Auxiliary subunit regulation of high-voltage activated calcium channels expressed in mammalian cells

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Abstract

The effects of auxiliary calcium channel subunits on the expression and functional properties of high-voltage activated (HVA) calcium channels have been studied extensively in the Xenopus oocyte expression system, but are less completely characterized in a mammalian cellular environment. Here, we provide the first systematic analysis of the effects of calcium channel b and a2–d subunits on expression levels and biophysical properties of three different types (Cav1.2, Cav2.1 and Cav2.3) of HVA calcium channels expressed in tsA-201 cells. Our data show that Cav1.2 and Cav2.3 channels yield significant barium current in the absence of any auxiliary subunits. Although calcium channel b subunits were in principle capable of increasing whole cell conductance, this effect was dependent on the type of calcium channel a1 subunit, and b3 subunits altogether failed to enhance current amplitude irrespective of channel subtype. Moreover, the a2–d subunit alone is capable of increasing current amplitude of each channel type examined, and at least for members of the Cav2 channel family, appears to act synergistically with b subunits. In general agreement with previous studies, channel activation and inactivation gating was regulated both by b and by a2–d subunits. However, whereas pronounced regulation of inactivation characteristics was seen with the majority of the auxiliary subunits, effects on voltage dependence of activation were only small (<5 mV). Overall, through a systematic approach, we have elucidated a previously underestimated role of the a2–d subunit with regard to current enhancement and kinetics. Moreover, the effects of each auxiliary subunit on whole cell conductance and channel gating appear to be specifically tailored to subsets of calcium channel subtypes.

Keywords: alpha2-delta subunit, beta subunit, current densities, inactivation, tsA-201 cells

Introduction

High-voltage activated (HVA) calcium channels are heteromultimeric complexes comprising a pore-forming α1 subunit, one of four different auxiliary α2–δ, and one of four β subunits (for a review see Catterall, 2000). Although the α1 subunits contain the essential molecular components to form a functional calcium channel (such as voltage sensors and pore-forming loops), coexpression with auxiliary subunits is thought to result in channels with properties that more closely resemble native calcium channel currents (Lacerda et al., 1991; Stea et al., 1993). Expression studies in Xenopus oocytes and mammalian cells suggest that coexpression of HVA calcium channels with any one of the four β subunits results in increased membrane expression of the channels, thus resulting in increased current densities (Mori et al., 1991; Williams et al., 1992; Brust et al., 1993; Castellano et al., 1993; Neely et al., 1993; Stea et al., 1993; De Waard et al., 1994; De Waard & Campbell, 1995; Kamp et al., 1996; Jones et al., 1998; Yamaguchi et al., 1998). It is thought that binding of the β subunit to the α1 subunit's domain I–II linker region masks an endoplasmic reticulum (ER) retention signal (Bichet et al., 2000), thus facilitating translocation of
the α₁ subunit to the plasma membrane (Chien et al., 1995; Yamaguchi et al., 1998; Gao et al., 1999). In addition, β subunits have been shown typically to mediate hyperpolarizing shifts in the voltage dependences of activation and inactivation, in addition to regulating inactivation kinetics (Birnbaumer et al., 1998; Walker & De Waard, 1998). More recently, a negative regulatory effect of the overexpressed β₃ subunit on Caᵥ2.2 (N-type) calcium channel current has been demonstrated in *Xenopus* oocytes (Yasuda et al., 2004). Compared with β subunits, the effect of α₂–δ subunits on HVA calcium channel currents is ambiguous. For instance, in the absence of β subunits, α₂–δ subunits reportedly mediate only a negligible effect on current densities of Caᵥ1.2, Caᵥ2.1, Caᵥ2.2 and Caᵥ2.3 channels (Stea et al., 1993; Tomlinson et al., 1993; De Waard & Campbell, 1995; Parent et al., 1997; Qin et al., 1998a) or significant (2–7.5-fold enhancement) in both oocytes and mammalian cells (Mori et al., 1991; Shistik et al., 1995; Felix et al., 1997; Jones et al., 1998; Wakamori et al., 1999). Finally, a number of putative calcium channel γ subunits have been identified; however, although some γ subunits appear to affect channel biophysics in oocytes (Kang et al., 2001), the physiological role of these subunits remains to be determined.

Over the years, the picture has emerged that a number of functional properties of calcium channels expressed in oocytes differ from those obtained in mammalian expression systems such as HEK-293 and COS-7 cells, including sensitivity to ω-agatoxins (Bourinet et al., 1999) and small organic blockers (Zamponi, 1999), and channel expression level when an α₁ subunit is injected/transfected alone. This is compounded by the notion that *Xenopus* oocytes endogenously express at least one calcium channel β subunit isoform (Tareilus et al., 1997), plus low levels of endogenous α₁ subunits. Considering that most of our current knowledge concerning the action of calcium channel auxiliary subunit is derived from oocyte studies, it is thus important to determine systematically how different calcium channels are regulated by auxiliary subunits in mammalian cells devoid of endogenous calcium channels.

Here, we report results from ~1700 whole cell patch clamp recordings to provide a comprehensive analysis of the effects of α₂–δ₁ and the four different β subunits on Caᵥ1.2 (L-type), Caᵥ2.2 (N-type) and Caᵥ2.3 (R-type) calcium channels expressed transiently in mammalian (tsA-201) cells, under identical experimental conditions. Our data show that for L-type and N-type channels, α₂–δ subunits appear as important or more important for regulating whole cell conductance compared with β subunits, and that for Caᵥ2 channels, β and α₂–δ subunit act concertedly to increase peak current amplitude. The majority of the auxiliary subunits only weakly regulate channel activation, whereas more pronounced effects on voltage-dependence and rates of inactivation were observed with α₂–δ and the majority of β subunits for each calcium channel type. Collectively, our data constitute the first systematic analysis of calcium channel subunit regulation in mammalian cells, and reveal that each auxiliary subunit exhibits channel isoform and function-specific regulation.

