue of L type channels (Jeziorski *et al.*, 1998). Like $\alpha 1G$, it only has minimal conservation of the β binding motif, although the WI motif is conserved (Table I). However, when the jellyfish $\alpha 1$ subunit is expressed in *Xenopus* oocytes, coexpression with either a jellyfish or a mammalian β subunit was reported to increase expression and hyperpolarize the voltage-dependence of activation of the current in the same way as occurs for other HVA $\alpha 1$ subunits (Jeziorski *et al.*, 1999). This suggests that other sites on $\alpha 1$ subunits are probably involved in interaction and mediating the effects of β subunits.

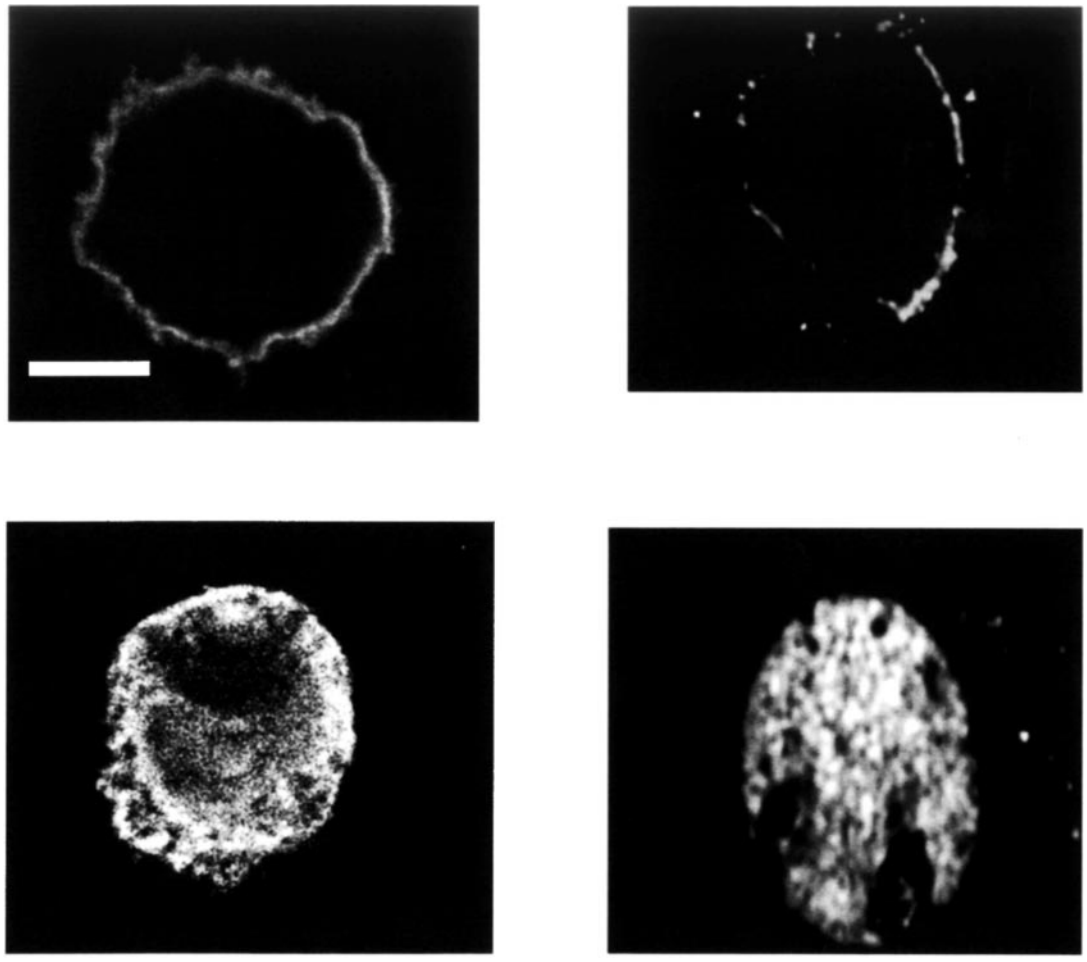

Fig. 4. Expression of $Ca_v\beta$ subunits at the plasma membrane in COS-7 cells. Rat β subunits were heterologously expressed in COS-7 cells, as described in Bogdanov *et al.* (2000). Upper row: β1b (left) and β2a (right) expressed alone in COS-7 cells both show plasma membrane localization. Lower row: β3 (left) and β4 (right) do not show plasma membrane localization when expressed alone in COS-7 cells.

SUBCELLULAR LOCALIZATION OF $CA_V\beta$ SUBUNITS

It was originally thought possible that one mechanism whereby β subunits produce trafficking of α1 subunits is that they themselves are targeted to the plasma membrane. However this does not appear to be the mechanism. Plasma membrane association has been observed for β2a when expressed alone (Bogdanov *et al.*, 2000; Chien *et al.*, 1995) (Fig. 4), and for β2a-GFP expressed in cardiac myocytes (Wei *et al.*, 2000). Rat, rabbit, and human β2a subunits are palmitoylated at the N terminus (for review see Birnbaumer *et al.*, 1998). A palmitoylation-deficient mutant of $Ca_v\beta$2a, when expressed alone, showed a modified subcellular distribution, with little at the plasma membrane (Bogdanov *et al.*, 2000; Chien *et al.*, 1996, 1998). However, this palmitoylation-deficient β2a is still able to traffic Ca_v1.2 to the plasma membrane (Birnbaumer *et al.*, 1998), and to enhance the current density for Ca_v2.2 (Canti *et al.*, 2000). Furthermore, we have observed that, while the β3 and β4 subunits are not themselves associated with the plasma membrane (Fig. 4), all β subunits are able to produce membrane trafficking of $Ca_v\alpha$1 subunits in COS-7 cells (Bogdanov *et al.*, 2000; Brice *et al.*, 1997). We have also observed membrane association for β1b (Brice *et al.*, 1997; Fig. 4), and in a recent study we have examined the basis for this. We have identified, by making chimeric β subunits, that the C terminal region of β1b, the region showing most diversity between β subunits, is responsible

for its plasma membrane association. Furthermore we have identified, by truncation mutations, an 11 amino acid motif present in the C terminus of β1b but not in β3 (amino acids 547–556 of β1b, WEEEEDYEEE), which when deleted, reduces membrane association of β1b (Bogdanov *et al.*, 2000). It is possible that such membrane association is important for the selective localization or clustering of particular calcium channels with which β1b is associated. It is also of interest here that membrane association of another β2 splice variant (β2e) that is not palmitoylated has also been observed (Takahashi *et al.*, 2003). The basis for this has not yet been determined.

Expression of $Ca_v\beta$ Subunits in a Polarized Cell Line

We have observed that β1b is also targeted to the plasma membrane when expressed alone in the polarized Madin Darby Canine Kidney (MDCK) epithelial cell line (Bogdanov *et al.*, 2000). In MDCK cells, β1b and β2a were both targeted to the basolateral membrane, when expressed alone, suggesting either that they possess a basolateral sorting signal, or that they bind to an endogenous protein that is targeted basolaterally. The basolateral sorting route is thought to require a specific signal which, if absent or deleted in transmembrane proteins, results in apical sorting by a default route (Hopkins, 1991). We have also shown in this cell line that $Ca_v2.1$ shows differential trafficking with different β subunits (Brice and Dolphin, 1999). Unsurprisingly, expression of $Ca_v2.1$ alone did not result in any membrane association (Fig. 5). Coexpression with β1b and β4 resulted in $Ca_v2.1$ transport to the apical membrane, and β2a caused trafficking to the basolateral membrane (Brice and Dolphin, 1999). The β3 subunit was not able to traffic $Ca_v2.1$ to any plasma membrane, agreeing with the much lower affinity of β3 than other β subunits for the $Ca_v2.1$ AID sequence (De Waard *et al.*, 1995). Conversely, the sorting of β1b is also influenced by the α1 subunit with which it is expressed. When coexpressed with $Ca_v2.1$, β1b was found primarily at the apical membrane of MDCK cells, the same location as $Ca_v2.1$ (Brice and Dolphin, 1999), when expressed in this combination. In contrast when coexpressed with $Ca_v1.2$, β1b was found primarily at the basolateral membrane, like $Ca_v1.2$ when coexpressed in this combination (Brice and Dolphin, 1999).