**Experimental Procedures**

**Transient expression of Ca channels**

HEK tsA-201 cells were maintained at 37 °C (5% CO₂) in Dulbecco's minimal essential medium (DMEM) supplemented with fetal bovine serum, penicillin and streptomycin. Cells were transfected with cDNAs encoding rat α₁ (Caᵥ1.2, Caᵥ2.2, Caᵥ2.3) alone, or in combination with β (β₁b, β₂a, β₃, β₄) and/or α₂ δ₁, and enhanced green fluorescent protein (EGFP) using a standard calcium phosphate protocol as described in detail by us previously (for example, see Stotz et al., 2004). For Caᵥ1.2 and Caᵥ2.2 channels, 7 µg of each calcium channel subunit plus 4 µg of EGFP cDNA was used for transfection. Owing to the robust expression of Caᵥ2.3 channels, an additional set of experiments was conducted with 1 µg Caᵥ2.3 cDNA to reduce whole cell conductance. Cells were moved to 28 °C 24 h after transfection and maintained for up to 7 days. The wild-type cDNA constructs used in this study were kindly provided by Dr Terry Snutch (University of British Columbia).

**Electrophysiological recordings**

Whole-cell patch-clamp recordings were performed with 20 mm barium external solution (comprising, in mm: 20 BaCl₂, 1 MgCl₂, 10 HEPES, 40 tetraethylammonium chloride, 10 glucose, 65 CsCl, pH 7.2) at room temperature. Borosilicate glass pipettes of ~3–4 MΩ resistance were filled with a cesium methane sulfonate-based internal solution (in mm: 108 CsCH₃SO₄, 4 MgCl₂, 9 EGTA, 9 HEPES, pH 7.2). Series resistance was compensated by 85%. Data were acquired and filtered at 1 kHz with an Axopatch 200B amplifier, linked to a personal computer equipped with pCLAMP 9.0 software (Axon Instruments). The effects of various calcium channel subunits on whole cell amplitude were examined in multiple transfections and, for each calcium channel α₁ subunit, included comparisons...
within the same batches of cells. Cells with similar capacitance were chosen for recordings (~20 pF). Current-voltage ($I-V$) relationships were obtained by step depolarization between −60 mV and +50 mV in 10 mV increments, from a typical holding potential of −100 mV. To assess steady-state inactivation properties, a 5-s conditioning pulse to various holding potentials preceded a test depolarization to +20 mV. Data were leak subtracted off-line and analysed with Clampfit 9.0 (Axon Instruments) and Prism 3.0 (GraphPad). $I-V$ relations were fitted with the equation:

$$I_{\text{peak}} = (V - E_{\text{rev}}) G_{\text{max}} [1/(1 + \exp(V_{1/2,\text{act}} - V)/k_{\text{act}})]$$

where $E_{\text{rev}}$ is the reversal potential, $V_{1/2,\text{act}}$ is the half-activation potential, $G_{\text{max}}$ is the maximum slope conductance and $k_{\text{act}}$ is the slope factor. To analyse faithfully the effects of auxiliary subunits on $G_{\text{max}}$ values, GFP-positive cells that did not express functional channels were included. Current-voltage data from cells with currents smaller than 10 pA could not be fitted, and thus we were unable to determine exact $G_{\text{max}}$ values. As it was essential that these data were included in Fig. 6, $G_{\text{max}}$ values in these experiments were arbitrarily assumed to be zero.

For the voltage-dependence of inactivation, we fitted the initial falling phase of normalized steady-state inactivation data with a modified Boltzmann relation:

$$I_{\text{peak}}(\text{normalized}) = C + (1 - C)[1 + \exp((V - V_{1/2,\text{inact}})/k_{\text{inact}})]$$

where $V$ is the holding potential, $V_{1/2,\text{inact}}$ is the half-inactivation potential, $C$ reflects the noninactivating fraction (i.e. the fraction of open state inactivation) and $k_{\text{inact}}$ is a slope factor.

**Reverse transcriptase and polymerase chain reaction (RT-PCR) analysis**

To detect low levels of calcium channel auxiliary subunits, RT-PCR using RNA from HEK cells and rat brain and retinal tissue was performed. The reaction consisted of 1 µg of RNA, 1 × RT buffer, 10 mm dNTPs, 5 units RT (Superscript, Gibco) and 10 pmol of each downstream oligonucleotide:

- $\beta_1$ (5'-CCCGGGACATGCTGTTCTC),
- $\beta_2$ (5'-GCTGGGTAAATTGAGA),
- $\beta_3$ (5'-CAGCCACGTGCGCCCTGTG),
- $\beta_4$ (5'-AATGACAGCAGCCCATTAG),
- $\alpha_2$-$\delta_1$ (5'-TGTCCTGTTTTCTTGTGTC),
- $\alpha_2$-$\delta_2$ (5'-CCTGCAGGATCTGGTCACTG),
- $\alpha_2$-$\delta_3$ (5'-GCTACTATAGTTAGGTTTCC),
- $\alpha_2$-$\delta_4$ (5'-ACTGTTGTAGTTAGGTTGGG)

at 42 °C for 90 min. From this reaction, 5 µL was removed and added to a 50-µL PCR composed of 1 × PCR buffer, 1.25 µm dNTPs, 2 units of Taq polymerase (Qiagen), 20 pmol of the 3’oligo described in the RT reaction and 20 pmol of the 5’oligonucleotide:

- [($\beta_1$ (5'-AGGAGGCACCACGGCAAGCC),
- $\beta_2$ (5'-AAGAAGCAGTCACAATAA),
- $\beta_3$ (5'-ACCTCAGAACCAGCAGCTG),
- $\beta_4$ (5'-ATTGAAGAAGCAGTTCT),
- $\alpha_2$-$\delta_1$ (5'-ACATAACCGGCAAATTTGAAA),
- $\alpha_2$-$\delta_2$ (5'-ACCTGACACAGGAGATGCCTG),
- $\alpha_2$-$\delta_3$ (5'-CAGCTCTCTCAGTGGCACA),
- $\alpha_2$-$\delta_4$ (5'-CAAGCTCTCAGCTGCGAG)]

The reactions were placed in a preheated PCR block for 15 min at 95 °C followed by 30 cycles (30 s at 94 °C, 40 s at 55 °C, 1 min at 94 °C). The PCR products were separated through a 1.5% agarose gel stained with ethidium bromide for visualization. All oligonucleotide sequences were directed towards the human sequence of the calcium channel subunits.
**Statistical analysis**

A Kruskal–Wallis test with a Dunn's test as a post test was used to evaluate scatter plots for $G_{\text{max}}$. One-way anova with Dunnett's test as a post test was used for all other comparisons. Asterisks indicate statistical significance +/-\(\beta\), number symbols indicate statistical significance +/-\(\alpha_2-\delta\). Single, double and triple symbols reflect significance at the 0.05, 0.01 and 0.001 levels, respectively.