THE ROLE OF ENDOGENOUS $CA_V\beta$ SUBUNITS IN HETEROLOGOUS EXPRESSION OF $CA_V\alpha1$ SUBUNITS

Much early work on the roles of $Ca_v\beta$ subunits in calcium channel expression was performed in *Xenopus*

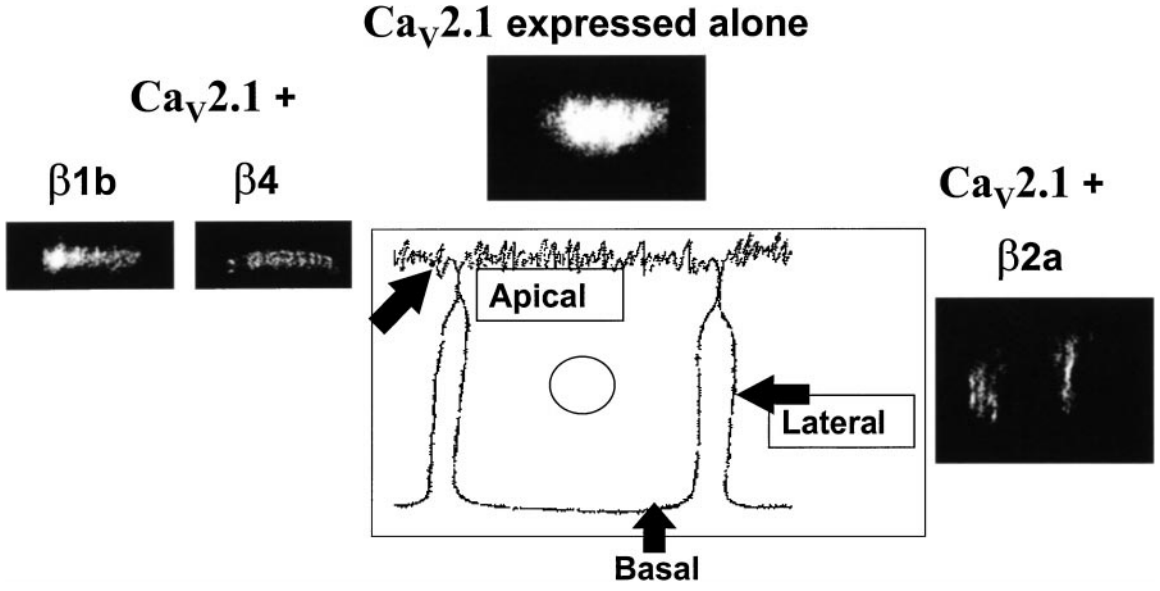

Fig. 5. Expression of $Ca_v2.1$ calcium channel subunit in polarized MDCK cells: Effect of β subunit coexpression. Central panel shows a schematic representation of a polarized MDCK cell. Top: Immunolocalization of $Ca_v2.1$ expressed alone. Left: apical localization of $Ca_v2.1$ expressed with either β1b or β4. Right: basolateral localization of $Ca_v2.1$ expressed with β2a. Details of experimental protocols are given elsewhere (Brice and Dolphin, 1999).

oocytes, but these cells are now known to contain an endogenous *Xenopus* β subunit that complicates the interpretation of these results (Tareilus *et al.*, 1997). This endogenous β subunit was found to be both necessary and able to traffic at least some heterologously expressed $Ca_v2.3$ calcium channel to the plasma membrane, since if endogenous β subunit expression was reduced or eliminated by injection of $\beta 3$ antisense oligonucleotides, $Ca_v2.3$ expression was lost. The endogenous *Xenopus* β subunit is 98% homologous to other cloned $\beta 3$ sequences (Tareilus *et al.*, 1997), and when the same endogenous *Xenopus* $\beta 3$ subunit was overexpressed in oocytes, it was able to regulate the biophysical properties of expressed $Ca_v2.3$ channels (Tareilus *et al.*, 1997). We have found a similar result for $Ca_v2.2$ expressed in *Xenopus* oocytes (Canti *et al.*, 2001).

When $\alpha 1$ subunits are expressed alone in *Xenopus* oocytes, it is unclear whether the low concentration of endogenous chaperoning β subunits remain associated with each expressed $\alpha 1$ subunit in the plasma membrane to form an irreversible complex, or whether the complex dissociates, so that free $Ca_v\alpha 1$ subunits are present in the plasma membrane, since there is no clear evidence from the biophysical properties of the expressed channel that it has a β subunit present (Canti *et al.*, 2001; Tareilus *et al.*, 1997). Also pertinent to this argument, injection of $\beta 3$ protein into *Xenopus* oocytes expressing $Ca_v1.2$ subunits alone has acute effects on their biophysical properties (Yamaguchi *et al.*, 1998). This result suggests either that $Ca_v1.2$ expressed alone may not be irreversibly bound to the endogenous β subunit responsible for its trafficking, or if it does form a complex, this may be able to associate with one or more additional β subunits, that are responsible for the β subunit-mediated voltage-dependent and kinetic effects.

The point has been made that we do not know how many β subunits bind physiologically to a functional calcium channel (Birnbaumer *et al.*, 1998). It is likely that, in the process responsible for trafficking, a $Ca_v\alpha 1$ subunit binds one $Ca_v\beta$ subunit with very high affinity. This interaction is presumably via the I–II linker, as this is the highest affinity binding site identified (De Waard *et al.*, 1995). However, it has recently been found that a mutation in the I–II linker of $Ca_v1.2$, which disrupts the binding of β subunits and trafficking to the plasma membrane, did not prevent modulation of the channel biophysical properties by β subunits (Gerster *et al.*, 1999). These data are in contrast to the earlier data of others, identifying the I–II linker as being primarily responsible for both effects (Pragnell *et al.*, 1994). Nevertheless the binding of a β subunit to the I–II linker site (responsible for trafficking) may be a prerequisite for the subsequent reversible binding, either of a second β subunit, or of the same β subunit, to other sites on the channel, responsible for the modulation of biophysical parameters.

We have contributed to this debate by performing an intracellular $Ca_v\beta$ subunit dose-response curve in *Xenopus* oocytes (Fig. 6(A)), using nuclear injection of between 0 and 720 pg $\beta 3$ cDNA (Canti *et al.*, 2001). We have obtained evidence that the effect of β subunits on the maximum conductance of expressed $Ca_v2.2$, which is a measure of the number of $Ca_v2.2$ calcium channels in the plasma membrane, occurs with a K_D of about 17 nM for $\beta 3$ (Fig. 6(B); Canti *et al.*, 2001). The site responsible for chaperoning the $\alpha 1$ subunit to the plasma membrane has a sufficiently high affinity for the β subunit, that significant binding is estimated to occur at around the endogenous concentration of $\beta 3$ protein in oocytes, which we measured to be about 16–20 nM. This correlates well with