**Results**

**Lack of expression of endogenous calcium channels and auxiliary subunits**

It has been shown that *Xenopus* oocytes express endogenous \(\beta_3\) subunits, which play a important role in \(\alpha_1\) subunit expression when the \(\alpha_1\) is injected alone (Tareilus *et al*., 1997; Canti *et al*., 2001). It has been suggested that HEK-293 cells may express two \(\alpha_2-\delta_1\) variants and one type of calcium channel \(\beta\) subunit (Brust *et al*., 1993). To determine whether our tsA-201 cell system (a cell line derived from HEK-293 cells) endogenously expresses calcium channel subunits, we therefore conducted an RT-PCR analysis of tsA-201 cell mRNA. As shown in Fig. 1A, we could not detect transcripts for any of the auxiliary \(\alpha_2-\delta\) or \(\beta\) subunits in tsA-201 cells, whereas positive control mRNA isolated from either rat brain or human retina (for \(\alpha_2-\delta_4\)) yielded robust PCR bands. Hence, we conclude that under our culturing conditions, tsA-201 cells do not express significant levels of auxiliary calcium channel subunit mRNA.

We also determined whether transfection of the ancillary subunits used in our study could result in barium currents in the absence of exogenously transfected calcium channel \(\alpha_1\) subunits. We detected no current activity when the channels were coexpressed with a combination of either \(\beta_1b/\alpha_2-\delta_1\) or \(\beta_3/\alpha_2-\delta_1\) (Fig 1B, \(n=10\) each). We did, however, detect very small (\(\approx 5-10\) pA) inward barium currents in some of the cells expressing \(\beta_3/\alpha_2-\delta_1\) or \(\beta_4/\alpha_2-\delta_1\); as we will show below, these were negligible compared with whole cell currents obtained in the presence of any of the \(\alpha_1\) subunits examined in this study. Hence, for all intents and purposes, the tsA-201 cell expression system is
devoid of endogenous calcium channel activity, and appears to lack auxiliary calcium channel subunit expression altogether.

**Auxiliary subunits are poor regulators of calcium channel activation**

To examine systematically the effects of auxiliary calcium channel subunits on the functional properties of selected calcium channel subtypes, Ca\textsubscript{v}1.2, Ca\textsubscript{v}2.2 and Ca\textsubscript{v}2.3 calcium channel α\textsubscript{1} subunits were transiently transfected into tsA-201 either alone or with various combinations of α\textsubscript{2}–δ\textsubscript{1} and β subunits, and resulting depolarization-activated barium currents were characterized using whole cell patch clamp recordings. For Ca\textsubscript{v}2.3 subunits, we noted that peak current amplitudes were substantially larger than those observed with the other calcium channel subtypes. Hence, to facilitate comparison, and to rule out the possibility that our observations could be skewed by an excess of Ca\textsubscript{v}2.3 α\textsubscript{1} subunits over any cotransfected auxiliary subunits, an additional set of experiments were carried out in which the amount of Ca\textsubscript{v}2.3 α\textsubscript{1} subunit cDNA was reduced from our typical value of 7 µg to 1 µg.

Figure 2 shows normalized ensemble \(I-V\) relations, recorded for each calcium channel subtype under identical experimental conditions (Fig.2A–D). As clearly evident from the figure, in comparison with previous findings obtained with channels expressed in *Xenopus* oocytes and mammalian cells, coexpression of auxiliary subunits in our tsA-201 expression system resulted in only minor (< 5 mV) if any changes in the voltage dependences of activation of the three calcium channel subtypes examined. Statistically significant changes were observed only for selected subunit combinations (see Tables 1–3). For example, coexpression of α\textsubscript{2}–δ\textsubscript{1}, β\textsubscript{2a} or β\textsubscript{4} subunits with Ca\textsubscript{v}1.2 channels resulted in a hyperpolarizing shift in half-activation potential that did not occur with Ca\textsubscript{v}2 channels. The lack of the effect of β\textsubscript{1} on Ca\textsubscript{v}1.2 channels is consistent with previous data obtained from HEK-293 cells (Kamp *et al.*, 1996) and oocytes (Stea *et al.*, 1993). Analogous effects of α\textsubscript{2}–δ\textsubscript{1} subunit on Ca\textsubscript{v}1.2, Ca\textsubscript{v}2.2 and Ca\textsubscript{v}2.3 channels have been shown in various cells including HEK-293 cells (Williams *et al.*, 1992; Brust *et al.*, 1993; Tomlinson *et al.*, 1993; Parent *et al.*, 1997; Jones *et al.*, 1998; Klugbauer *et al.*, 1999; Stephens *et al.*, 2000). By contrast, β\textsubscript{1b} subunits appeared preferentially to regulate the voltage dependence of activation of Ca\textsubscript{v}2.3 channels irrespective of the amount of Ca\textsubscript{v}2.3 α\textsubscript{1} subunit cDNA used for transfection. Surprisingly, β\textsubscript{3} subunits did not cause significant hyperpolarizing shifts for any of the calcium channels tested. Qualitatively similar findings with β\textsubscript{3} subunits have been reported for Ca\textsubscript{v}1.2 channels expressed in tsA-201 cells (Gerster *et al.*, 1999) and for Ca\textsubscript{v}2.2 channels expressed in *Xenopus* oocytes (Lin *et al.*, 1997). Collectively, these data indicate that subunit effects on the activation characteristics of calcium channels expressed in tsA-201 cells are not only subtle, but also dependent on the type of calcium channel α\textsubscript{1} subunit.
Auxiliary subunits regulate inactivation kinetics of voltage-gated calcium channels