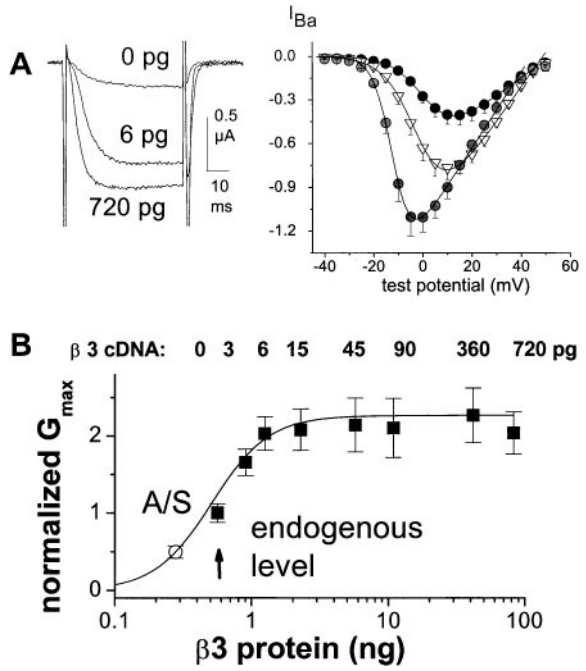

Fig. 6. Modulation of $Ca_v2.2$ expression by coexpression of VDCC β subunits. The $Ca_v2.2$ channel was expressed with 0–720 pg $\beta 3$ cDNA. The data are taken from (Canti *et al.*, 2001). (A) Left: example currents at 0 mV for 0, 6, and 720 pg $\beta 3$ cDNA, as given. Right: IV relationships (mean $\pm$ sem) for 0 (closed circles), 6 (open triangles), and 720 pg (grey circles) $\beta 3$ cDNA. (B) The G_{max} was determined as the slope conductance from the linear region of all IV relationships, normalized to the value in the absence of coexpressed $Ca_v\beta 3$, and plotted against the amount of $\beta 3$ protein calculated from a standard curve. The open circle represents the G_{max} determined following the A/S ODN injection (Canti *et al.*, 2001). The data are fit by a logistic function with a midpoint of 0.54 ng of $\beta 3$ protein (approximately 17 nM). The arrow represents the measured endogenous $\beta 3$ protein level. The amount of $\beta 3$ cDNA injected is indicated above each point.

the affinity of the I–II linker of $Ca_v2.2$ for the $\beta 3$ subunit, determined from Biacore experiments to be about 20 nM (Canti *et al.*, 2000; Fig. 2).

The large increase in β subunit concentration (17 nM–2 μM) produced by expression of increasing concentrations of $\beta 3$ cDNA only results in a two-fold increase in the $Ca_v2.2$ G_{max}, pointing to a vast excess of $\beta 3$ subunits over $Ca_v2.2$ channels (Canti *et al.*, 2001). Furthermore, a $\beta 3$ antisense oligonucleotide reduced the number of oocytes expressing calcium currents, and in those oocytes that did express currents, reduced the maximum conductance by about 50%. This is in close correlation with the estimated 47% reduction of endogenous $\beta 3$ protein by the $\beta 3$ antisense oligonucleotide injection found in those cells expressing calcium currents. It is tempting to interpret these and the previous β antisense results (Tareilus *et al.*, 1997), as indicating that the β subunit is obligatory for the functional expression of these HVA channels.

EFFECT OF $CA_V\beta$ SUBUNITS ON CALCIUM CHANNEL PROPERTIES

The increase in current density brought about by β subunits can be attributed to a number of effects on biophysical properties, as well as the important effect on trafficking.

Voltage-Dependence

All β subunits hyperpolarize the voltage-dependence of activation of all HVA VDCCs. In contrast, for steady state inactivation differences are apparent, both with regard to different $\alpha 1$ subunits and different β subunits. For $Ca_v1.2$ there is little difference between different β subunits and splice variants in their ability to hyperpolarize steady-state inactivation (Jones *et al.*, 1998; Takahashi *et al.*, 2003). In contrast, for $Ca_v2.3$ and $Ca_v2.2$ all except palmitoylated $\beta 2$a hyperpolarize the voltage-dependence of steady-state inactivation (Birnbaumer *et al.*, 1998; Canti *et al.*, 2000; Jones *et al.*, 1998). The hyperpolarizing shift for activation of $Ca_v1.2$ with $\beta 2$a, measured in *Xenopus* oocytes, was about -50 mV, whereas that for $Ca_v2.3$ was only -15 mV (Olcese *et al.*, 1996). In the light of our data on the concentration-dependence of the effects of β subunits (Canti *et al.*, 2001), one possible interpretation of these results is that $Ca_v2.3$ channels expressed alone in oocytes have a higher affinity for endogenous β subunit than $Ca_v1.2$, but this remains to be determined.

Where it has been studied, the $Ca_v\beta$ subunits all produce an increase in mean open time that is at least in part due to a hyperpolarizing shift in the voltage-dependence of the mean open time (Meir *et al.*, 2000; Wakamori *et al.*, 1999). The effect of expression of increasing concentrations of the $\beta 3$ subunit revealed that the concentration-dependence of the hyperpolarization of the V_{50} for activation and steady-state inactivation was right-shifted, compared to the concentration-dependence of the increase in maximum conductance (Canti *et al.*, 2001). At intermediate concentrations of $\beta 3$, two components of inactivation could be observed corresponding to $\beta 2$a with or without a β subunit (Fig. 7(A), (B)). The K_D for the effect of $\beta 3$ on the steady-state inactivation was about 120 nM (Fig. 7(C)). This is a seven-fold higher concentration of expressed $\beta 3$ subunit than is required for the effect on the maximum conductance (Canti *et al.*, 2001). One explanation for this difference is that the affinity of the channel for the β subunit is greater while it is being trafficked to the plasma membrane, but is reduced when the channel is in the polarized plasma membrane (see Fig. 8 and the related discussion).

Kinetics

The view initially prevailed that β subunits affected kinetic transitions close to the open state (Neely *et al.*, 1993). However, a more recent study indicates that they influence all kinetic processes (Colecraft *et al.*, 2002), and have a marked effect on open probability, largely by reducing the mean closed time.

The kinetics of current activation are little affected for any of the β subunits coexpressed with $Ca_v2.3$ (Jones *et al.*, 1998). We have also recently observed that for $Ca_v2.2$ single channels, the distribution of latencies to first opening of $Ca_v2.2$ channels and the mean open and closed times were similar for both $\beta 1$b and $\beta 2$a subunits. However, the inclusion of the $\beta 2$a subunit led to channels with an additional prominent slow activation phase (Meir and Dolphin, 2002), which may represent slow exit from an inactivated state.

Whilst VDCC $\alpha 1$ subunits contain inherent determinants of voltage-dependent inactivation (Cens *et al.*, 1999; Herlitze *et al.*, 1997; Spaetgens and Zamponi, 1999; Zhang *et al.*, 1994), association with different β subunit isoforms dictates their overall inactivation rate (Meir and Dolphin, 2002; Olcese *et al.*, 1994). Although the precise mechanism regarding voltage-dependent inactivation has been the subject of recent controversy (Jones *et al.*, 1999; Shirokov, 1999), it is clear that β subunit composition differentially affects inactivation properties.

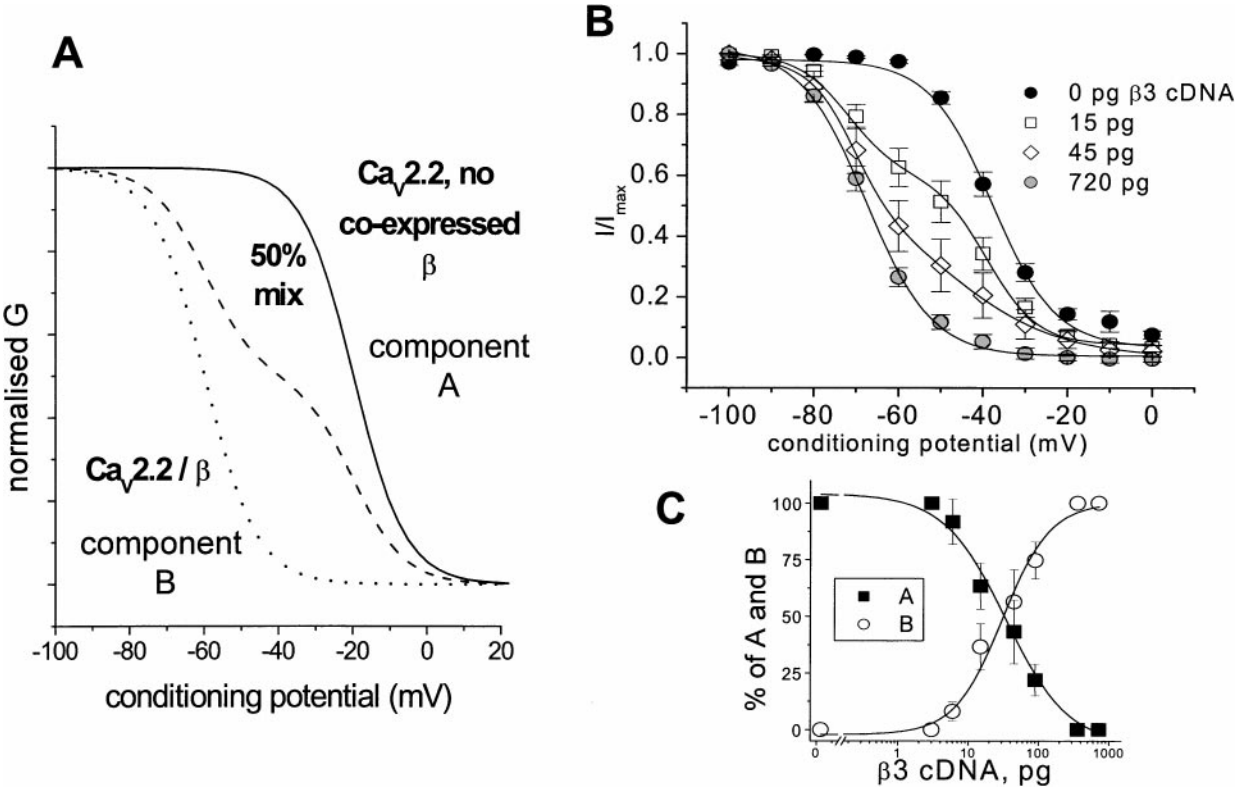

Fig. 7. Effect of β3 subunit coexpression on steady-state inactivation of $Ca_v2.2$. Steady-state inactivation curves were obtained from 100 ms duration test pulses to 0 mV, preceded by a 25 s prepulse to the conditioning potential given (−100–0 mV). The fits are to a single or double Boltzmann function (Canti *et al.*, 2001). (A) Simulated steady-state inactivation for three conditions, no added β subunit (solid line), a maximally effective amount of β subunit (dotted line), and sufficient β subunit to produce 50% of each component (dashed line). (B) Mean steady-state inactivation curves for $Ca_v2.2$ coexpressed with 0 (closed circles), 15 (open squares), 45 (open diamonds) or 720 (grey circles) pg of β3 cDNA. (C) The mean % of the two components of steady-state inactivation A (closed squares, with $V_{50,\text{inact,a}}$, approx −40 mV) and B (open circles, with $V_{50,\text{inact,b}}$, approx −70 mV) are given for each concentration of β3 cDNA injected. The data are fit to logistic functions with midpoints corresponding to about 4.3 ng β3 protein.

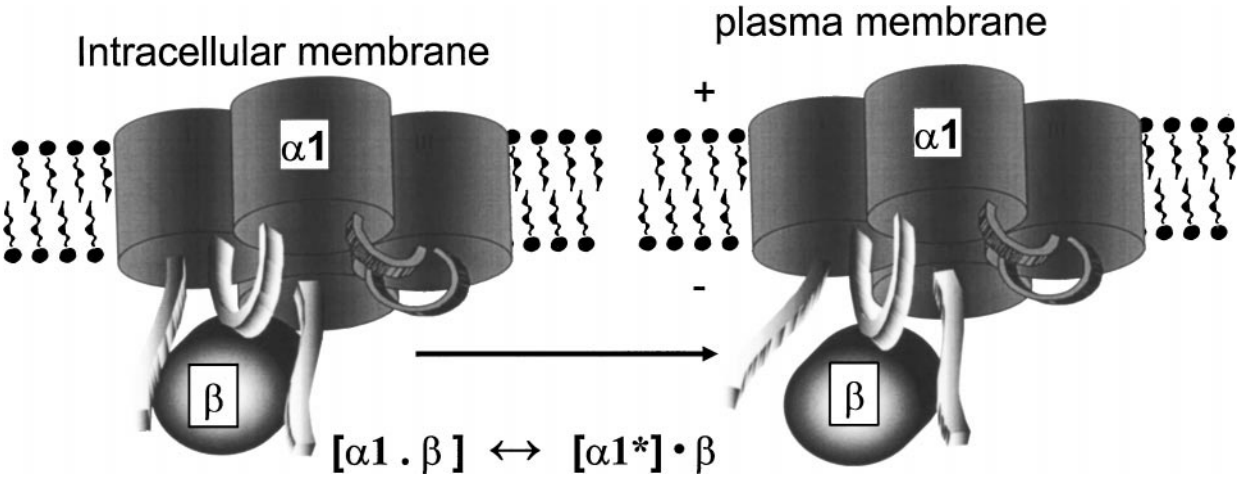

Fig. 8. Binding of $Ca_v\beta$ subunits. One β subunit potentially binds to a complex site including the I–II linker, the N terminus and the C terminus of $Ca_v\alpha1$. On the left is the model for the high affinity association involved in trafficking the $Ca_v\alpha1$ subunit to the plasma membrane, and on the right, there may be a conformational change at the plasma membrane, decreasing the affinity for the same $Ca_v\beta$ subunit.

At the whole cell level, coexpression of β1b, β2a, β2e, or β4 subunits with HVA α1 subunits generally decreased the inactivation rate, whereas β3 enhanced inactivation, compared to the α1 subunit expressed alone. Effects are particularly dramatic for both β2a and β2e subunits expressed with $Ca_v1.2$ (Chien and Hosey, 1998; Takahashi *et al.*, 2003) and for β2a with $Ca_v2.2$ (Bogdanov *et al.*, 2000; Stephens *et al.*, 2000) or $Ca_v2.3$ (Qin *et al.*, 1998). Mutation of the two cysteine residues involved in palmitoylation of β2a caused a significant reduction of the β2a-mediated retardation in inactivation kinetics and introduced a resolvable fast component of inactivation. Inactivation properties of (C3,4S)-β2a were closer to those seen with the more inactivating β1b, β3, and β4 isoforms (Stephens *et al.*, 2000). It has been suggested that the VDCC I–II loop itself may dictate voltage-dependent inactivation properties, acting as a blocking particle analogous to the amino terminal inactivation ball in voltage-dependent potassium channels (Cens *et al.*, 1999). In this scenario, the membrane-associated palmitoylated β2a would render the I–II loop unable to participate in inactivation by binding to it and anchoring in an inaccessible conformation. Additional evidence for this "hinged lid" hypothesis has been obtained recently (Stotz *et al.*, 2000). It has also been shown recently that disruption of the interaction between the I–II linker and the III–IV linker of $Ca_v2.1$ enhances inactivation, but only in the absence of a coexpressed β subunit (Geib *et al.*, 2002). These authors prefer the hypothesis that the interaction of the C terminal ends of the S6 segments of each domain of the α1 subunit controls inactivation, and that this can be modified by the domain linkers, since these are attached to the S6 segments.

We have investigated the role of the N terminus of $Ca_v2.2$ in the kinetics of voltage-dependent inactivation. In a similar manner to removal of the palmitoylation sites on β2a, deletion of the $Ca_v2.2$ amino terminus markedly opposed the β2a-mediated retardation of inactivation. In contrast, deletion of the $Ca_v2.2$ amino terminus had little effect on β3- or β4-mediated inactivation properties. Furthermore, transfer of the $Ca_v2.2$ amino terminus into a $Ca_v1.2$ backbone resulted in a gain-of-function chimera with slower inactivation properties than $Ca_v1.2$, which were no different from those of parental $Ca_v2.2$ in the presence of β2a. Mutations within the $Ca_v2.2$ amino terminal sequence also opposed the β2a-mediated slowing of inactivation (Stephens *et al.*, 2000). Using a similar approach, a loss-of-function construct, where the $Ca_v2.1$ amino terminus was replaced by the corresponding $Ca_v1.2$ region, was used to demonstrate a functional interaction between $Ca_v\beta$ subunits and the $Ca_v1.2$ amino terminus (Walker *et al.*, 1999).