The data shown in Fig. 2 could be consistent with a relative lack of functional expression of the various auxiliary subunits used in our experiments. However, Figs 3 and 4 clearly show that this is not the case. As seen from the raw current records in Fig. 3 and from the ensemble data shown in Fig. 4, coexpression with α2-δ1 and/or most of the individual β subunits resulted in a significant change in the inactivation kinetics of CaV2 calcium channels. In particular, β3, which did not affect activation of the channels, mediated a pronounced speeding of CaV2.2 and CaV2.3 channel inactivation, in general agreement with previous recordings of CaV2.3 channels in Xenopus oocytes (Olcese et al., 1994; Parent et al., 1997). As also reported previously on numerous occasions (Olcese et al., 1994; Parent et al., 1997; Stephens et al., 2000), β2a subunits slowed inactivation of all calcium channel subtypes examined. The β1b subunit preferentially accelerated inactivation kinetics of CaV2.2 channels. By contrast, β4 subunits did not appear to be able to regulate inactivation kinetics of the three calcium channel subtypes examined. Unlike with CaV2.2 and CaV2.3 channels, the inactivation kinetics of CaV1.2 channels were, with the exception of β2a, not effectively regulated by the β subunit, similar to published data (Gerster et al., 1999). It should be noted that in COS-7 cells all β subunits slowed inactivation kinetics of CaV2.2 channels (Stephens et al., 2000).
The $\alpha_2$–$\delta_1$ subunit tended to accelerate inactivation kinetics of all calcium channels subtypes both in the absence and in the presence of $\beta$ subunits. This is consistent with previous reports for Ca$_{1.2}$ (Felix et al., 1997; Klugbauer et al., 1999), Ca$_{2.2}$ (Gao et al., 2000) and Ca$_{2.3}$ channels (Qin et al., 1998a) but not Ca$_{2.2}$ channels in HEK-293 cells (Williams et al., 1992; Brust et al., 1993). Interestingly, $\alpha_2$–$\delta$ subunit regulation of Ca$_{2.3}$ channel inactivation was lost when the amount of Ca$_{2.3}$ expression was reduced (compare Fig. 4C and D), and at this point, we do not have a convincing mechanistic explanation for this result. Interestingly, $\alpha_2$–$\delta_1$ could not accelerate inactivation of the three calcium channels when coexpressed with $\beta_{2a}$, suggesting that the $\beta_{2a}$-subunit-mediated slowing of inactivation dominates over $\alpha_2$–$\delta_1$-induced acceleration. Our collective data are again consistent with the idea that $\beta$ subunit regulation may be specifically tailored to subsets of calcium channel subtypes in cooperation with $\alpha_2$–$\delta_1$ subunits.
Figure 5 illustrates the steady-state inactivation behaviour of the three calcium channel isoforms in the absence and the presence of various auxiliary subunits. Ca\textsubscript{v}2.2 (Fig. 5B) and Ca\textsubscript{v}2.3 (Fig. 5C and D), but not Ca\textsubscript{v}1.2 (Fig. 5A), exhibited distinguishable closed- and open-state inactivation in the absence of \(\beta\) subunits, as seen from the biphasic voltage-dependence of inactivation. The separation of closed- and open-state inactivation was augmented following coexpression of the \(\beta\) subunit, such that the relative contribution of open-state inactivation to the overall inactivation process became dramatically increased (Fig. 5B–D– unfitted portion of the data). As a result, closed-state inactivation was diminished to less than 20% of total inactivation, precluding a detailed analysis of the half-inactivation potentials under these conditions. For Ca\textsubscript{v}1.2 + \(\alpha_2\delta\) channels (see Table 1), coexpression with the \(\beta_2a\) subunit mediated a discernible 7 mV depolarizing shift in half-inactivation potential, consistent with previous findings (Olcese \textit{et al.}, 1994; Qin \textit{et al.}, 1998b; Gerster \textit{et al.}, 1999). By contrast, the remaining calcium channel \(\beta\) subunit isoforms tended to mediate hyperpolarizing shifts in half-inactivation potential, which in the case of Ca\textsubscript{v}2.3 channels did not reach significance as frequently as those seen with the other calcium channel subtypes (see Tables 1–3), and in disagreement with previous results in oocytes (Stea \textit{et al.}, 1993; Wakamori \textit{et al.}, 1999; Canti \textit{et al.}, 2001). The \(\alpha_2\delta\) subunit mediated negative shifts in half-inactivation potential for Ca\textsubscript{v}1.2, Ca\textsubscript{v}2.2 and Ca\textsubscript{v}2.3 (1 \(\mu\)g) channels, but the effects were typically not additive with those of the \(\beta\) subunits. This suggests that the \(\alpha_2\delta\) subunit is capable of regulating the voltage-dependence of calcium channel inactivation by a mechanism that may perhaps converge with that of \(\beta\) subunit regulation. Altogether, however, with the exception of the \(\beta_2a\) subunit, auxiliary subunit regulation of half-inactivation potentials is relatively weak, with shifts in \(V_{1/2,\text{inact}}\) of typically less than 10 mV.