Conductance

No effect of β subunits has yet been found on the main, single channel conductance for any calcium channel, but we have found that for the HVA α1 subunits, expressed in the absence of β subunits, there is an increase in the frequency of observation of a small conductance mode of the channel (Meir and Dolphin, 1998). This mode is also more prevalent at low depolarizations, and we can speculate that this might represent a mode or subconductance state in which all voltage sensors have not moved in response to depolarization, as has been proposed for certain K^+ channels (Chapman *et al.*, 1998; Zheng and Sigworth, 1998).

HOW MANY $CA_V\beta$ SUBUNITS ARE BOUND TO AN α1 SUBUNIT?

Our evidence supports the hypothesis that there are two distinct binding processes for β subunits on $Ca_v2.2$ (Canti *et al.*, 2001). One explanation is that a single binding site undergoes a marked reduction in affinity for $Ca_v\beta$ subunits once the $Ca_v\alpha1$ subunits have been trafficked from the endoplasmic reticulum and are inserted in the polarized plasma membrane (Fig. 8). Alternatively one might postulate the coexistence of two separate $Ca_v\beta$ subunit binding sites on each $Ca_v2.2$ molecule, although some evidence argues against this hypothesis. The isolated I–II linker of α1 subunits has a high affinity binding site for β subunits (De Waard *et al.*, 1995; Pragnell *et al.*, 1994), and there are two other low affinity sites on the N and C-termini of various α1 subunits (Qin *et al.*, 1997; Walker *et al.*, 1998, 1999). However none of the *in vitro* binding studies has been able to address whether there is one complex binding pocket in an intact channel, such that the same β subunit binds with high affinity to the I–II linker and with lower affinity to the N and C-termini, or whether more than one β subunit can bind to a single channel. Certainly when two β subunits with differing properties are expressed together (β2 and β3), the channels behave as if they are a mixed population, rather than having an intermediate behavior (Jones *et al.*, 1998).

A mutation in the I–II linker of $Ca_v2.1$ (Y392S) not only reduced the binding affinity for β subunits *in vitro* and markedly lowered the expression of $Ca_v2.1$ currents, but also prevented the β subunit-induced hyperpolarization of current activation (Pragnell *et al.*, 1994). Similar data have been obtained for other mutations in the I–II linker of $Ca_v2.1$ and $Ca_v2.3$ (Berrou *et al.*, 2001; Herlitze *et al.*, 1997). Furthermore, the I49A mutation in the N terminus of $Ca_v2.2$ not only affects the ability of β3 to influence

the facilitation rate of $Ca_v2.2$, but also affects the maximum conductance, both indicating an increased affinity for $Ca_v2.2$ (Canti *et al.*, 2001). However, we have not found measurable binding of $\beta 3$ to the isolated $Ca_v2.2$ N-terminus in the Biacore system suggesting that the N terminal motif may be a β binding-site modifier, rather than an independent binding site. This supports the interpretation of our data that the differing concentration-dependence of the effects of $\beta 3$ subunits on trafficking and on biophysical properties of $Ca_v2.2$ do not depend on physically separate binding sites, but rather on one complex binding site, whose affinity for β subunits is high for the nascent calcium channel, but is reduced once the channel has reached the polarized plasma membrane, and is transiently enhanced by depolarization. This is also supported by purification studies reporting a 1:1 stoichiometry for $\alpha 1$ and β subunits (Hosey *et al.*, 1989; Tanabe *et al.*, 1987; Witcher *et al.*, 1993). The existence of multiple $\alpha 1$-β interaction sites is also supported by a recent paper describing inhibition of this interaction by the AID peptide (Hohaus *et al.*, 2000). Nevertheless, it still remains possible that the first β subunit binds in the complex binding pocket outlined above, and the second β subunit subsequently binds with lower affinity, in part via interaction with the first β subunit, and is responsible for modifying the biophysical properties. In this regard other MAGUK family proteins have been found to form intermolecular interactions within subfamilies (Wu *et al.*, 2000).

The hypothesis proposed in Canti *et al.* (2001) was that at a physiological membrane potential, the $\alpha 1$ subunits expressed at the plasma membrane are in equilibrium with the β subunits. This is not at odds with a 1:1 stoichiometry in the channel complex, since under most conditions there may be a sufficient excess of β subunit that almost all $\alpha 1$ subunits are associated with a bound β subunit. It remains to be determined whether this is always the case for native calcium channels, or whether there are conditions under which $Ca_v\beta$ subunits are limiting. However, N type channels of smaller conductance, and showing voltage-independent G protein modulation, have been observed at the dendrites of cultured sympathetic neurons, that might represent N type channels without an associated β subunit (Delmas *et al.*, 2000). Furthermore, during the purification process of the $\alpha 1$ subunit, the potential difference across the channel, and its native conformation, would rapidly be lost, and the affinity of β subunits for the channels might thus be increased, ensuring 1:1 stoichiometry.

It is possible that in certain native systems the concentration of $Ca_v\beta$ subunits is limiting. We showed that heterologous expression of $Ca_v\beta 2a$ subunits in undifferentiated NG108-15 cells induced the appearance of an HVA current with the characteristics of an L type current (Wyatt *et al.*, 1998), which might be partially an effect on pre-existing L type calcium channels since the calcium channel agonist BayK8644 could induce a similar enhancement of current. Furthermore, it has recently been shown that heterologous expression of β subunits in ventricular myocytes increased the expression of native calcium currents (Colecraft *et al.*, 2002). Although it can always be argued that heterologous expression of the β subunit can result in further synthesis of calcium channels, it has recently been shown that β subunit protein injected into *Xenopus* oocytes increased $Ca_v1.2$ calcium currents (Yamaguchi *et al.*, 1998) and that β subunit protein influenced skeletal muscle calcium channels in a cell-free system (Garcia *et al.*, 2002).

EFFECT OF β SUBUNITS ON PHARMACOLOGY OF CALCIUM CHANNELS

Although DHP agonists produce enhancement of L type $Ca_v2.2$ subunits when expressed alone, $Ca_v\beta$ subunits influence calcium channel ligand binding sites (Welling *et al.*, 1995), generally by producing an increase in the number of high affinity binding sites, consistent with an increased number of channels. Many drugs, such as mibefradil and verapamil, bind preferentially to inactivated calcium channels, and therefore their ability to inhibit the channels will be indirectly affected by the β subunit complement because of their differential effects on inactivation (Berjukow *et al.*, 2000; Lacinova *et al.*, 1995; Welling *et al.*, 1995; Zamponi *et al.*, 1996). It has also been found in one study that coexpression of $Ca_v\beta$ subunits with $Ca_v1.1$ reduced the agonist response to BayK8644 (Varadi *et al.*, 1991). However, coexpression of $Ca_v\beta$ subunits has been found to promote the agonist phase of a calcium channel "antagonist" mibefradil, that is most evident at low drug concentrations (Welling *et al.*, 1995).

A yeast two hybrid screen has been used to identify novel drugs that interfere with the binding of β subunits to the I–II linker of $Ca_v1.2$ (Young *et al.*, 1998). Furthermore, it has been found that the schistosome β subunits inhibit mammalian $Ca_v2.3$ current and that this inhibition is reversed by the antischistosome drug praziquantel (Kohn *et al.*, 2001). It is proposed that this is the mechanism of action of this drug.

EFFECT OF β SUBUNITS ON FACILITATION OF CALCIUM CHANNELS

Facilitation is a term that refers to the reversible enhancement of calcium current, often by manipulation of the membrane potential. Facilitation by depolarizing prepulses was first identified in bovine chromaffin cells by

Neher and colleagues (Fenwick *et al.*, 1982). Voltage-dependent facilitation of L type channels has also been studied in skeletal, smooth, and cardiac muscle, where a number of studies have sought to elucidate the mechanism(s) involved (Feldmeyer *et al.*, 1992; Sculptoreanu *et al.*, 1993b; Wang *et al.*, 1995).

Facilitation has been observed at the single channel level, in chromaffin (Hoshi and Smith, 1987) and cardiac (Pietrobon and Hess, 1990) cells, where mode 2 gating (long openings) of calcium channels is strongly promoted following large depolarizations. Voltage-dependent facilitation is also observed if $Ca_v\alpha1$ subunits are expressed heterologously (Bourinet *et al.*, 1994; Kleppisch *et al.*, 1994; Sculptoreanu *et al.*, 1993a). Although it has been reported that facilitation is a property of cloned $Ca_v\alpha1$ subunits (Kleppisch *et al.*, 1994), another report has suggested that it does not occur in the absence of coexpressed $Ca_v\beta$ subunits (Bourinet *et al.*, 1994), despite the fact that β subunits mimic to some extent the process of facilitation (Dolphin, 1996; Herlitze *et al.*, 2001). Facilitation of L-type channels, as originally observed in native tissues is likely to involve multiple processes, including Ca^{2+} entry and phosphorylation (Dzhura *et al.*, 1994; Sculptoreanu *et al.*, 1993a).

Facilitation is also observed for non-L-type channels, and is the term often used for relief of G-protein-mediated inhibition by a depolarizing prepulse (see below). The slow component of activation observed with β2a is itself a manifestation of facilitation, as it occurs more rapidly at more depolarized voltages (Meir and Dolphin, 2002). Possibly the depolarizing prepulse increases the coupling of $Ca_v\alpha1$ and β, and this is observed as facilitation (Canti *et al.*, 2001; Dolphin, 1996). There are several lines of evidence suggesting that β subunits bind more tightly to calcium channels during a large depolarization, and consequently are involved in the process of facilitation. It is therefore possible that the effects of a prepulse observed in Canti *et al.* (2001) reflect a depolarization state-dependent increase in affinity of $Ca_v2.2$ channels for $Ca_v\beta$ subunits, resulting in a temporary disruption of the equilibrium between $Ca_v\alpha1$ and $Ca_v\beta$. However, although there are reports that $Ca_v\beta$ subunits promote the occurrence of long openings (Hohaus *et al.*, 2000) and mode 2 gating (Cuadra *et al.*, 2001), which is associated with facilitation, this has not universally been observed (Colecraft *et al.*, 2002).

EFFECT OF $CA_V\beta$ SUBUNITS ON G PROTEIN MODULATION OF CALCIUM CHANNELS

Calcium channel β subunits play an important role in G-protein-mediated calcium current inhibition. When they were partially depleted by antisense microinjection in DRGs, agonist inhibition of calcium current was enhanced (Campbell *et al.*, 1995). We first suggested that there may be competition or allosteric interaction between the activated G protein moiety and the $Ca_v\beta$ subunit for their effect on the $\alpha1$ subunit.

A role for β subunits in G-protein inhibition of heterologously expressed calcium channels has now been extensively examined (Bourinet *et al.*, 1996; Canti *et al.*, 2000, 2001; Meir *et al.*, 2000; Qin *et al.*, 1997; Roche and Treistman, 1998). In initial studies in *Xenopus* oocytes, there was reported to be less, or even a loss of G protein inhibition following coexpression of a β subunit (Bourinet *et al.*, 1996; Qin *et al.*, 1997), although these studies only examined inhibition at a single potential, and need to be interpreted with caution because of the presence of an endogenous oocyte β subunit. The result was interpreted in terms of a competitive interaction between $Ca_v\beta$ and $G\beta\gamma$ at an overlapping binding site (Bourinet *et al.*, 1996). However, by studying the voltage-dependence of the effect, we have shown that, although at certain potentials there is a decrease in G protein inhibition in the presence of coexpressed β subunits, this cannot represent a simple competition between β subunits and $G\beta\gamma$ dimers, but the interaction is dynamic, depending on the membrane voltage (Canti *et al.*, 2000).

Prepulse facilitation, a characteristic of G protein-modulated $Ca_v2.2$ calcium channels, is thought to involve $G\beta\gamma$ unbinding from the channel, induced by depolarization. The main evidence that actual unbinding occurs is that reinhibition following a prepulse is $G\beta\gamma$ concentration-dependent (Stephens *et al.*, 1998; Zamponi and Snutch, 1998). In *Xenopus* oocytes, the facilitation rate, representing $G\beta\gamma$ dissociation, was markedly increased by heterologous expression of $Ca_v\beta$ subunits (Canti *et al.*, 2000; Roche and Treistman, 1998). We have further determined the concentration-dependence of this effect. We observed that at low-intermediate levels of $Ca_v\beta$ expressed, the facilitation rate has both a fast and a slow component (Fig. 9). We interpret the fast component, which has an invariant τ, as representing $G\beta\gamma$ dissociation from $Ca_v\beta$-bound channels. We hypothesize that the process with the slow time constant represents $Ca_v\beta$ binding during the depolarizing prepulse to the component of channels that are free in the membrane, as it is dependent on the concentration of $Ca_v\beta$. This implies that the affinity for the $Ca_v\beta$ subunit increases during a depolarizing prepulse (Canti *et al.*, 2001). This model does not support the idea that G protein modulation results from a simple competition between $Ca_v\beta$ and $G\beta\gamma$ for binding to the channel, or that at the usual high levels of β subunit there is any dissociation of $Ca_v\beta$ from the channel during G protein modulation, but rather that under normal conditions where the channels all have a $Ca_v\beta$