**Calcium channel \(\beta\) and \(\alpha_2\delta\) subunits regulate steady-state inactivation**

Figure 5 illustrates the steady-state inactivation behaviour of the three calcium channel isoforms in the absence and the presence of various auxiliary subunits. Ca\textsubscript{v}2.2 (Fig. 5B) and Ca\textsubscript{v}2.3 (Fig. 5C and D), but not Ca\textsubscript{v}1.2 (Fig. 5A), exhibited distinguishable closed- and open-state inactivation in the absence of \(\beta\) subunits, as seen from the biphasic voltage-dependence of inactivation. The separation of closed- and open-state inactivation was augmented following coexpression of the \(\beta_2a\) subunit, such that the relative contribution of open-state inactivation to the overall inactivation process became dramatically increased (Fig. 5B–D– unfitted portion of the data). As a result, closed-state inactivation was diminished to less than 20% of total inactivation, precluding a detailed analysis of the half-inactivation potentials under these conditions. For Ca\textsubscript{v}1.2 + \(\alpha_2\delta\) channels (see Table 1), coexpression with the \(\beta_2a\) subunit mediated a discernible 7 mV depolarizing shift in half-inactivation potential, consistent with previous findings (Olcese \textit{et al.}, 1994; Qin \textit{et al.}, 1998b; Gerster \textit{et al.}, 1999). By contrast, the remaining calcium channel \(\beta\) subunit isoforms tended to mediate hyperpolarizing shifts in half-inactivation potential, which in the case of Ca\textsubscript{v}2.3 channels did not reach significance as frequently as those seen with the other calcium channel subtypes (see Tables 1–3), and in disagreement with previous results in oocytes (Stea \textit{et al.}, 1993; Wakamori \textit{et al.}, 1999; Canti \textit{et al.}, 2001). The \(\alpha_2\delta\) subunit mediated negative shifts in half-inactivation potential for Ca\textsubscript{v}1.2, Ca\textsubscript{v}2.2 and Ca\textsubscript{v}2.3 (1 \(\mu\)g) channels, but the effects were typically not additive with those of the \(\beta\) subunits. This suggests that the \(\alpha_2\delta\) subunit is capable of regulating the voltage-dependence of calcium channel inactivation by a mechanism that may perhaps converge with that of \(\beta\) subunit regulation. Altogether, however, with the exception of the \(\beta_2a\) subunit, auxiliary subunit regulation of half-inactivation potentials is relatively weak, with shifts in \(V_{1/2,\text{inact}}\) of typically less than 10 mV.
FIG. 5. Voltage dependence of inactivation in the form of steady-state inactivation curves obtained for Ca_{1.2} (A), Ca_{2.2} (B) and Ca_{2.3} (7 µg cDNA, C; 1 µg cDNA, D) in the absence and the presence of various auxiliary subunits. The individual data points are means of 5–37 experiments (see Tables 1–3). Error bars reflect standard errors. The solid lines reflect fits via the Boltzmann equation that have been restricted to the closed state inactivation portion.

### TABLE 1. Effects of auxiliary subunits on activation and steady-state inactivation of Ca_{1.2} calcium channels

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<th>Subunit</th>
<th>Activation</th>
<th>Inactivation (steady-state)</th>
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<tr>
<td></td>
<td>$V_{1/2}$(mV)</td>
<td>$E_{m}$(mV)</td>
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<tr>
<td>Ca_{1.2}</td>
<td>5.9 ± 0.9</td>
<td>48.7 ± 0.9</td>
</tr>
<tr>
<td>Ca_{1.2}/βb</td>
<td>6.4 ± 0.8</td>
<td>49.2 ± 0.9</td>
</tr>
<tr>
<td>Ca_{1.2}/β2a</td>
<td>1.7 ± 0.7**</td>
<td>45.6 ± 1.4</td>
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<tr>
<td>Ca_{1.2}/β3</td>
<td>6.7 ± 0.6</td>
<td>49.4 ± 0.6</td>
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<tr>
<td>Ca_{1.2}/β4</td>
<td>0.5 ± 1.1***</td>
<td>48.4 ± 1.0</td>
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<tr>
<td>Ca_{1.2}/γs/β1b</td>
<td>1.9 ± 0.4***</td>
<td>46.5 ± 0.4</td>
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<tr>
<td>Ca_{1.2}/γs/β2a</td>
<td>1.3 ± 0.5***</td>
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<td>Ca_{1.2}/γs/β3</td>
<td>2.5 ± 0.7***</td>
<td>48.3 ± 0.5</td>
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<tr>
<td>Ca_{1.2}/γs/β4</td>
<td>-0.9 ± 0.8*</td>
<td>46.0 ± 0.8*</td>
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* Asterisks and number symbols reflect statistical significance as described in the Materials and methods section.

### TABLE 2. Effects of auxiliary subunits on activation and steady-state inactivation of Ca_{2.2} calcium channels

<table>
<thead>
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<th>Subunit</th>
<th>Activation</th>
<th>Inactivation (steady-state)</th>
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<tr>
<td></td>
<td>$V_{1/2}$(mV)</td>
<td>$E_{m}$(mV)</td>
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<td>Ca_{2.2}</td>
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<td>Ca_{2.2}/βb</td>
<td>146 ± 0.5</td>
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<td>Ca_{2.2}/β2a</td>
<td>126 ± 1.1</td>
<td>58.4 ± 0.9</td>
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<td>Ca_{2.2}/β3</td>
<td>156 ± 1.0</td>
<td>51.8 ± 0.9</td>
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<td>Ca_{2.2}/β4</td>
<td>122 ± 0.6</td>
<td>56.1 ± 0.6</td>
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<td>Ca_{2.2}/γs/β1b</td>
<td>131 ± 0.7</td>
<td>57.4 ± 0.7***</td>
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<td>Ca_{2.2}/γs/β2a</td>
<td>90 ± 0.6***</td>
<td>57.7 ± 0.6 **</td>
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<td>Ca_{2.2}/γs/β3</td>
<td>118 ± 0.9</td>
<td>58.9 ± 0.6***</td>
</tr>
<tr>
<td>Ca_{2.2}/γs/β4</td>
<td>105 ± 1.0*</td>
<td>58.6 ± 1.0</td>
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* Asterisks and number symbols reflect statistical significance as described in the Materials and methods section.
Regulation of whole cell conductance by calcium channel α2–δ1 and β subunits

It has been suggested that one major function of calcium channel β subunits is to promote functional expression of calcium channels in the plasma membrane, as reflected by a dramatic increase in whole cell current amplitudes following β subunit coexpression (Chien et al., 1995; Yamaguchi et al., 1998; Gao et al., 1999). To determine whether a similar phenomenon occurs in tsA-201 cells, we analysed $G_{\text{max}}$ values for the individual calcium channel subtypes in the absence or presence of various auxiliary subunits (see Fig. 6). In each case, we evaluated their effects on both median and mean $G_{\text{max}}$ values, and our interpretations of the data are based on considering the statistical analysis of means and median values.