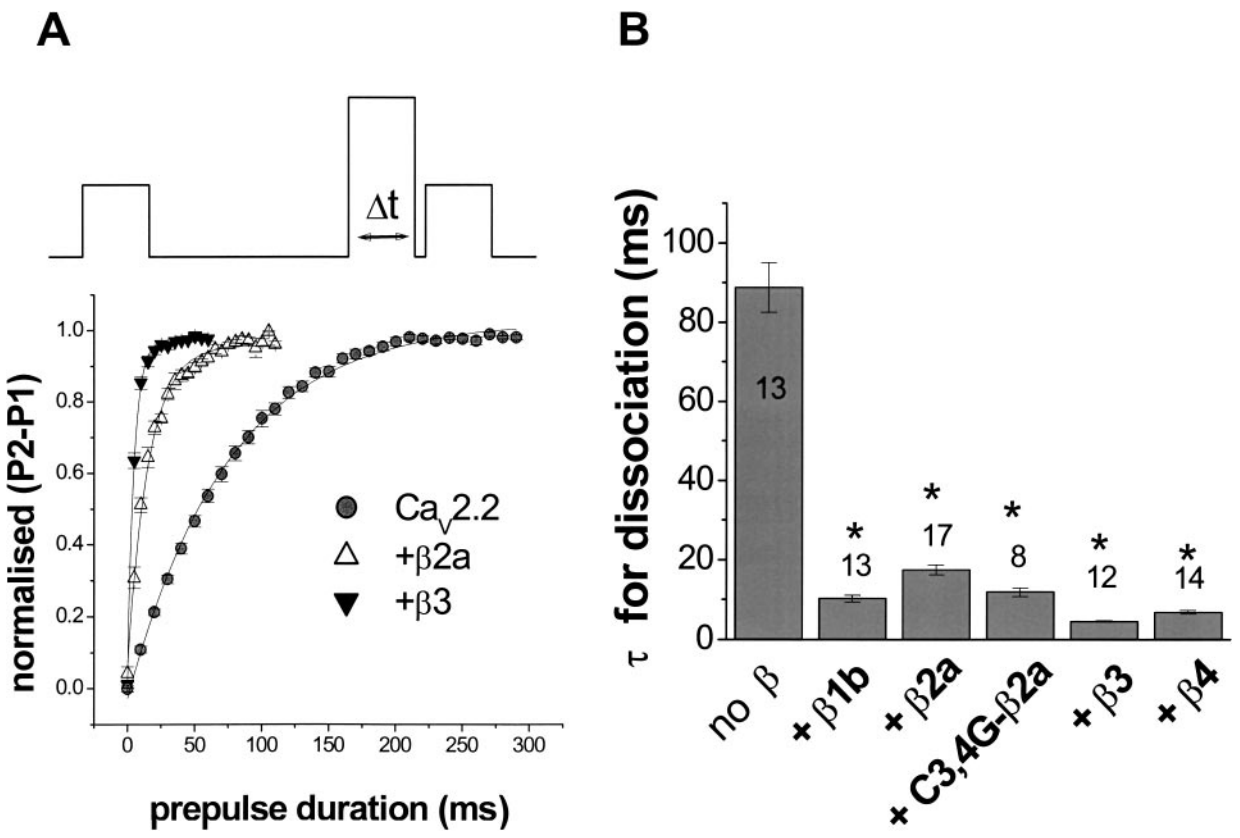

Fig. 9. Effect of $Ca_v\beta$ subunits on prepulse facilitation during G protein modulation. (A) Top panel: voltage waveform for measurement of facilitation rate, by increasing the prepulse duration. The graph shows the facilitation rate for $Ca_v2.2$ with $Ca_v\beta2a$ (open triangles), $Ca_v2.2$ with $Ca_v\beta3$ (closed inverted triangles) and $Ca_v2.2$ without heterologously expressed $Ca_v\beta$ (grey circles) during application of quinpirole. Continuous lines are the result of single exponential fits to the data. Experimental details are given elsewhere (Canti *et al.*, 2000). (B) The histogram gives the τ values for facilitation for all the β subunits examined including the palmitoylation-deficient mutant of $\beta2a$ (C3, 4S-$\beta2a$), determined from exponential fits to individual data, for the subunit combinations shown beneath the histogram bars.

bound, depolarization results in a conformational change between $Ca_v\alpha1$ and β which decreases the stability of $G\beta\gamma$ binding.

This also explains the observation that at some potentials G protein modulation is enhanced in oocytes in the absence of overexpressed $Ca_v\beta$, or following antisense depletion of $Ca_v\beta$ subunits, by the following reasoning. The slowed current activation in the presence of $G\beta\gamma$ results from $G\beta\gamma$ dissociation and is a reflection of the fact that $G\beta\gamma$-bound channels either do not open upon depolarization until $G\beta\gamma$ dissociates, or show a very brief "reluctant" openings (Lee and Elmslie, 2000; Patil *et al.*, 1996). It is likely that reducing $Ca_v\beta$ slows the overall facilitation rate during the test pulse as well as the prepulse, and this is likely to be the reason that a reduction in $Ca_v\beta$ levels results in enhanced inhibition. The direct effects of $Ca_v\beta$ subunits on inactivation will also influence the net amount of G protein inhibition exhibited (Toselli *et al.*, 1999) although β-mediated inactivation is not directly affected by G protein modulation (Meir and Dolphin, 2002). This is further evidence that $Ca_v\beta$ does not dissociate during the process of G protein modulation. This subject is reviewed in more detail elsewhere (Dolphin, in press).

In an expression system (COS-7 cells) in which no endogenous β subunit protein was detected by immunocytochemistry, the presence of heterologously expressed $Ca_v\beta$ subunits was essential for the relief of $G\beta\gamma$-mediated inhibition by a depolarizing prepulse (Meir *et al.*, 2000). We concluded from that study that $Ca_v\beta$ subunits were essential for the process of facilitation, or $G\beta\gamma$ dissociation. In the same system, receptor-mediated inhibition via activation of the D2 dopamine receptor was not completely abolished in the absence of coexpressed β subunits, but reversal of this inhibition by a 100 ms prepulse was lost, implying that in the absence of $Ca_v\beta$ subunits, $G\beta\gamma$

dimers are able to bind and produce a small non-voltage-dependent inhibition of the $Ca_v2.2$ current (Meir *et al.*, 2000).

Potential Overlap of Determinants for VDCC β Subunit and G$\beta\gamma$ Subunit Function

The G$\beta\gamma$ binding site on the $Ca_v\alpha$l subunit intracellular I–II loop (De Waard *et al.*, 1997) partially coincides with binding sites for auxiliary β subunits (Pragnell *et al.*, 1994). However, other studies showed that the three amino acids critical for β subunit interaction are not within, but adjacent to, the Q$\times\times$ER consensus sequence implicated in G$\beta\gamma$ binding (De Waard *et al.*, 1997; Herlitze *et al.*, 1996). A partial overlap in VDCC β subunit- and G$\beta\gamma$ binding sites has also been proposed for the $Ca_v2.3$ carboxyl terminal site (Qin *et al.*, 1997). However, deletion of the majority of this C terminal $Ca_v2.3$ site affected G$\beta\gamma$ modulation, but allowed retention of full sensitivity to β2a, suggesting that another binding site is the prime mediator of the β subunit response (see also Jones *et al.*, 1998).

We have shown that, within the N terminus of $Ca_v2.2$, between amino acids 45 and 55, combined mutation of two arginines to alanines (R52A,R54A) prevented modulation of $Ca_v2.2$ by G proteins. Furthermore, 4 individual point mutations (S48A, I49A, R52A and R54A) were isolated which significantly impaired modulation (Canti *et al.*, 1999). We have subsequently shown that both the α1B-R52,54A and α1B-R52A constructs also exhibited compromised β2a retardation of inactivation, as did α1B-Q47A, which was shown previously to undergo normal G$\beta\gamma$ modulation (Stephens *et al.*, 2000). Taken together with our initial study that identified this site (Canti *et al.*, 1999), the results indicate that the $Ca_v2.2$ amino terminus contribute determinants for both $Ca_v\beta$2a subunit and G$\beta\gamma$ dimer function. However, the differentiating effect of α1B-Q47A indicates that although the overall region involved may partially coincide, the determinants are not identical.

POST-TRANSLATIONAL PROCESSING OF $CA_V\beta$ SUBUNITS

Phosphorylation

Initial studies showed that the purified DHP receptor from skeletal muscle is rapidly phosphorylated by protein kinase A (PKA) (Röhrkasten *et al.*, 1988). An RRPTP sequence was identified as a motif in β1a that is phosphorylated by cAMP (De Jongh *et al.*, 1989). In intact cardiac myocytes, agents increasing cyclic AMP cause phosphorylation primarily of $Ca_v\beta$ subunits (Haase *et al.*, 1993), which have a number of consensus sequences for cyclic AMP-dependent phosphorylation. It has been proposed that $Ca_v\beta$2 subunits are involved via their phosphorylation in the cyclic AMP-mediated signal transduction pathway to the upregulation of L type calcium channels (Puri *et al.*, 1997). However, there is also evidence that cardiac $Ca_v1.2$ subunits are phosphorylation targets (De Jongh *et al.*, 1996; Gao *et al.*, 1997). The mechanism remains unclear, as it has been difficult to reproduce the effect of β-adrenergic receptor activation on $Ca_v1.2$ in heterologous expression systems, but it is clear that targeting of the kinase to the channel requires an A kinase anchoring protein (Gao *et al.*, 1997; Johnson *et al.*, 1997).

PKC and Other Phosphorylation Sites

There are numerous predicted phosphorylation sites for PKC and other protein kinases on β subunits, either conserved in all species of β subunit, or specific for certain β subunits. However, whether a predicted site is actually a substrate for phosphorylation depends on whether it is appropriately exposed to the relevant kinase. It is generally not sufficient to show that this is the case using *in vitro* methods such as phosphorylation of peptides derived from the sequence, as these may fold in a very different way. There are two PKC phosphorylation sites in the BID linker sequence between the SH3 and GK domains, that are predicted to be exposed and are conserved between β subunits. Mutation of the first of these PKC sites (Ser228) to an arginine in β1b reduced the enhancement of $Ca_v2.1$ currents compared to wild type β1b, but had no effect on the voltage-dependence of activation or steady-state inactivation (De Waard *et al.*, 1994). It is of interest that the cloned schistosome β subunits both contain a cysteine and alanine in place of the conserved serines that represent putative phosphorylation sites in the consensus sequences identified above. Furthermore, these β subunits do not enhance the expression in *Xenopus* oocytes of two α1 subunits tested, either $Ca_v2.3$ or jellyfish α1 subunit (Kohn *et al.*, 2001).

There is evidence that microtubule-associated protein kinase (MAP kinase) is able to phosphorylate and potentiate L and N type calcium channels in dorsal root ganglion neurons (Fitzgerald, 2000), but it is unknown whether this phosphorylation is direct, either on the α1 or β subunit. However, it has recently been shown that the effect requires the presence of a β subunit (Fitzgerald, 2002). It has also been shown that activation of the lipid

kinase phosphatidylinositol 3 (PI3)-kinase results in enhancement of calcium channels in smooth muscle (Viard *et al.*, 1999) and cerebellar granule neurons (Blair and Marshall, 1997). We have observed that this results in activation of downstream kinases that then affect specific β subunits (Viard *et al.*, unpublished results).

Palmitoylation

Only β2a is palmitoylated at its N terminal dicysteine motif (Chien *et al.*, 1996). The mechanism of palmitoylation and depalmitoylation of proteins has recently been reviewed (Resh, 1999). Palmitoylation of other proteins results in their localization to lipid microdomains in the plasma membrane (Resh, 1999), and if this is also true for β2a, it may represent a mechanism for targeting of calcium channels to these particular domains.

Turnover

Using a β subunit antisense sequence common to all β subunits, injected into rat dorsal root ganglion neurons, we showed that the β subunit level, as assessed immunocytochemically with an antibody that recognised all β subunits, was maximally reduced after 108 h, giving an approximate half life of about 50 h. The calcium channel currents in these cells were maximally reduced by 47% with a corresponding +7 mV shift in current activation (Berrow *et al.*, 1995). We have also observed that the β3 subunit, when expressed alone in COS-7 cells, was rapidly turned over, since immunostaining was completely lost after 2–6 h treatment with the protein synthesis inhibitor cycloheximide (Bogdanov *et al.*, 2000). In contrast, plasma membrane-associated β1b is much more stable, as its immunolocalization is not affected by inhibition of protein synthesis for up to 6 h. It is possible that the association of β1b with a specific membrane bound protein increases its stability. We identified a largely acidic motif (amino acids 547–556, WEEEEDYEEE) that is involved in the membrane-association of β1b. Acidic motifs in other proteins have been found to bind to pleckstrin homology (PH) domains (Burks *et al.*, 1998). However, the binding partner target of this β1b motif remains to be determined.

GENE-TARGETING OF $CA_V\beta$ SUBUNITS

Knock-out of the β1 isoform (*CACNB1*), present in skeletal muscle as β1a and in heart and brain as β1b, resulted in a lethal phenotype. Homozygous β1 -/- mice showed reduced skeletal muscle mass, with disorganisation of muscle structure, and they died at birth from asphyxiation. There was a 3.9-fold reduction in the number of DHP binding sites (Strube *et al.*, 1996), indicating reduced expression of $Ca_v1.1$ in neonatal myotubes. There was a corresponding 10–20 fold reduction, although not a complete loss of skeletal muscle calcium currents, together with marked impairment in excitation-contraction coupling (Strube *et al.*, 1996). Of interest is the finding that heterozygotes were asymptomatic, indicating that there is normally a sufficient excess of β1 subunit, such that loss of 50% has no effect.

Deletion of the $Ca_v\beta$2 gene results (*CACNB2*) in an embryonic lethal phenotype, underlining the essential role of β2 in cardiac contraction. If these mice were rescued by transgenic expression of β2 in cardiac tissue, a retinal phenotype was observed with reduced sensitivity to light. There was a loss of expression of α1F ($Ca_v1.4$) and loss of ribbon synapses of the photoreceptor terminals as β2 is normally expressed in photoreceptors (Ball *et al.*, 2002).

In contrast, knock-out of the β3 isoform (*CACNB3*) (normally found in brain, heart, and aorta) did not result in a major phenotype, indicating that other β subunits are able to substitute for its function. It is likely that the phenotype of the β1 knock-out is due to the fact that no other β subunits are expressed in skeletal muscle. However, the loss of β3 did result in an altered balance between Ca_v channel types in sympathetic neurons (Namkung *et al.*, 1998). It also reduced response to specific noxious stimuli, by reducing the expression of $Ca_v2.2$ channels in sensory neurons (Murakami *et al.*, 2002).

INVOLVEMENT OF $CA_V\beta$ SUBUNITS IN DISEASE

In addition to mutations in the $Ca_v2.1$ gene (*CACNA1A*), mutation in the $Ca_v\beta$4 subunit gene have also been found in patients with idiopathic generalized epilepsy and episodic ataxia (Escayg *et al.*, 2000). These include a missense mutation C104F, and a mutation inducing premature truncation at R482, which is very near the C terminus of $Ca_v\beta$4. Both β4 mutants were active, producing an increase in expression of $Ca_v2.1$, compared to wild type β4. Therefore it remains unclear why these mutations produce such a marked phenotype. The similarity of the phenotypes for the $Ca_v2.1$ and β4 mutations reinforces the view that these two subunits interact physiologically.

The group of spontaneously arising calcium channel mutations in mice that result in a phenotype of absence epilepsy and cerebellar ataxia include a β subunit mutant, as well as mutants in $Ca_v2.1$ (tottering and leaner;

Fletcher *et al.*, 1996) and $\alpha 2\delta$-2 (ducky, which has a truncation mutation in $\alpha 2\delta$-2; Barclay *et al.*, 2001; Brodbeck *et al.*, 2002). The mouse mutant, lethargic, has a frameshift mutation in the gene for $\beta 4$, that results in a truncated $\beta 4$ subunit message and no $\beta 4$ protein (Burgess *et al.*, 1997). It is essentially a spontaneous $\beta 4$ knock-out. This has been shown to result in β subunit reshuffling during development, and an overexpression of $\beta 1$b, which may be an adaptive developmental response to the lack of $\beta 4$ (McEnery *et al.*, 1998). Physiologically, there is no reduction in Purkinje cell calcium currents, presumably because of compensation by $\beta 1$b, but there is an alteration in thalamic excitatory synaptic currents (Caddick *et al.*, 1999). These results suggest that the lethargic phenotype stems from effects only in certain cell types where other β subunits cannot adequately substitute for the function of the missing $\beta 4$.

Our results in MDCK cells (Brice and Dolphin, 1999; discussed above) suggest that $Ca_v\beta$ subunits can exert an effect on the targeting of the VDCC complex, particularly for the $Ca_v2.1$ subunit. This provides a possible mechanism for the major cerebellar deficit found in the lethargic mutant mouse. Since in expression studies, all β subunits are able to interact with the $Ca_v2.1$ subunit (Brice *et al.*, 1997; De Waard and Campbell, 1995), until now it has been unclear how the absence only of $\beta 4$ could produce such effects. However, if $\beta 4$ is one of only two β subunits able to target $Ca_v2.1$ to presynaptic sites, and the other, $\beta 1$b, has only low expression in the adult brain (Ludwig *et al.*, 1997; McEnery *et al.*, 1998), it is quite conceivable that the loss of $\beta 4$ could produce aberrant targeting of $Ca_v2.1$, and result in major defects in cerebellar development and function.

There is also evidence for altered calcium channel activity in human heart failure (Schroder *et al.*, 1998). In cardiac myopathy associated with failed cardiac myografts, there was a large reduction in $Ca_v\beta$ subunit mRNA and protein by up to 80%, and the major species detected was $\beta 1$b (Hullin *et al.*, 1999). There was also an increase in the amount of truncated relative to full-length $\beta 3$ transcript in human left ventricular tissue showing ischaemic cardiomyopathy, compared to nonfailing tissue (Hullin *et al.*, 2003). The pathophysiological consequences of these changes are yet to be fully understood.

CONCLUSIONS

It is now clear that the $Ca_v\beta$ subunits are much more than a passive structural subunit of HVA calcium channels. Their levels in tissue can be dynamically and developmentally regulated, and there is evidence for a reversible interaction with $\alpha 1$ subunits. There is also much evidence that $Ca_v\beta$ subunits are substrates for dynamic and reversible phosphorylation by a number of different protein kinases, strongly influencing calcium channel function. Furthermore $Ca_v\beta$ subunits have been implicated in a number of disease states, including cardiac myopathy and cerebellar ataxia.

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