The whole cell conductance data shown in Fig. 6 reveal a number of surprises. First, with Ca v1.2 and Ca v2.3 channels, but not with Ca v2.2, robust current activity was observed even in the absence of any auxiliary subunit (Fig. 6A, C and D–left panels). Second, in the absence of α2–δ1, calcium channel β subunits did not affect the whole cell conductance of Ca v1.2 channels. For Ca v2.2 and Cav2.3 (1 µg) channels lacking α2–δ1, coexpression of β subunits significantly affected median values, but not the overall means and, strikingly, coexpression with β3 never resulted in current enhancement. Finally, for Ca v1.2 channels, the coexpression of α2–δ1 significantly increased whole cell conductance without further enhancement of current activity by β subunit coexpression. By contrast, whereas the presence of α2–δ1 also enhanced Cav2.2 current activity, the presence of β subunits resulted in further conductance increases that were larger than expected from a simple additive effect (Fig. 6B), suggesting that α2–δ1 and β subunits act synergistically to promote Ca v2.2 activity. A similar trend was also observed with Ca v2.3, here mainly β1b and β4 appeared to act in concert with α2–δ1. Analogous findings were reported previously in HEK-239 cells (Williams et al., 1992; Brust et al., 1993; but not Jones et al., 1998) and oocytes (Shistik et al., 1995; Parent et al., 1997; but not Wakamori et al., 1999). It should be noted here that coexpression of α2–δ1 with each of the three channel types frequently resulted in substantially higher current amplitudes than those observed when this subunit was absent (compare left and right panels in Fig. 6A–D). Thus, in contrast to what was previously thought, the α2–δ1 subunit may perhaps play a more pronounced role in regulating current amplitudes than the different types of β subunits. Finally, we note that there was no correlation between the effects of β subunits on inactivation kinetics and on whole cell conductance (data not shown), suggesting that these two processes are governed by distinct mechanisms (see also Jones, 2002). Moreover, this further supports the idea that a lack of effect of a particular subunit on a given functional property is not due to a lack of subunit expression (for example β1 subunits did not affect current levels, but mediated a pronounced effect on inactivation kinetics).
Figure 7 examines the fraction of GFP-positive cells that did not express detectable membrane currents. For Ca,1.2 calcium channels (Fig. 7A), we typically observed an \( \approx 80\% \) success rate, irrespective of the subunit that was coexpressed (with exception of \( \beta_{1b} \), which appeared to increase the fraction of expressing cells further). Hence, the observation that \( \beta \) subunits did not regulate current densities (Fig. 6A) is not secondarily due to an increase in cells with no detectable current. With Ca,2.3 calcium channels (Fig. 7C and D), an 85–100\% success rate was obtained, indicating that these channels express very effectively in tsA-201, consistent with the large \( G_{\text{max}} \) values observed with this Ca,2.3. The data obtained with Ca,2.2 are perhaps the most striking. In the absence of \( \beta \) subunits, the percentage of cells with no detectable current was \( \approx 45\% \) and \( \approx 35\% \) in the absence and the presence of \( \alpha_{2-3} \), respectively.
respectively (Fig. 7B). Coexpression with $\beta_{1b}, \beta_{2a}$ or $\beta_4$ subunits dramatically enhanced the fraction of expressing cells, whereas $\beta_3$ subunits did not. These data indicate that the small enhancements of median $G_{\text{max}}$ values in the absence of $\alpha_2-\delta_1$ in Fig. 6B were, at least in a part, due to increased success rate. The finding that $> 95\%$ success rate was seen with Ca,2,2 channels in the presence of $\beta_{1b}, \beta_{2a}$ and $\beta_4$ irrespective of the presence of $\alpha_2-\delta_1$ indicates that the increase in $G_{\text{max}}$ in the concomitant presence of $\alpha_2-\delta_1$ and either one of these $\beta$ subunits (Fig. 6B) is due to a true increase in current amplitude rather than the number of expressing cells. This is consistent with the substantial population of large current amplitudes in the scatter plots shown in Fig. 6B (top right panel). Williams et al. (1992) reported a similar finding using the HEK cell expression system. Currents from cells expressing Ca,2,2$\alpha_1$ alone or with $\alpha_2-\delta_1$ were negligible and the percentages of detectable current-expressing cells were less than 10% in both cases. Cells cotransfected with Ca,2,2$\alpha_1$ and $\beta_{1c}$ showed robust currents and the current-expressing cells were increased up to $\approx 35\%$. This percentage was not changed by further cotransfection of $\alpha_2-\delta_1$ with Ca,2,2$\alpha_1$ and $\beta_{1c}$, although current amplitude was enormously enhanced due to increase in the number of large current cells.

Collectively, $\beta_{1b}, \beta_{2a}$ and $\beta_3$ subunits are capable of increasing the fraction of cells that express functional currents as well as current amplitude of Ca,2 channels, especially Ca,2,2. By contrast, $\alpha_2-\delta_1$ subunits solely enhance current amplitude without affecting the fraction of cells expressing detectable barium currents. Pronounced $\beta$ subunit effects on the fraction of expressing cells may contribute to synergistic effects between $\beta$ and $\alpha_2-\delta_1$ subunits on Ca,2,2 channels.

**Discussion**

Since the first purification of L-type native calcium channels, it has been known that these channels are heteromultimers that contain a large (190–250 kDa) primary subunit (termed $\alpha_1$), plus several lower molecular weight auxiliary subunits (for a review see Catterall, 2000). Early studies in Xenopus oocytes indicated that expression of the $\alpha_1$ alone subunit can result in functional channels, but that coexpression with $\beta$ subunits results in biophysical properties that more closely resemble native channels (Lacerda et al., 1991; Stea et al., 1993). It is now known that vertebrates express four different $\beta$ subunits, four different types of $\alpha_2-\delta$ and eight different isoforms of $\gamma$ subunits. The mRNA or protein expression of $\alpha_1$, $\beta$ and $\alpha_2-\delta$ subunits changes during neural development (Jones et al., 1997; Vance et al., 1998) and the expression of those subunits appears to be individually controlled (Vance et al., 1998), thus providing a mechanism by which calcium channel activity can be fine tuned during neurogenesis.
In expression systems, γ subunits can exert pronounced effects on calcium channel function (Rousset et al., 2001; Moss et al., 2002), but their precise roles for channel function need to be explored, and it is not universally accepted that γ subunits do biochemically interact with neuronal calcium channel complexes. By contrast, it is well established that α₂–δ and β are bona fide subunits of neuronal HVA calcium channels. In particular, the mutual interaction sites for binding between α₁ and β subunits have been identified at the single amino acid level (Pragnell et al., 1994; De Waard et al., 1995; Witcher et al., 1995). With the exception of the Ca₉3 family and Ca₁.4 channels (McRory et al., 2004), all known α₁ subunits of voltage-activated calcium channels contain an alpha interaction domain (AID), a highly conserved signature sequence that is critical for β subunit binding. Interaction site(s) for the α₂–δ complex have been proposed to be located in extracellular region(s) but have not been precisely identified (Gurnett et al., 1997).

In Xenopus oocytes, the coexpression of β subunits has been reported to regulate α₁ subunit function potently, including dramatic effects on channel activation and inactivation, and notably, a significant increase in whole cell conductance (Mori et al., 1991; Castellano et al., 1993; Neely et al., 1993; Stea et al., 1993; De Waard et al., 1994; De Waard & Campbell, 1995; Yamaguchi et al., 1998). Typically, β₁ and β₃ subunits have been reported to accelerate inactivation, and to mediate substantial hyperpolarizing shifts in the voltage-dependences of activation and inactivation, whereas β₂a subunits are thought to mediate depolarizing shifts in channel gating, and to slow the time course of inactivation, the latter effect being due to palmitoylation of two unique cysteine residues in the N-terminus region (Birnbaumer et al., 1998; Walker & De Waard, 1998). By contrast, based on oocyte work, α₂–δ subunits have been considered to be relatively unimportant compared with the roles of the β subunits. However, Xenopus oocytes endogenously express calcium channel subunits (Tareilus et al., 1997), and are thought to exhibit unique post-translational modification of mammalian membrane proteins. Hence, it was important to perform a detailed analysis of subunit regulation of calcium channels in a mammalian cellular background.

As shown here, certain aspects of subunit regulation appear to be qualitatively similar between Xenopus oocytes and tsA-201 cells. These include β subunit regulation of inactivation kinetics, as well as of the voltage-dependences of activation and inactivation. However, a number of striking differences to previous results were observed. First, β and α₂–δ subunits mediated little, if any, effect on the voltage dependence of activation for the three channel types examined. For Ca₁.2, Ca₂.2 and Ca₂.3 channels, half-activation voltages across the entire set of different subunit combinations varied by only 7 mV, 6 mV and 10 mV, respectively. Although a 10 mV shift in half-activation potential would in principle be expected to have pronounced physiological effects in an intact neuron, the majority of individual subunit effects were not statistically significant. In previous studies, it has been shown that all four β subunits negatively shift half-activation voltages of various types of Ca channels by 2–17 mV in Xenopus oocytes (Castellano et al., 1993; Neely et al., 1993; Stea et al., 1993; De Waard et al., 1994; De Waard & Campbell, 1995; Lin et al., 1997; Yamaguchi et al., 1998; Wakamori et al., 1999; Canti et al., 2001) (or Yasuda et al., 2004), 0–10 mV in HEK-293/HEK tsA-201 cells (Kamp et al., 1996; Jones et al., 1998; Gerster et al., 1999) and 15 mV in COS-7 cells (Stephens et al., 2000). Although the < 5 mV hyperpolarizing shifts of activation by β₁₁, β₂a and β₃ subunits observed in this study are generally much lower than for the majority of previous results, we note that the effects of individual β subunits largely vary with the three calcium channels examined. Secondly, only β₂a subunits appeared to mediate dramatic effects on steady-state inactivation, notably by increasing the fraction of open state inactivation of Ca₂.2 channels. By contrast, the remaining β subunits were capable of inducing a only a < 10 mV negative shift in half-inactivation potential, which was further attenuated in the presence of α₂–δ subunits. These small shifts in steady-state inactivation are consistent with the 0–15 mV shifts reported previously for channels expressed in HEK-293/HEK tsA-201 cells (Jones et al., 1998; Gerster et al., 1999), but are much smaller than the 15–25 mV hyperpolarizing shifts reported for β₁₁, β₁ and β₃ subunits in the Xenopus oocyte expression system (Stea et al., 1993; De Waard et al., 1994; De Waard & Campbell, 1995; Wakamori et al., 1999). We note that in Xenopus oocytes, even larger (30–40 mV) hyperpolarizing shifts in the voltage-dependence of inactivation can be observed following injection of high concentrations of β subunit cDNA/cRNA, following long conditioning pulses of 25 s to 3 min that induce slow inactivation (Canti et al., 2001; Yasuda et al., 2004). Our experimental paradigms were designed to isolate the fast inactivated state, and further experimentation will be required to test the effect of different α₁/β/α₂–δ combinations on slow inactivation in our system. Finally, for Ca₁.2 channels, there was a striking lack of β subunit regulation of whole cell conductance. This contrasts with previous observations in Xenopus oocytes (Neely et al., 1993; Yamaguchi et al., 1998) and HEK-293/HEK tsA-201 cells (Kamp et al., 1996; Gerster et al., 1999). Instead, in agreement with a three-fold enhancement of Ca₁.2 channel currents by α₂–δ₁ in HEK-298 cells (Bangalore et al., 1996), the α₂–δ subunit appeared to be more effective in increasing G_max values compared with β subunits. It is unlikely that the weak regulation of calcium channel activity was due to inefficient expression of auxiliary subunits for several reasons. First, coexpression of channels with either α₂–δ, β₁ or β₂a subunits resulted in robust and highly reproducible effects on inactivation rates, indicating that these subunits were
functionally expressed in our experiments. The expression of β1b subunits resulted in a significant increase in $G_{\text{max}}$ for Ca,2,2 + $\alpha_2$–$\delta$ and Ca,2,3 + $\alpha_2$–$\delta$ calcium channels and substantially increased the fraction of Ca,2,2-expressing cells. In addition, β1b protein expression is readily confirmed via Western blots (S. E. Jarvis et al., unpublished observations). Finally, although coexpression with $\beta_4$ subunits mediated little effect on calcium channel function, this subunit also potently increased the fraction of Ca,2,2-expressing cells, and we have demonstrated recently that Ca,2,2 effectively coimmunoprecipitate with $\beta_4$ subunits from tsA-201 cell lysate (Stotz et al., 2004), thus confirming their functional expression and association with calcium channels in our system. Hence, we can conclude that all subunits are functionally expressed in our experiments.

The lack of consistent effects of β subunits on whole cell conductance is surprising in light of previous suggestions that β subunits mask an ER retention signal on the calcium channel α1 subunit to allow its efficient translocation to the plasma membrane (Bichet et al., 2000). However, trafficking of the α1 subunit to the plasma membrane does not necessarily imply whole cell current enhancement (Neuhuber et al., 1998; Gerster et al., 1999). It is important to note that while whole cell conductance not only reflects the numbers of functional channels in the plasma membrane, but also the maximum open probability of the channel, as well as single channel conductance. Whereas β subunits are not thought to alter single channel conductance, channel open probability at the plateau of the activation curve may well be affected by auxiliary subunits (Neely et al., 1993; Wakamori et al., 1993, 1999; Jones et al., 1998; Gerster et al., 1999; Hohaus et al., 2000; but see Meir & Dolphin, 1998). Without a comprehensive biochemical approach and/or detailed single channel analysis, we cannot at this stage distinguish between subunit effects on membrane expression and open probability; however, our experiments indicate that β subunits do not efficiently regulate Ca,1,2 channel activity, and that they mediate a less than two-fold increase in $G_{\text{max}}$ for Ca,2,3 channels. Together with the observation that the voltage-dependences of activation and inactivation were for the most part only weakly dependent on $\alpha_2$–$\delta$ and β subunit expression, regulating the overall amount of calcium entry may perhaps not be the primary function of these subunits. We note that it is unlikely that the lack of effect of β subunit coexpression on Ca,1,2 current density is due to lack of β subunit expression, because all four β subunits were able to regulate inactivation kinetics of Ca,2,2 and Ca,2,3 channels, in addition to affecting the position of the steady-state inactivation curve of Ca,1,2 channels.

Yet, the role of calcium channel subunits as important regulatory elements of calcium channel function is undeniable. Even in tsA-201 cells, calcium channel β subunits dramatically regulate G protein inhibition of Ca,2,2 calcium channels (Feng et al., 2001). Moreover, there is compelling evidence that β and α2–δ subunits are critical for neuronal and/or muscle physiology. A point mutation leading to a premature stop codon in the mouse β4 subunit gives rise to the lethargic mouse phenotype. This mouse is characterized by seizures and ataxia (Burgess et al., 1997); however, N-type and P/Q-type channel activity in Schaffer collateral synapses does not appear to be affected (Qian & Noebels, 2000). Knockout of the β3 subunit reduces L-type and N-type channel activity in neurons (Namkung et al., 1998), and knockdown of the calcium channel β4 subunit prevents excitation contraction coupling and alters L-type calcium channel kinetics (Gregg et al., 1996). Mice lacking β2 die during embryogenesis due to absence of heartbeat; however, restoration of β2a expression in the heart rescues the lethal phenotype such that homozygous mice lacking β2a in other organs (including the brain) are behaviorally normal (Ball et al., 2002). A premature stop codon in the α2–δ subunit (also known as the ducky mouse mutation) results in an ataxic phenotype accompanied by cerebellar atrophy (Barclay et al., 2001). In this mouse, P/Q-type current activity is reduced without major effects on single channel amplitude or kinetics, suggesting that this subunit is critical for regulating membrane expression of the channel. Taken together, whereas in some cases, absence of functional auxiliary subunits appears to alter calcium current activity per se, in other instances severe phenotypes are observed without gross alterations of calcium channel function. That said, even small changes in whole cell conductance (such as those observed in our study for Ca,1,2 and Ca,2,3 channels) may have profound implications for neuronal function. In fact, the amplitude and duration of macroscopic calcium channel current, and hence intracellular Ca$^{2+}$ concentration during action potentials, has a significant effect on neurotransmitter release due to the power relation between transmitter release and intracellular Ca$^{2+}$ concentration (see Wu & Saggau, 1997). It is also likely that auxiliary subunits contribute to targeting of calcium channels to specific subcellular compartments such as presynaptic nerve termini (Wittmann et al., 2000), a feature which we cannot address using tsA-201 cells. Hence, the poor ability of auxiliary subunits to alter current activity in our expression system in no way undermines the significance of their expression in native cells.

Overall, our data constitute the first truly systematic analysis of the effects of auxiliary calcium channel subunits on various calcium channel subtypes expressed in mammalian cells. Through a systematic approach, we could elucidate a previously underestimated role of the α2–δ1 subunit with regard to current enhancement and kinetics. Moreover, the effects of each auxiliary subunit on whole cell conductance and channel gating appear to be tailored to a given calcium channel subtype. Yet, in contrast to previous findings from numerous other studies,
auxiliary subunits are not absolutely required for functional expression, and overall only weakly regulate basic calcium channel biophysics.

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Abbreviations

AID, alpha interaction domain; ER, endoplasmic reticulum; HVA, high-voltage activated.

References


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